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Antimicrobial and Anti-Biofilm Activities of Bee Venom against some Dental pathogens

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ABSTRACT

Dental plaque is a poly-microbial biofilm that forms in the mouth and is the source of oral infections. However, when these microbes or their components enter the connective tissues or circulation, they may increase the risk for some systemic diseases. Plaque contains a variety of microorganisms embedded in an extracellular matrix of polymers that can also develop between the teeth and the gingival crevice making it difficult to be reached by antibiotics in case of an infection. Treatment with traditional antibiotics becomes more challenging due to biofilm formation. Bee venom (BV) has anti-inflammatory, anti-apoptotic, anti-fibrotic, anti-atherosclerotic, antibacterial, antiviral, antifungal, and anticancer effects.

Our study was conducted using 150 oral swap samples of dental plaque patients from the Oral medicine and periodontitis department – Faculty of Dentistry –at Alexandria University. After purification and identification by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) biotype. Only 39 bacterial isolates were taken. Two different honeybee strains, *Apis mellifera yamentica,* and *Apis mellifera carnica* from a private farm in the Ganaklis area at Nobarya, Behaira were used in this study. Bee venom was analyzed by HPLC. The disc diffusion method was used to test the effect of bee venom on various bacterial isolates, yielding a mean of inhibition zones of 3 mm. The minimum inhibitory concentrations (MIC) of BV were determined with values ranging from 31.3 – 44.4 µg/ml. while the minimum bactericidal concentration (MBC) was almost 30 µg/ml for all samples. Biofilms are dense micro-communities that grow on inert surfaces and encapsulate themselves with secreted polymers, where microbes can adapt to environmental changes and can protect the microbes from disinfectant agents or antibiotics. Bee venom was used as an antibiofilm agent. Sub-MIC concentrations of 25 and 20 µl of B.V. virtually completely suppressed biofilm formation in a representative sample of *Staphylococcus sciuri* and *Streptococcus mutans* biofilm, respectively.

1. Introduction

Dental caries and periodontal diseases are known as the top oral health burden in both developing and developed nations affecting around 20–50% of the population worldwide and are the main reason for tooth loss [1]. Actinomyces, Streptococcus, and Lactobacillus species were considered to be the main causal agents of root caries [2]. As a survival strategy of microbes, they evolve from the planktonic state and associate together as "communities" to form complex matrix-like structures known as biofilms. Biofilms are dense micro-communities that grow on inert surfaces and encapsulate themselves with secreted polymers, where microbes can adapt to environmental change by altering their gene expression patterns. This biofilm structure and corresponding changes in gene expression can protect the microbes from disinfectant agents or antibiotics [3]. A Strong symbiosis between *Streptococcus mutans* and *Candida albicans* has led to an increase in biofilm mass and cell density, as well as enhanced virulence [4]. The attachment of the microorganism to a solid surface begins with the adhesion of the microorganism to the tooth enamel and other materials such as the tooth root or dental implant. This attachment could occur via two mechanisms: sucrose-dependent (based on the activity of glycosyltransferases and glucan binding proteins) and sucrose-independent (based on the activity of glycosyltransferases and glucan binding proteins) (using interactions between adhesion particles of microorganisms and saliva agglutinins) [5].

The second phase is Exopolysaccharide (EPS) matrix formation which starts with the irreversible connection of bacteria with the surface. This matrix is considered as the open architecture of nutritional channels, spaces, and other properties, including heterogeneity of the environment (pH and oxygen gradients, co-adhesion) that forms the protection from the host defense factors and desiccation [6]. The third stage is Biofilm maturation, which takes place when the matrix is still being developed and other bacterial species join the biofilm [7].

The fourth stage is bacterial succession. Where newly occurring species of bacteria adhere to the previously attached pioneering species. The presence of one microorganism creates ecological niches for other microorganisms, which facilitates their survival in the new favorable conditions [8]. The fifth stage is the formation of a mature biofilm associated with the growth-rate reduction of particular bacteria. The interactions between microorganisms play the most important role not only in the formation of mature biofilm structure but also in the disconnection of bacterial species from such formed structure, occupying subsequent ecological niches within the oral cavity microbiome [9].

There are several important aspects to the formation of biofilm including, biocompatibility during microorganism adhesion, nutritional conditions [10], hydrodynamic conditions [11], surface type (smooth, rough, and their combinations) [12], and many other unexplained and undiscovered factors. To counteract the natural resistance/tolerance of microbial biofilms against antimicrobial agents and to mitigate their pathologic consequences, new strategies are being considered and studied.

They can be classified into four main categories according to Bjarnsholt *et al* [13]:

(a) prevention, of biofilm formation through antibiotic prophylaxis, or modifying surface characteristics using antimicrobial or anti-adhesive coatings. For instance, silver nanoparticles on titanium surfaces are currently being evaluated for use in dentistry [14].

(b) Weakening, by interfering with signaling molecules, virulence factors, and/or biofilm-forming properties to make the biofilm more susceptible to conventional antimicrobial agents and the natural host defense system.

(c) Disruption, through disorganizing the biofilm structure disrupts the communication network between its cells and makes them more susceptible to antimicrobials. For example, Alhede *et al* [15] showed that vortexing *Pseudomonas aeruginosa* biofilms were a valuable *in vitro* methods of increasing their sensitivity towards tobramycin.

(d) Killing, which involves destroying biofilm cells by specific and/or nonspecific anti-infective means.

Bee stings are probably one of the first natural cures for arthritis, in the ancient civilization of China, India, Egypt, Babylon, and Greece, where bee venom was used for apitherapy [16]. The bioactive compound of BV termed apitoxin can be divided into

(i) proteins such as Melittin, apamin, MCD-peptide (Mast Cell Degranulating peptide), and adolapin. Melittin has an MW of 2840 Daltons but it can reach up to 12500 Daltons because of its tetrameric form [17].

(ii) enzymes, like phospholipase A2 (PLA2), hyaluronidase, α-glucosidase, acid phosphomonoesterase, lysophospholipase, and

(iii) amino acids, phospholipids, and volatile compounds [18].

The fact that extensive use of antibiotics over the past six decades in our hospitals and communities has led to an increased prevalence of bacteria with acquired resistance to the antibiotics. [19], giving rise to the development of new approaches for the treatment of bacterial infections.

The majority of bacterial genera, such as *Streptococcus, Pseudomonas, Salmonella, Escherichia*, and *Burkholderia*, have developed several ways to resist antibiotics. Such bacteria are becoming a serious clinical problem throughout the world. Thus, new effective antibacterial agents with new antibacterial mechanisms need to be continuously developed [20]. The antimicrobial activity of BV has been documented against both gram-negative bacteria including, *Escherichia coli, Salmonella spp, Enterobacter cloacae* and *Citrobacter freundii* and gram-positive bacteria such as coagulase-positive and negative *Staphylococci*.

It is well known that BV and its two major components [melittin and phospholipase A2 (PLA2)] present antimicrobial activities and thus can be used as complementary anti-bacterial agents [21]. These compounds exert their effects against bacteria by inducing pores through their membranes leading to their cleavage and then lysis [22].

This study aims to test the antibacterial effect of serial dilutions from the selected bee venoms in comparison with the effect of standard antibiotics against the well-identified bacterial strains, isolated from patients with dental plaque, using (MALDI-TOFF-MS) bio-typing. Calculate the minimum inhibitory and bactericidal concentrations of the selected bee venoms. Biofilm formation as well as antibiofilm treatment with bee venom will be analyzed *in vitro* using the scanning electron microscope.

2. Material and methods

2.1 Sample collection

This study was conducted using 150 oral swap samples of dental plaque patients from the Oral medicine and periodontitis department – the faculty of Dentistry –at Alexandria University. The samples were taken by a sterile cotton swap from the gingiva.

Two different honeybees strains, *Apis mellifera yamentica,* and *Apis mellifera carnica* from a private farm in the Ganaklis area at Nobarya, Behaira were used in the study. The bee venom was collected by a local electric shock device at an alternating current and fixed current.

Only five types of bee venoms were enrolled in this study; venom from mixed bee types, venom from Carnica bee (during fall), Carnica bee (during winter), venom from mixed bee using alternate current, and venom from New Zealand bees. The electric shock device comprises a bee venom collection frame with wire electrodes installed parallel to each other. Electrical current goes through them in the form of impulses. Bee venom frames are mounted on the top or under every hive and are then connected to an electro-stimulator; we used an alternate and fixed current. For the fixed current 27 volts were applied for 90 min to collect 40% of the venom and then the same voltage was applied for 60 min to collect 60% of the venom. Using electrical impulses to stimulate the bee workers to sting through latex or fiber sheets placed on a glass plate [19]. Bees that come into contact with the wires received a mild electrical shock and are stung onto the glass sheet. The odor that evaporated from the venom mobilized and irritated the other bees and they also started to sting. The bee venom collected dries on the glass. The frames with fresh dried bee venom on them were carefully packed into a special container for transportation to the laboratory. The processing of bee venom was then started right after the frames are brought back to the laboratory. After that bee venom was packed up in dark glass jars and stored in a cool and dry place [23]. At the laboratory the samples were dissolved in distilled water and filtered to get rid of impurities, frozen at -80°C then lyophilized.

2.2 Bee Venom chromatographic identification

The bee venom solution for preparative HPLC (20 A, Shimadzu, Japan) studies was prepared by dissolving 5 mg of that lyophilized venom product in 1ml of deionized water. Before loading onto the column, the solution was filtered through a 4.5 µm membrane filter. This instrument is available at the "City of Scientific Research and Technology Application (SRTA-City)" in the Center of Pharmaceutical & Fermentation Industries Development (New Borg El-Arab City – Alexandria – Egypt). Stainless steel column (25cm x 4.6mm) was packed with prontosil kromaplus C18 (5µm) with a flow rate detector (1 ml/min) and injection volume of 20 µl, running was optimized for 60 minutes at a wavelength of 220 nm using sample conc. (15 mg/ml) the mobile phase (A) consists of 0.1% Tri floro acetic acid in water and the mobile phase (B) is only acetonitrile [24].

Bee venom components collected at different seasons and from different bee strains Table **1**.

Table 1. Different bee venom types used in the study

V1	venom from mixed bee types
V2	venom from Carnica bee (during fall)
V3	venom extracted from mixed bee using alternate current
V4	venom from Carnica bee (during winter)
V5	venom from New Zealand bee

2.3 Microbiological Analysis

A loop full of each sample was taken aseptically into a 5 ml brain heart infusion (BHI) broth medium (Oxoid-UK) and was shaken. Dilutions were prepared in 1ml of (BHI) broth medium. Two consecutive dilutions 10^4 and 10^5 were plated for each sample. Samples were plated in Mitis Salivarius agar media (Becton-Dickinson, Franklin Lakes, NJ, USA) and then incubated for 24 hrs at 37°C. Bacterial colonies were then counted and expressed as number of colony-forming units per milliliter (CFU/ml). Different colonies were selected based on morphology using standard microbiological criteria, with special emphasis on color, shape, size, and form. Microbial cultures were stocked in the isolation medium supplemented with 60 % glycerol and kept at -80°C [25].

The Gram staining technique was used to visualize cell shape and categorize the isolates into gram-negative and gram-positive [26]. Bacterial samples were identified using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) biotype (Bruker Logo Bruker Daltonik MALDI Biotyper) [27].

2.4 Antimicrobial Susceptibility Testing

The disc diffusion method (Kirby-Bauer) was employed to conduct susceptibility testing. Antibiotic discs were obtained from i2a (Montpellier, France). Susceptibility testing was done on Mueller-Hinton agar (Becton-Dickinson, Franklin Lakes, NJ, USA) using MacFarland 0.5 from overnight cultures followed by incubation at 35°C for 16-18h [28].

Penicillin G (P-10), Kanamycin (K-30), Chloramphenicol (C-30), Streptomycin (S-10), and Ampicillin (AM-10) were used against representative bacterial strains from plaque patient swabs, and inhibition zone diameters were measured and recorded manually, according to Clinical & Laboratory Standards Institute guidelines [29] and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) 1.3 guidelines.

2.5 Antimicrobial activities of Bee Venom

Using Gentamycin (have identical antibiotic disc loads in both CLSI and EUCAST guidelines) as positive control and bee venom at concentrations of (10, 20, 30, 40, and 50 µg/ml). All 39 bacterial samples were tested in triplicate, and the average of inhibition zones for each sample was displayed in our results, using Pearson correlation between groups as a statistical relationships.

2.6 Minimum inhibitory concentrations (MICs) and minimal Bactericidal concentration (MBCs)

The MIC was performed as described in M27-A2 (CLSI) with modifications [30]. The broth microdilution method uses 96-well microplates (Corning-Merck-Germany). The bacterial inoculum concentration of ($5x10^5$ CFU/ml) was applied in each well. Bee Venom sample (5.0 mg) was dissolved in distilled H_2O (1 mL) to obtain a 5000 µg/mL stock solution then five dilutions were prepared as 50, 40, 20, 10, and 5 µg/mL and applied against bacteria in Mueller-Hinton media (Becton-Dickinson, Franklin Lakes, NJ, USA).

Three wells containing bacterial suspension without Bee Venom were used as (Growth control) and the (background control) are three wells containing media without bacterial inoculum. The optical density (O.D.) was measured after incubation for 24 hr at 37°C using an absorbance microtiter plate reader at Medical Biotechnology Dep., Genetic Engineering Institute, City of Scientific Research And Applied Technology (SPECTROSTAR Nano-BMG LABTECH, Germany) at wavelength 620 nm.

The lowest concentration showing no growth was recorded as the (MIC). 0.1 ml collected from the well that recorded lowest concentration showing no growth after sub-cultured in (LB agar) plates and incubated overnight at 37 °C. The lowest concentration which produced a viable count of the original inoculums ($5x10^{\wedge 5}$ CFU/ml) was determined as the minimal bactericidal concentration (MBC).

2.7 Antibacterial Effect of Bee Venom Component

Using the most abundant bee venom fractions, Melittin and Phospholipase A2. Disk diffusion method was done using Melittin and phospholipase A2 concentrations of (10, 20, 30, 40, and 50 µg/ml)on Mueller-Hinton agar plates against overnight cultures (MacFarland 0.5) of the tested bacterial isolates followed by incubation at 35°C for 16-18h [28]. All the examined concentrations were inoculated in triplicates.

2.8 Biofilm formation assay

The detected bacterial strains were tested to determine their ability to form the biofilm (quantitatively) using the tissue culture plate method (TCP) as described by Bekir *et al* [31]. Isolates were cultivated overnight in 96-well polystyrene tissue culture microtiter plates at 37°C, in trypticase, soy broth (OXOID, UK) supplemented with 1% glucose as the growth medium. The culture medium was removed and the attached bacteria were then fixed using 95% ethanol, and stained with 1% crystal violet (1 ml of crystal violet stock solution with 10 ml H_2O and 40 ml of oxalate stock solution). Optical density was measured at 570 nm using a plate reader (SPECTROSTAR Nano-BMG LABTECH, Germany). Isolates exhibiting O.D.570 nm > 0.1 were considered positive for biofilm production. Biofilm production was interpreted as strong, moderate, or low, according to StepanoviĆ *et al* [32]. The experiment was performed in triplicate.

2.9 Antibiofilm activity of Bee Venom

Antibiofilm activities of Bee Venom were determined at five concentrations as (30, 20, 10, 5, and 2.5) µg/mL against selected biofilm-producing strains, where the isolates were incubated with Bee Venom in microtiter plates, and the procedure was performed as previously described according to [31]. For each isolate, the results presented are averages of at least 3 replicate wells. The effect of Bee Venom on biofilm formation was observed by analytical Scanning Electron Microscope (SEM) (JEOL, JSM-6360LA, JEOL USA, Inc.) at the central laboratory of the City of Scientific Research & Applied Technology.

3. RESULTS

3.1 Sample collection

A total of thirty-nine microbial isolates were recovered from oral swabs collected from the dental plaque of 150 patients under treatment in the clinic of Oral medicine and periodontitis department – Faculty of Dentistry – Alexandria University. Bee venom was extracted using a local electric shock device from two separate honeybee strains, Apis mellifera yamentica, and Apis mellifera carnica, housed on a private farm in the Ganaklis district of Nobarya, Behaira. Only samples from Apis mellifera carnica and mixed bee venom could give representative and fixed samples and enrolled in this study.

3.2 Chromatographic Identification

On preparative HPLC, melittin and phospholipase A2 standards were utilized to create a standard curve for identifying differences between bee venom components collected at different seasons and from different bee strains Table 2. From Fig. 1 Comparing our bee venom standards, we discovered that V2 had the highest concentration of melittin (50.38%) and V3 had the highest concentration of phospholipase A2 (38.53%).

3.3 Microbiological analysis

3.3.1. Antibacterial effect of standard antibiotics (Disc Diffusion method)

Penicillin G (P-10) (10 ug/ml), Kanamycin (K-30) (30 mg/ml), Chloramphenicol (C-30) (30 mg/ml), Streptomycin (S-10) (10 mg/ml), and Ampicillin (AM-10) (10 mg/ml) were used in concentrations that were effective on both logarithmic and stationary phase cells of 7 representative strains from patients with dental plaque, and inhibition zone diameters were measured and recorded manually, according to Clinical & Laboratory As seen in Fig. 2: Ampicillin and Penicillin G have no bactericidal action (except Bacillus subtilis which affected by Penicillin G). The single Gram-negative bacteria detected in our samples, Pseudomonas auregenosa, was exclusively impacted by Chloramphenicol and Streptomycin, with a clear zone of 6 mm and 3 mm, respectively.

Table 2. The calculated concentrations of different Bee Venom according to HPLC results

Material	Melittin		Phospholipase A2	
	Area (mg/ml)	Concentration (%)	Area (mg/ml)	Concentration (%)
Melittin Stand.	----	65 %	Nil	---
Phospholipase A2 Stand.	Nil	---	---	100 %
V1	2.1	41.67 %	1.3	25.97 %
V2	**2.5**	**50.38 %**	**1.7**	**34.32 %**
V3	2.3	46.1 %	1.9	38.53 %
V4	2.6	49.39 %	1.6	32.51 %
V5	**2.3**	**45.39 %**	**1.3**	**26.22 %**

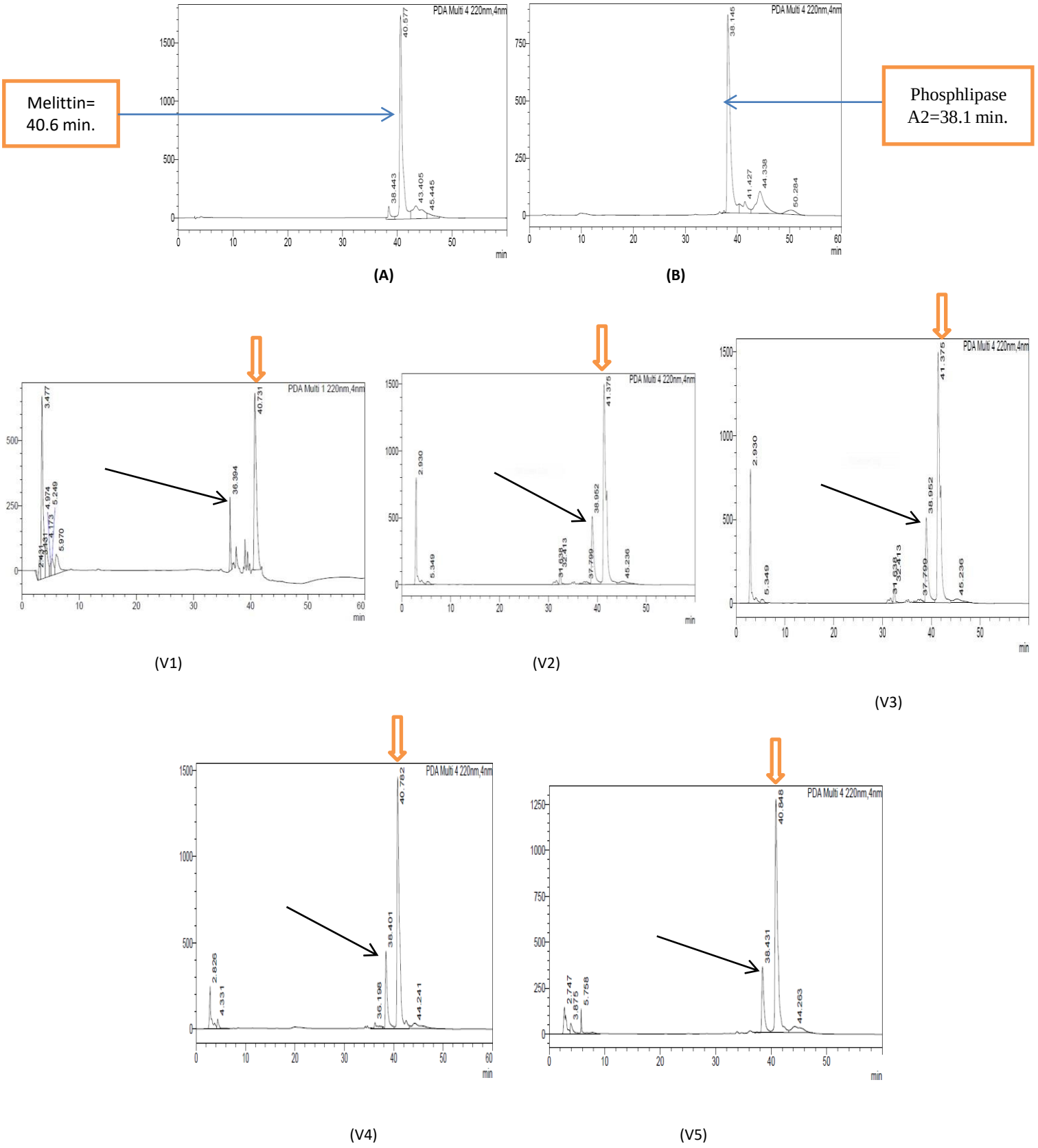

Fig. 1 HPLC results of bee venom standards: Melittin Standard (A) and Phospholipase A2 Standard (B) and bee venom samples from V1 to V5 showing the peak at which melittin was detected and referred by and the peak of phospholipase A2 by minutes

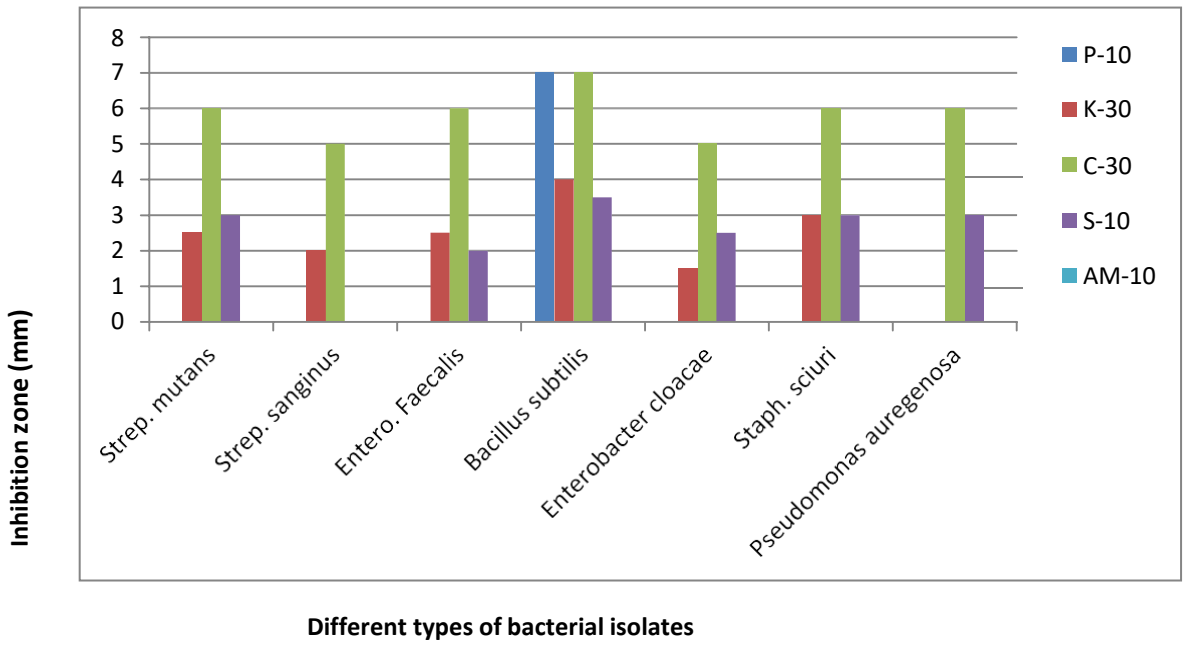

Fig. 2 The effect of standard antibiotics on different bacterial isolates. Where, P-10 is Penicillin G; K-30 is Kanamycin 30 mcg; C-30 is Chloramphenicol 30 mcg; S-10 is Streptomycin 10 mcg, and AM-10 is Ampicillin 10 mcg.

3.3.2. Antibacterial effect of Bee Venom (Disc Diffusion method)

The effect of bee venom on different bacterial isolates was determined using the disc diffusion method according to Kirby-Bauer method [28]. Our results are shown in Table 3**.** where, mixed bee venom (V1), Carnica bee (V2) & New Zealand type (V5) was the most effective on Streptococcus sp. with a clear zone of 3 mm. at a concentration of 50 µg/ml, Pearson correlation between groups showed a non-significant positive relationship (P-value > 0.05) between groups (V1, V2, V3, V4, and V5).

Enterococcus sp. was strongly affected by the venom collected from Carnica bee (V2), and by the venom collected from bees using Alternative current (V3) giving clear zones of 4 & 3.5 mm. respectably at a concentration of 50 µg/ml with a non-significant negative relationship.

Isolates from Bacillus sp. showed good resistance to bee venom except with V3 and V4 give a clear zone of 1.5 mm. with 50 µg/ml. Unfortunately, Bee venom has a small effect on Staphylococcus sp. except with (V1) and (V2) giving clear zones up to 3 mm. at a concentration of 50 µg/ml. Three isolates from Enterobacter sp. were included in our study and were all affected by bee venoms especially for V1, V3, and V4 giving clear zones of 2 mm. at 50 µg/ml.

3.3.3 Antibacterial effect of Melittin

We discovered that phospholipase A2 has no effect on bacterial growth when different concentrations of Melittin and Phospholipase A2 standards were tested on different bacterial cultures. Melittin alone, on the other hand, had a 3 mm diameter inhibitory impact.

3.3.4. Minimum Inhibitory Concentration (MIC)

As shown in Fig. 3, representative samples from three different bacterial strains with MIC values of 31.31 µg/ml for *Enterococcus faecium*, 39.81 µg/ml for *Staphylococcus sciuri*, and 44.39 µg/ml for *Streptococcus mitis*. While the Minimum Bactericidal Concentration (MBC) for all 3 strains was almost the same (30 µg/ml).

3.3.5. Biofilm formation by different microbial isolates

As shown in Fig. 4, values are expressed as the percentage of absorbance (570 nm) of cells in treated wells compared with that of untreated wells. Among the five tested isolates, biofilm formation was strong in three, namely, *Enterococcus faecium* (88%), *Staphylococcus sciuri* (67%), and *Streptococcus mitis* (50%), moderate with *Streptococcus mutans* (34%), and absent in *Streptococcus sanginus*.

Table 3. The effect of bee venom on different bacterial isolates expressed as inhibition zones in mm.

No.	(V1) Mixed B.V. (µg/ml)					(V2) Carnica B.V. (µg/ml)					(V3) Alternative current B.V. (µg/ml)					(V4) Carnica B.V.(µg/ml)					(V5) New Zealand B.V. (µg/ml)					Bacterial isolates
	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	
1	0.5	0.5	1	1.5	2	0.5	0.5	1	2	2.5	R	0.5	1	1.5	2	0.5	0.5	1	1	1.5	0.5	1	1	1.5	2	Streptococcus salivarius
2	0.5	0.5	1	1.5	2	R	0.5	1	1.5	2	R	0.25	0.5	1	1.5	R	0.5	1	1.5	2	0.25	0.5	0.5	1	1.5	Streptococcus vestibularis
3	٤	0.25	0.5	1	2	R	0.5	1	1.5	2	R	0.25	0.5	1	1.5	R	R	0.5	1	1.5	R	0.5	1	1.5	2	Streptococcus pneumoniae
4	0.5	0.5	1	2	3	0.25	0.5	2	2.5	3	0.5	0.5	1	1	1.5	R	0.5	1	1	1.5	R	0.5	1	2	3	Streptococcus oralis
9	R	0.5	0.5	1	1	0.25	0.25	0.5	1	1.5	R	0.25	0.5	0.5	1	R	0.25	0.25	0.5	1	R	0.25	0.5	1	1.5	Enterococcus faecium
10	R	1	2	2	3	R	0.5	1.5	3	4	R	1	1	1.5	3.5	0.5	0.5	1	2	2	0.25	0.5	1.5	1.5	2	Enterococcus fecalis
11	R	0.25	0.5	0.5	1	R	0.25	0.5	0.5	1	0.5	0.5	1	1	1.5	0.5	0.5	1	1	1.5	R	0.25	0.25	0.5	0.5	Bacillus cereus
12	R	R	0.25	0.25	0.5	R	R	0.25	0.25	0.5	R	R	0.25	0.25	0.5	R	R	0.25	0.25	0.5	R	R	0.25	0.25	0.5	Bacillus subtilis
13	1	1	1.5	2	3	1	1	1.5	2	2.5	R	0.5	1	1	1.5	R	0.5	1	1	1.5	R	R	0.25	0.25	0.5	Staphylococcus aureus
14	0.25	0.5	0.5	1	2	R	0.25	0.5	0.5	1	R	0.25	0.5	0.5	1	R	0.25	0.5	1	1	0.25	0.25	0.5	0.5	1	Staphylococcus sciuri
15	R	0.5	1	1.5	2	0.5	0.5	1	1	1.5	R	0.5	1	1	1.5	0.25	0.25	1	1	1.5	0.25	0.5	1	1	1.5	Enterobacter cloacae

R= Resistance

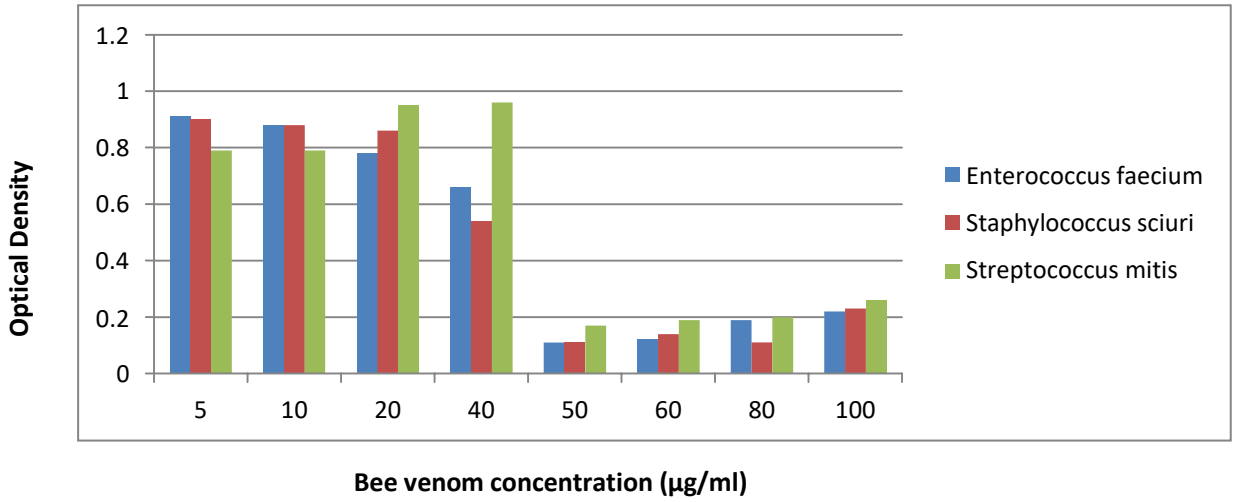

Fig. 3 The effect of different B.V. concentrations on the tested bacterial growth

3.3.6. Anti-biofilm formation effect of Bee Venom

Biofilm formation was nearly inhibited in a representative sample from *Staphylococcus sciuri and Streptococcus mutans* by sub-MIC concentrations of 25 µl /ml and 20 µl/ml of B.V. respectively as shown in Fig. 5.

3.3.7. Scanning Electron Microscope of Biofilm samples

The effect of different concentrations of bee venom on biofilm formation was observed by an analytical Scanning Microscope (JEOL, JSM-6360LA, JEOL USA, Inc.) at the central lab. Of City of Scientific Research & Applied Technology. Fig. 6 shows that concentrations of (2.5, 5 & 10) µl/ml of mixed bee venom gives decrease in biofilm formation of *Streptococcus mitis* and higher concentrations as (10 & 20) µl/ml of mixed bee venom on *Staphylococcus sciuri* gives greater effect on biofilm formation.

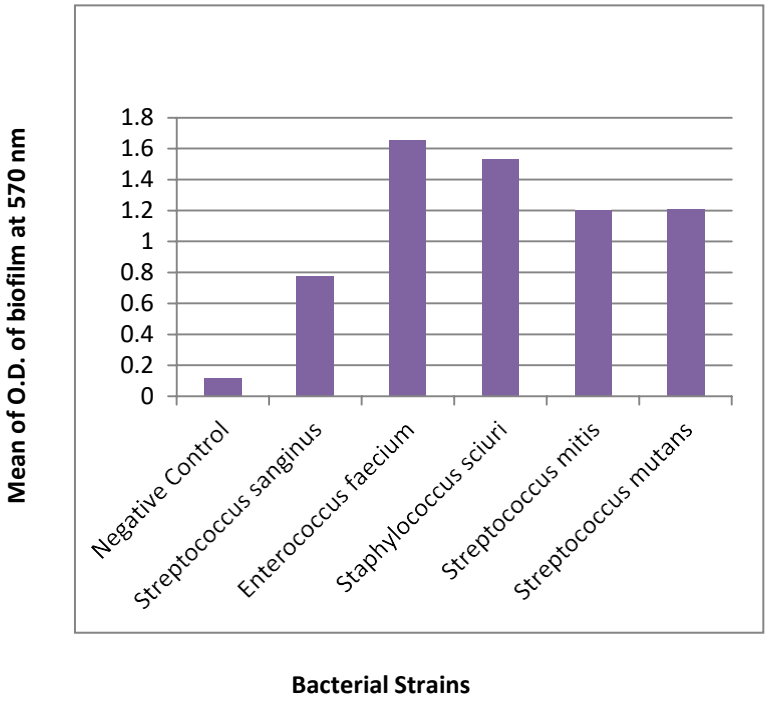

Fig. 4 Biofilm formation of tested isolates. Where, values are expressed as % of absorbance (570 nm) of cells in treated wells compared with that in untreated wells

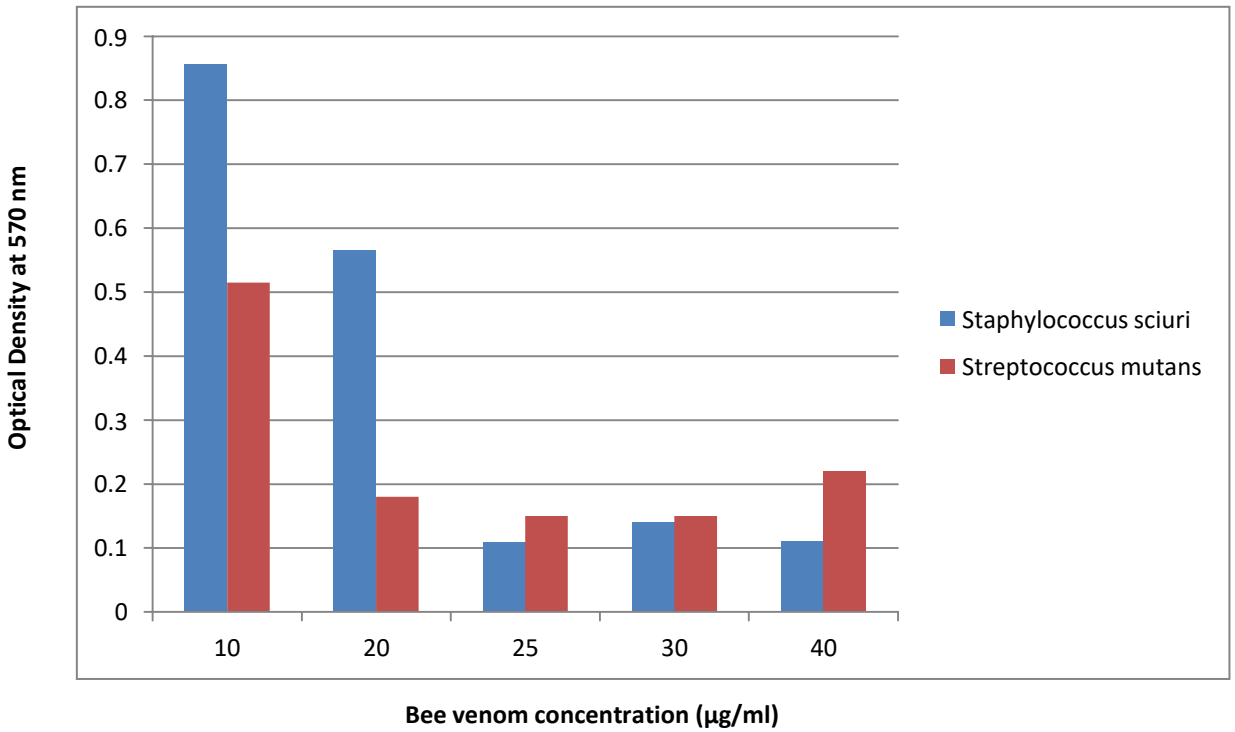

Fig. 5 Effect of sub-MIC of Bee Venom on biofilm formation by *Staphylococcus sciuri* and *Streptococcus mutans*

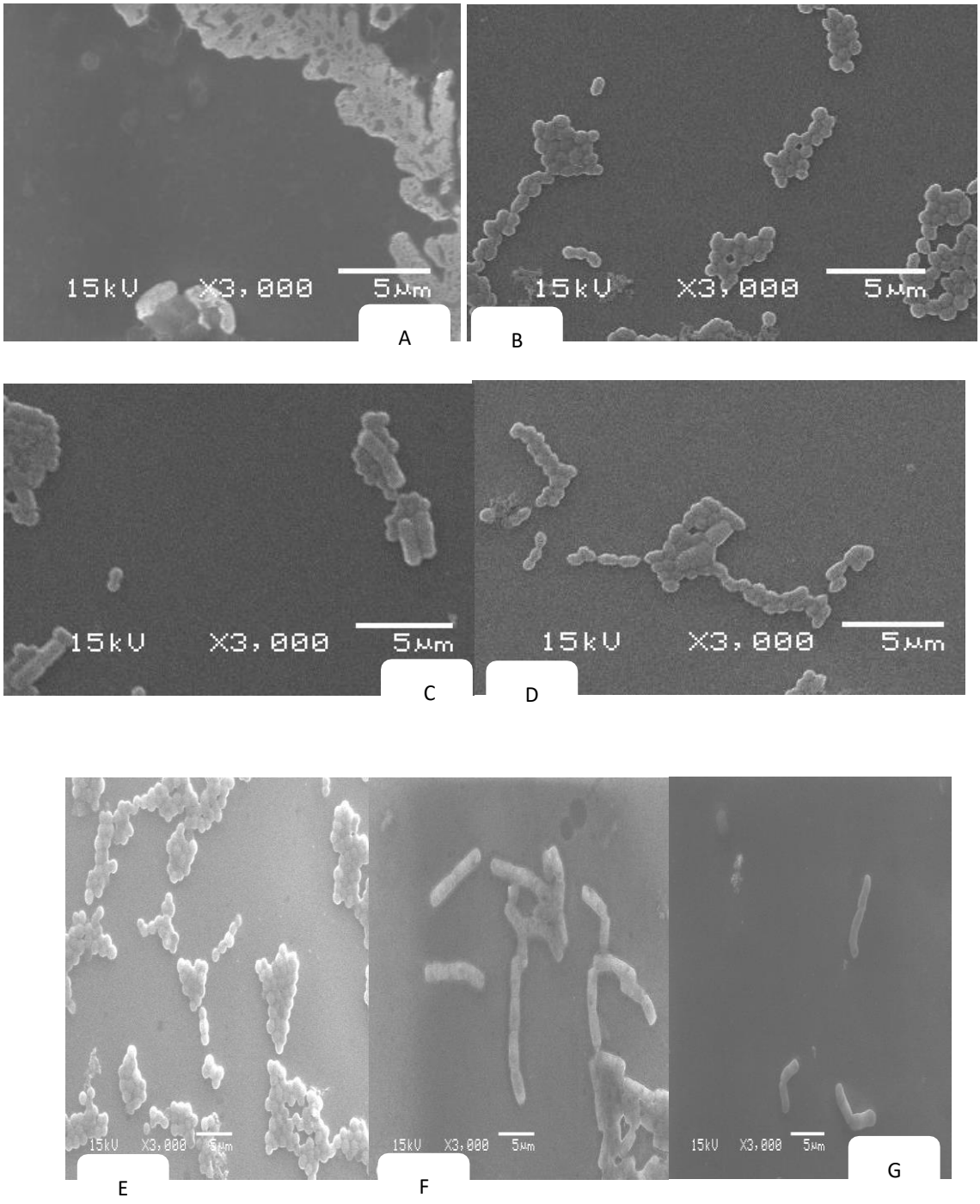

Fig. 6 SEM micrographs at X 3,000 of *Streptococcus mitis* (A), grown with 2.5 µL B.V. (B), 5 µL B.V.(C), and 10 µL B.V.(D) *Staphylococcus sciuri* (E), grown with 10 µL B.V. (F), and 20 µL B.V.(G)

4. Discussion

The oral ecosystem is a good culture for the growth of a variety of microorganisms which ranges from mycoplasmas to bacteria and viruses in certain extreme cases. Some microbes continue to survive deeply on the surfaces with the help of their biofilm-forming abilities leading to the resident oral microbiome which resides in synchronization with the host.Marsh and Zaura, [33] have validated the extent of proximity among the microorganisms within the dental biofilms. Complex levels of interactions have also been discovered in studies, which could lead to a favorable or bad effect. Changes may result in altered microbial interactions, which may have a synergistic or antagonistic effect on the host, raising the potential for oral symptoms [34].

The quick breakdown and generation of acids caused by eating sugar results in a lower pH within the biofilm, which is linked to the creation of dental plaques. Reduced pH harms the microflorae that contribute to the quality of enamel, which leads to the start of dental disorders. Microbial diversity is also diminished as a result of this situation [35].

The content of melittin in bee venom samples collected during the different seasons and types ranged from 41.67 to 50.38% with a mean value of 46.03% Table 2. The average content of phospholipase A2 was 32.25% (25.97-38.53 %). The results on the principal bee venom constituents obtained in this study differ from those measured by Rybak-Chmielewska and Szezesna [22] found that the bee venom consists of melittin with a mean value of 64.61% and phospholipase A2 of only 12.98%. Variation in results between the two studies is probably due to the different origins of samples and the use of different sampling methods.

Khalafallah [36] Mentioned that the heaviest poison sac was obtained during summer, autumn, and then winter. This is because the spring season is the start of bee activities season, the clover and citrus crops are full of pollens spread in, thus considered as natural feeding full of protein content, so the venom quantity increased during the spring season when compared to other seasons. In the summer season, cotton and corn are more available providing pollen, on the other hand, the high temperature during summer reduced the production of venom. In the autumn season, when the crops reduced, increasing the need for external nutrition. In winter, no working bees were found due to the reduction of temperature and the queen stops laying eggs and no nurse bees, all these reasons reduced the quantity and the quality of venom.

Bee venom collection was affected by the month of collection and different day times; results concluded that July was the best month, due to the availability of corn pollen grains and collection between 7-9 pm was the best time for bee venom collection. Additionally, venom gland and venom sac were affected also by honeybee feeding and gave higher amounts of venom [37]. Several antimicrobials, such as ampicillin, chlorhexidine, sanguinarine, metronidazole, phenolic antiseptics, and quaternary ammonium antiseptics, among others, effectively prevent dental caries [38]. However, various adverse effects such as tooth and restoration staining, increased calculus formation, diarrhea, and disarrangements of the oral and intestinal flora has been associated with the use of these chemicals [39]. These drawbacks justify the search for new effective anti-cariogenic compounds that could aid in caries prevention [40].

Only three of the standard antibiotics (Kanamycin, Chloramphenicol, and Streptomycin) out of the five affected our two isolates of *Streptococcus sp.* (*Streptococcus mutans* and *Streptococcus sanginues*), while all five bee venom types (V1, V2, V3, V4, and V5) had an inhibitory effect on the different isolates of *Streptococcus sp.* at three different concentrations 30, 40 and 50 µg/ml.

The same was recorded for *Enterococcus sp.*, although it is not the main cause of endocarditis, and when comparing the mean effects of the five bee venoms at a concentration of 50 µg/ml to the three standard antibiotics, the mean values were 53.4% for Kanamycin & Streptomycin, and 24.4% for Chloramphenicol. The possibility that patients with enterococcal endocarditis may infect themselves via gastrointestinal translocation would obviate a number of issues in identifying the source of infection in many clinical cases: antibiotic and/or systemic stress-mediated gut permeability to enterococci are common conditions in both outpatient and inpatient settings. Additionally, the lack of an obvious systemic, cell-mediated immune response seen in some - though not all - endovascular infection models, suggesting that *E. faecalis* may be able to evade the host immune system for lengthy periods, adds a further wrinkle to establishing concrete relationships between the onset of (perhaps transient) bacteremia and endovascular colonization. This area of research requires further investigation to understand both the potential and actual routes of patient self-infection [41].

Only New Zealand bee venom out of the five tested venoms did not affect *Bacillus sp.* and Ampicillin is the only antibiotic that did not affect *Bacillus sp.* A comparison of the mean effects of the other four bee venoms at a concentration of 50 µg/ml to the other four standard antibiotics gave mean values of 14.5% of Penicillin G, 24% of Kanamycin, 12.5% of Chloramphenicol, and 27.8% of Streptomycin.

From all collected samples, only one isolate of *Pediococcus pentosaceus* was found with almost the same stimuli from all bee venom types with an inhibition zone of 2 mm at 50 µg/ml also, *Pseudomonas auregenosa* (Gram-negative bacteria "GNB") had an inhibition zone of 2 mm with 50 µg/ml of Carnica bee venom. *Lactobacillus plantarum* was resistant to almost all different concentrations of bee venom.

Our results demonstrated that Gram-positive bacteria (GPB) were more sensitive at lower concentrations of Bee Venom than GNB. These results are in harmony with another study by Hegazi *et al* [20] who reported that the antimicrobial activity of Bee Venom has been documented for both GPB and GNB, including *Escherichia coli (E. coli)*, *Salmonella spp, E. cloacae, C. freundii,* coagulase- positive and negative *Staphylococcus*. Park *et al* [42] demonstrated that honeybee venom inhibited the growth of seventeen Gram-positive and partially two Gram-negative out of 44 bacterial strains isolated from bovine mastitis in Korea. Honeybee Venom's antimicrobial action can a result of the presence of several peptides, such as adolapin, apamin, melittin, mast-cell-degranulating peptides, biologically active amines enzymes, and non-peptide components [22]. Where antimicrobial peptides (AMPs) that exhibit antimicrobial activity can be obtained from the poisons of various animals, such as bees [43].

AMPs have a broad antimicrobial spectrum and are not affected by classical mechanisms of resistance to conventional antibiotics. AMPs interact primarily with the lipids of cytoplasmic membranes or cell walls, leading to membrane permeabilization, cell lysis, and death [44]. AMP interaction with the lipid monolayer as described by Brogden [44] can cause peptide aggregation forming pores, lipid and peptide combination forming a toroidal pore, or direct membrane disruption [45]. AMPs work on bacteria at various stages of biofilm, like structure, attachment, and dispersion [46]. Čujová *et al* [47] reported that honeybee venom contained melittin, which is more active against GPB than GNB.

In this study, it was found that Phospholipase A2 did not affect bacterial growth at all. On the other hand, Melittin alone had an effect reaching a 3 mm inhibition zone. Although the effect of melittin on *Streptococcus sp.* is much higher than any type of bee venom as a whole and the effect of melittin is clear even with very low concentrations, there is a non-significant negative correlation between them. Different MIC values were recorded in this study as, *Enterococcus faecium* was 31.31 µg/ml, *Staphylococcus sciuri* = 39.81 µg/ml and *Streptococcus mitis*= 44.39 µg/ml. while the Minimum Bactericidal Concentration (MBC) for the three strains was almost the same at 30 µg/ml.

Concerning the antimicrobial assays of compounds isolated from natural sources, some authors [48, 49] have established MIC value criteria for the determination of their antimicrobial potential. These authors suggested that MIC values lower than 100.0µg/mL is considered very promising in the search for new anti-infection agents.

Kim *et al* [50] employed MIC and Minimum Bactericidal Concentration (MBC) to assess the activity of apitoxin against *S. mutans* (ATCC 25175); they obtained a value of 64 µg/mL in both cases. Here, we used *S. mitis*, one of the main cariogenic bacteria, that participates in the onset of the tooth decay process [38]. The MIC results obtained for the commercially available apitoxin and the apitoxin in nature for *Streptococcus salivarius, S. sobrinus, S. mutans, S. mitis, S. sanguinis, Lactobacillus casei, and Enterococcus faecalis* were close and lay between 20 and 40 µg / mL, which indicated good antibacterial activity [22].

Biofilm production makes the treatment using conventional antibiotics more difficult. Bacteria within biofilms are more resistant than those in the planktonic or sessile state. Studies have shown that biofilm cells can withstand up to 1000 times as many antibiotic concentrations as their planktonic peers and are even prepared to endure in biocidal and UV-exposed settings [51]. This makes it very hard to eradicate them once they have reached their biofilm form [52]. Biofilm production was interpreted as strong, moderate, or low, according to StepanoviĆ *et al* [32]. Values are expressed as the percentage of absorbance (570 nm) of cells in treated wells compared with that in untreated wells (considered to be 100%). Among the five tested isolates, biofilm formation was strong in *Enterococcus faecium* (88%), *Staphylococcus sciuri* (67%), *Streptococcus mitis* (50%), and moderate in the case of *Streptococcus mutans* (34%) with optical densities (O.D.570nm): 1.65, 1.53, 1.2, and 1.21 respectively. Only, *Streptococcus sanginus* gave no biofilm pattern.

Results obtained by Scherr *et al* [53] demonstrated that among the tested isolates biofilm formation was strong in 66.7% of MDR-isolates, namely; *E. cloacae, P. aeruginosa, S. aureus, S. haemolyticus, and E. faecalis.* Bacteria generate biofilm because the virulence factor performs a major part in infection by protecting against many clearance mechanisms. The biofilm a matrix can hinder certain immunological defenses, like macrophages that show unfinished entry into the biofilm matrix and "frustrated phagocytosis" [53].

Biofilm photos were detected by an analytical Scanning Microscope (JEOL, JSM-6360LA, JEOL USA, Inc.) at the central lab. Of City of Scientific Research & Applied Technology showing the effect of different concentrations of bee venom on biofilm formation. Bee Venom sub-MICs nearly totally inhibited *Staphylococcus sciuri and Streptococcus mitis* biofilm by concentrations of 25 and 20 µl of B.V. respectively. These results are in agreement with Sofy *et al* [54] that bee venom exhibited a strong antibiofilm effect against tested MDR-GNB and GPB.

5. Conclusion

In this study, BV was found to inhibit the growth and survival of oral bacterial strains, Gram-positive bacteria are more sensitive, even at lower concentrations of bee venom than Gram-negative bacteria; thus, one can conclude that BV may be an effective complementary antimicrobial agent for use against oral pathogenic bacteria. Moreover, bee venom showed a significantly decreasing effect on bacteria resistant to antibiotics within biofilms more than those in the planktonic or sessile state. Mellitin and phospholipase A2 contents of bee venom are affected by many factors like the season of extraction, time of collection, method of extraction, and the pollen grains that the bee eats. Phospholipase A2 has no antibacterial effect but, mellitin alone has an antibacterial effect more than bee venom as a whole.

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