Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 239-246, 2023
DOI: 10.9775/kvfd.2023.29132

Research Article

Evaluation of Antimicrobial and Antibiofilm Efficacy of Bee Venom and Exosome Against *Escherichia coli* K99 Strain

Demet CELEBI [1] (*) Ozgur CELEBI [2] Sumeyye BASER [2] Ali TAGHIZADEHGHALEHJOUGHI [3]

[1] Ataturk University, Faculty of Veterinary Medicine, Department of Microbiology, TR-25240 Erzurum - TÜRKİYE
[2] Ataturk University, Faculty of Medicine, Department of Medical Microbiology, TR-25240 Erzurum - TÜRKİYE
[3] University of Seyh Edebali, Faculty of Medicine, Department of Pharmacology, TR-11000 Bilecik - TÜRKİYE

ORCIDs: D.C. 0000-0002-2355-0561; O.C. 0000-0003-4578-9474; S.B. 0000-0003-2391-8191; A.T. 0000-0002-3506-0324

Article ID: KVFD-2023-29132 Received: 01.02.2023 Accepted: 28.04.2023 Published Online: 03.05.2023

Abstract: *Escherichia coli* K99 (F5) strain is one of the bacterial agents that cause calf deaths. F5 is an adhesin that allows pathogenic *E. coli* attach to the small intestine cells and colonize there. The presence of F5 in *E. coli* strains in isolated bacteria is classified as Enterotoxigenic. Bee venom and bee venom-derived exosomes are bioactive compounds that exhibit antimicrobial and antibiofilm activity. The aim of this study is to demonstrate the antimicrobial and antibiofilm activity of bee venom and bee venom-derived exosomes against *E. coli*, which cause calf diarrhea. Bee venom-derived exosomes and bee venom effects against *E. coli* strains were determined by using Minimal inhibition concentration (MIC), antibiofilm activity, fractional inhibition concentrations (FIC), and measurement of L929 cells viability ratio. Cell damage was examined under a fluorescent microscope by an immunohistochemical method. In our study, the MIC value of the bee venom-derived exosome was determined as 1.95 µg/mL. A synergistic effect was detected with a value of 0.44 in combinations of amoxicillin with clavulanic acid. Antibiofilm activity was determined at the rate of 48.8% in bee venom, while bee venom-derived exosomes inhibited the biofilm layer by 60.4%. In L929 cell lines, combination groups have been reported to reduce viability. Bee venom-derived exosomes are more effective on bacteria than pure bee venom. In conclusion; It is important that the bee venom-derived exosome, which is a biocompatible molecule and acts as a cargo element, exhibits antimicrobial and especially antibiofilm activity and is an alternative approach against increasing antibiotic resistance.

Keywords: *Antibacterial activity, Antibiofilm activity, Escherichia coli, Exosome, Fractional inhibition concentration, Synergistic effect*

Escherichia coli K99 Suşuna Karşı Arı Zehiri ve Arı Zehrinden İzole Edilen Eksozomun Antimikrobiyal ve Antibiyofilm Etkinliğinin Değerlendirilmesi

Öz: *Escherichia coli* K99 (F5) suşu buzağı ölümlerine sebep olan bakteriyal etkenlerden biridir. F5, patojenik *E. coli* suşlarının bağırsak hücrelerine yapışmasını ve ince bağırsağı kolonize etmesini sağlayan bir adezindir. F5'in varlığı, bakteri izolatının Enterotoksijenik *E. coli* olarak sınıflandırılmasını sağlar. Arı zehiri ve arı zehrinden izole edilen eksozomlar, antimikrobiyal ve antibiyofilm aktivite sergileyen bioaktif bileşiklerdir. Bu çalışmanın amacı buzağı ishaline sebep olan *E. coli,'ye* karşı arı zehiri ve arı zehrinden izole edilen eksozomların antimikrobiyal ve antibiyofilm aktivitesini ortaya koymaktır. *E. coli* suşlarına karşı hem eksozom hem de arı zehirinin minimum inhibisyon konsantrasyonu (MIC), antibiyofilm aktivitesi ve fraksiyonel inhibisyon konsantrasyonları (FIC) ve L929 hücrelerinde canlılık oranları belirlendi. İmmünohistokimyasal olarak hücre hasarı floresan mikroskop altında incelendi. Çalışmamızda arı zehirinden izole edilen eksozomların MIC değeri 1.95 µg/mL olarak tespit edildi. Amoksisilin klavulonik asit ile yapılan kombinasyonlarda 0.44 değer ile sinerjik etki tespit edildi. Antibiyofilm aktivitesi arı zehrinde %48.8 oranında belirlenirken arı zehri eksozomu % 60.4 oranında biyofilm tabakasını inhibe ettiği tespit edildi. L929 hücre hatlarında kombinasyon grupların canlılık oranını düşürdüğü rapor edildi. Arı zehri eksozomları arı zehrinden daha fazla bakteriler üzerinde etkili olmaktadır. Sonuç olarak; biyouyumlu molekül olan ve kargo elemanı olarak görev yapan arı zehiri eksozomunun antimikrobiyal ve özellikle antibiyofilm aktivite sergilemesi artan antibiyotik direncine karşı alternatif bir yaklaşım olması önem arz etmektedir.

Anahtar sözcükler: *Antibakteriyel aktivite, Antibiyofilm aktivitesi, Eksozom, Escherichia coli, Fraksiyonel inhibisyon konsantrasyonu, Sinerjistik etki*

How to cite this article?
Celebi D, Celebi O, Baser S, Taghizadehghalehjoughi A: Evaluation of antimicrobial and antibiofilm efficacy of bee venom and exosome against *Escherichia coli* K99 strain. *Kafkas Univ Vet Fak Derg,* 29 (3): 239-246, 2023.
DOI: 10.9775/kvfd.2023.29132

(*) Corresponding author: Demet CELEBI
Phone: +90 532 666 6938
E-mail: celebiidil@atauni.edu.tr

This article is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0)

Introduction

Resistance to antimicrobials has reached the level of red alert all over the world [1]. Although humanity won a great victory against microorganisms in the early days of the struggle that started with the discovery of antibiotics, this victory gained a great momentum towards defeat with factors such as resistance developed by microorganisms and internal mutations [2]. Unconscious, and excessive use of antibiotics, leads to appear resistance of microorganisms. Biofilm prevented antimicrobial drugs from penetrating at an effective dose [1,2]. This is how they managed to stay alive. In addition, they allowed the spread of mutant strains with their biofilm properties, which are a silent communication community [1,2]. Multi-drug resistance struggle, which threatens human health, has recently started to be seen as a major threat in terms of animal health. Among these factors, *Eschericia coli* strains are among the effective pathogens in the transfer of antimicrobial resistance genes in cattle and milk *E. coli* strains, which cause calf deaths in particular [3,4]. Resistance to β-lactams and fluoroquinolones, which are widely used in human and veterinary fields, causes alternative searches. The resistance to the carbapenem antibiotics in human medicine reveals the importance of its precautions once again. In addition, reasons such as increased resistance with mutant strains and the inadequacy of available antimicrobials led to the search for new antimicrobial candidates with the same mode of action. Among the candidates in these searches, many bee products rich in bioactive compounds were also of interest. Data showing that bee venom (BV) and other natural products exhibit remarkable activity against various diseases have taken their place in the literature [3-6]. Bee venom, called apitoxin, has been used in the treatment of arthritis, rheumatism, pain, cancer, skin diseases and in the field of traditional medicine. Studies have shown that it has anti-inflammatory, antimicrobial and antioxidant activities [7-11]. The peptides determined in the bee venom have antimicrobial activity against some gram-negative and gram-positive bacteria. It has been determined that the bee venom has a synergistic effect, especially in studies conducted with combinations with antimicrobials. The synergy that exists in poison combinations with antibiotics such as vancomycin and amikacin is promising [5,12,13]. Bee venom contains biologically active amines, enzymes, peptides, and non-peptide components. 50% of the dry weight of bee venom is a peptide component called melittin. Melittin is a characteristic component, especially with its strong cytotoxic properties and antimicrobial activity [14]. Exosomes with a double lipid layer and nanoscale membrane vesicles are involved in intercellular signal trafficking involving protein regulation mechanisms. They are secreted from almost all cells and have the characteristics of the cell of origin [15,16]. They were detected in biological fluids, isolated from cell culture media, and have cell-specific cargo properties [17-19]. The cargo molecules in them are composed of lipids, protein, DNA, mRNA, miRNA, and sRNA (small RNA) [19]. In addition to all these cargo elements, exosomes also contain tetraspanins, which play an important role in cell penetration and fusion [20]. Thanks to all these cargo elements that mediate signalling to recipient cells or tissues, exosomes are promising to become a biomarker and therapeutic tool in the treatment of cancer and pathogens with their role in intercellular signalling, cell-cell communication, immune responses, cellular homeostasis and autophagy. Recently, in addition to mortality rates due to various cancer types, the high mortality rates caused by microorganisms with multidrug resistance make it necessary to develop new treatment methods urgently. At this point, more research is needed on the mechanisms of action of exosomes in order to use them as biomarkers in the diagnosis, prognosis, and surveillance of multidrug pathogens. In addition, the antimicrobial properties and carrier capacities of the vesicles need to be determined in order to use drug-delivery vesicles without undesirable side effects. Based on all these concerns and information, we aimed to determine the antimicrobial activity of bee venom and exosome and to examine its antibiofilm ability. We planned to examine the synergistic effects due to the Fractional Inhibitory Concentration Index-Combination FIC indices with antibiotics approved by EUCAST. We designed to investigate the MTT values formed in the cells according to the synergy concentrations and cell damage immunohistochemically.

Material and Methods

Ethical Approval

Since the *E. coli* F82 (O101:K-:F5(K99)+) bacterial strain used in our study is the reference strain, ethical approval is not required.

Bacterial Strain Production

Bacteria to small intestinal epithelial cells K99 (F5) fimbrial antigen in classical Enterotoxigenic *E. coli* isolates isolated from calves is the most commonly detected antigenic structure. *E. coli* F82 (O101:K-:F5(K99)$^+$) strain was stored in trypticase soy agar at room temperature. Standard bacteriological methods were used to isolate and identify the *E.coli* strain. *E. coli* strain was inoculated into Eosin Methylene Blue (EMB) medium and incubated at 37°C for 24 h. Then, 10^8 CFU/mL suspension was prepared from the growing colonies according to McFarland 0.5 chart.

MIC Values

MIC values of bee venom and bee venom-derived exosome compounds against *E. coli* were determined using microdilution method. Bee venom and bee venom-derived exosome were traditionally determined in triplicate by the microdilution broth method. Serial dilutions of both bee venom and bee venom-derived exosome were prepared in microdilution at concentrations ranging from 1028-32 µg/mL. Bacterial colonies prepared according to McFarland 0.5 scale (10^8 CFU/mL) with serial dilutions were inoculated into all wells as 100 µL. Then, 100 µL of Mueller Hinton Broth (MHB) (MilliporeSigma) medium and a bee venom and exosome were added to the wells by dilution. The sample was incubated at 37°C for 24 h. MIC values were determined depending on the formation of agglutination [21].

Biofilm Analysis

The bacteria strain was incubated in MHB medium at 37°C for 18-24 h. Bacterial suspensions were prepared by standardizing them according to the McFarland 0.5 chart; 100 µL were added to the flat-bottomed wells and incubated at 37°C for 24 h. At the end of the incubation period, the wells were washed with distilled water and the cell residues associated with the biofilm were stained with 1% crystal violet (MilliporeSigma) for 37°C for 15 min. Biofilms observed in bacteria were photographed after the excess dye was washed off with water. To quantitatively determine biofilm formation, optical densities were measured on an ELISA reader (Biotek ELX800; BioTek Instruments, Inc.) at OD 570 - OD 630 nm. During the test, sterile TSB was used as a negative control [22]. After these procedures, bee venom and bee venom-derived exosome were added to each well and the antibiofilm activity was determined according to the formula below.

$$SBF = (AB-CW)/G$$

SBF: Specific biofilm formation; AB: Absorbance of 570 nm the attached end stained bacteria; CW: Absorbance of 570 nm of stained control wells containing only bacteria-free medium; G: Absorbance of 630 nm of cell growth in broth

Fractional Inhibitor Concentration Index-Combination (FIC)

When the in-vitro effectiveness of antibiotic combinations based on the Clinical and Laboratory Standards Institute (CLSI) and European Committee for Antimicrobial Susceptibility Test (EUCAST) standards are performed, if the effect is higher than the sum of the effect obtained when the same drugs are used alone, it is synergistic interaction, if it is equal to the sum, additive interaction. It is the test principle in which it is defined as indifference if the result obtained with one drug is equal, and as antagonism if it is lower than the effect of both drugs. Bee venom and bee venom-derived exosome with amoxicillin-clavulanic acid on bacterial strain checkerboard to test the effect of the combination (checkerboard) method was applied. This test is one of the synergy tests based on microdilution. Combination activity of antimicrobial agent has been tested on 96-well plate. 4xMIC and 1/32xMIC dilution was determined. First, each test tube was containing cation-regulated MHB. Graded dilutions from specified concentrations of the agent were prepared. Solutions of amoxicillin clavulanic acid plaque vertically, bee venom and bee venom-derived exosome were placed in the horizontal plane from right to left. Bacterial inoculum 0.5 McFarland ($1x10^8$ CFU/mL) in sterile 0.9% NaCl solution by standard density prepared. The final bacterial concentration in the wells 10 µL was added to each well at a rate of 5 $x10^5$ CFU/mL. Microdilution plates was incubate at 37°C for 24 h.

It was applied according to the FIC index formula used to determine the effectiveness of the combinations. And the results were determined according to the formula.

Calculation of the FIC index:

$$\text{FIC A} = \frac{\text{MIC numerical value of A in the presence of B}}{\text{MIC numerical value of A alone}}$$

$$\text{FIC B} = \frac{\text{MIC numerical value of B in the presence of A}}{\text{MIC numerical value of B alone}}$$

A: Antimicrobial 1 used in combination; B: Antimicrobial 2 used in combination

Σ FIC index = FIC A + FIC B

Σ FIC index ≤ 0.5: synergy

Σ FIC index >0.5 and <1: additive

Σ FIC index ≥1 and 4 ≤: ineffective (indifference)

Σ FIC index >4: antagonism

Bee Venom and Bee Venom-Derived Exosome Isolation

Bee venom New Techniques Laboratory Ltd. (Certificate No: 1543, Batch#1-5, Mtskheta Str. Tbilisi, 0149. Georgia). It was first centrifuged at 1000 g to remove debris. After the exosome isolation kit procedure (Total Exosome Isolation Reagent; Thermo Fisher; Massachusetts, USA) was applied, isolation was performed by centrifugation at 10.000 g for 30 min.

Scanning Electron Microscopes Analysis

The dimensions of the exosomes were evaluated by scanning electron microscopy (SEM) and images were taken under high vacuum and 20 kV EHT with the Carl ZeissEvo 40 SEM device (Jena, Germany).

Cell Culture

The L929 (CCL-1, ATCC) cell line was obtained from the medical pharmacology department of Bilecik Seyh Edebali University (Bilecik, Turkey). Briefly, the cell suspension was centrifuged at 1200 rpm for 5 min. Cells were resuspended in fresh medium (% Dulbeco-modified eagle medium (DMEM-F12), Fetal bovine serum (FBS) 10%, and antibiotic 1% (penicillin, streptomycin, and amphotericin B) and seeded in 25 cm^2 flask (Corning, USA) planted [21].

MTT Test

Control (cell medium only), *E. coli*, Amoxicilin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE (Bee venom-derived exosome) 1.95 µg/mL, Amoxicilin 4 mg/mL + *E. coli*, BV 62.5 µg For the determination of cytotoxicity of /mL + Amoxicilin 4 mg/mL + *E.coli*, BVE 1.95 µg/mL + Amoxicilin 4 mg/mL + *E. coli* groups, 'direct contact test method' will be applied, 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltatrazilium bromide containing MTT material (Sigma Aldrich inc, St.Louis, USA) will be evaluated. In order to determine the cytotoxicity with the MTT test, the mixture to be prepared with 5 mg of MTT powder in 1 mL of PBS will be passed through a sterile 0.20 µm filter (Corning, Wiessbaden, Germany) and kept at +4°C until the time of use, after its outer surface is covered with aluminium foil. After the medium liquids of the incubated cells are withdrawn, the previously prepared samples will be placed in each well and left to incubate again for 24 h at 37°C in an environment containing 5% CO_2. Thus, the cytotoxic effects of the groups at the end of the 24th h will be evaluated. In order to solubilize the formazan crystals formed as a result of the application of MTT, 99.4 mL dimethylsulfoxide (DMSO), 0.6 mL (HCl) and 10 g sodium laurylsulfate (SDS) will be added to the mixture as 100 µL/well and allowed to incubate again for 4 h. After this, the absorbance (optical density) will be measured in a spectrophotometer (µQuant, Bad Friedrichshall, Biotek, CA, United States) at a wavelength of 570 nm.

Immunofluorescence Analysis

Cells cultivated in cell culture were incubated for 30 min in paraformaldehyde solution for 30 min. The cells were then incubated in 3% H_2O_2 for 5 min. 0.1% Triton-X solution was dripped onto the cells washed with PBS and left for 15 min. After the incubation period, protein blocks were dripped onto the cells and kept in the dark for 5 min. Then, the primary antibody (8-OHdG cat no: sc-66036, Dilution Ratio: 1/100 US) was dropped and incubated in accordance with the instructions for use. Immunofluorescence secondary antibody was used as a secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/500. UK) and kept in the dark for 45 min. Then, DAPI with mounting medium (Cat no: D1306 Dilution Ratio: 1/200 UK) was dripped onto the sections and kept in the dark for 5 min, then the sections were closed with a coverslip. The stained sections were examined under a fluorescent microscope (Zeiss AXIO GERMANY) [21].

Statistical Analysis

In order to determine the intensity of positive staining from the pictures obtained as a result of the dyeing; 5 random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program. Data were statistically defined as mean and standard deviation (mean±SD) for % area. Mann-Whitney U test was performed to compare positive immunoreactive cells and immunopositively stained areas with healthy controls. As a result of the test, an AP value of <0.05 was considered significant and the data were presented as mean ± SD.

Results

Microbiological Results

In our study, minimal inhibition concentrations of bee venom and the obtained bee venom-derived exosome were determined against *E. coli* K99 (F5) strain. Amoxicillin clavulonic acid, one of the β-lactam antibiotics, was included in the study as a positive control in the MIC range determined by EUCAST. Minimal inhibition concentration value of bee venom, bee venom-derived exosome and amoxicillin-clavulanic acid against *E.coli* respectively, it was determined as 62.5 µg/mL 1.95 µg/mL and 4000 µg/mL. The MIC concentration of bee venom and exosome against *E. coli* is shown in *Table 1*.

Bee venom, bee venom-derived exosome and antibiotic concentrations prepared according to MIC values were determined by the checkerboard method to determine the FIC index. According to the FIC index formula, the synergistic effect of bee venom and exosome with amoxicillin clavulonic acid was observed. All these values are shown in *Table 2*.

Antibiofilm activity against biofilm ability was measured at a wavelength of 570 nm. And the results are summarized in *Table 3* and *Table 4*. In the results obtained, it was determined that the exosome structure inhibited the formation of biofilm. In our study results, while the antibiofilm activity was determined at the rate of 48.8% in bee venom, it was determined that the bee venom-

Table 1. *MIC values of bee venom, bee venom exosome and antibiotics against reference bacteria strains*

Bacteria Strains ATCC No	Bee Venom MIC µg/mL	Bee Venom Exosome MIC µg/mL	Antibiotics MIC mg/L
E. coli K99 (F5)	62.5 µg/mL	1.95 µg/mL	4 mg/L[a]

[a] *Amoxicillin-clavulanic acid*

Table 2. Results of the checkerboard assay with fractional inhibitory concentration and FIC indices of two-drug combinations

Bacteria Strains ATCC No	Agent	FIC	Interpretation
E. coli K99 (F5)	Bee venom Amoxicillin-clavulanic acid	0.33	Synergy
E. coli K99 (F5)	Bee venom exosome Amoxicillin-clavulanic acid	0.44	Synergy

Table 3. Biofilm OD values for bee venom at 570 nm wavelength

Bacteria Strains ATCC No	Positive Control	Negative Control	Highest OD Value
E. coli K99 (F5)	0.795	0.426	2.700
E. coli K99 (F5) + Bee venom			1.381

Table 4. Biofilm OD values for bee venom exosome at 570 nm wavelength

Bacteria Strains ATCC No	Positive Control	Negative Control	Highest OD Value
E. coli K99 (F5)	0.149	0.079	0.500
E. coli K99 (F5)+Bee venom exosome			0.198

derived exosome inhibited the biofilm layer by 60.4%. *E. coli* ATCC 25922 strain was used as positive control in our study.

Scanning Electron Microscopes Results

The obtained data are shown in *Fig.1*. Looking at the data obtained, it was determined that the particle sizes were between 67.47 nm and 105.9 nm.

MTT Results

Control (cell medium only), *E. coli*, Amoxicillin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE (Bee venom-derived exosome) 1.95 µg/mL, Amoxicillin 4 mg/mL + *E. coli*, BV 62.5 µg. The cytotoxic effects of BV 62.5 µg/mL + Amoxicillin 4 mg/mL + *E. coli and* BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups were determined after 24 h using the MTT method *(Fig. 2)*. *E. coli*, Amoxicillin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE data were compared with the control group. The cell viability rate of the control group was 100%. Amoxicillin 4 mg/mL + *E. coli*, BV 62.5 µg/mL + Amoxicillin 4 mg/mL + *E. coli*, BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups were compared with the *E. coli* group. The *E. coli* group was compared with the control group (## P<0.001). The lowest viability was observed at Amoxicillin 4 mg/mL + *E. coli* (viability rate was 158%) (P<0.05). BV 62.5 µg/mL + Amoxicillin 4 mg/mL + *E. coli*, BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups showed more toxicity (P<0.001) *(Fig. 2)*.

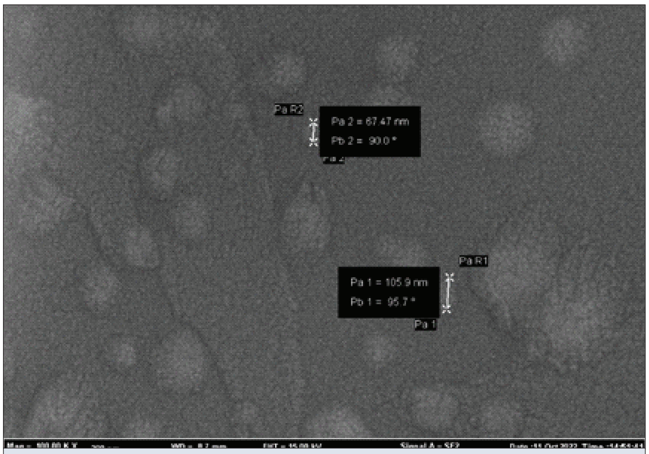

Fig 1. SEM evaluation of bee venom exosomes

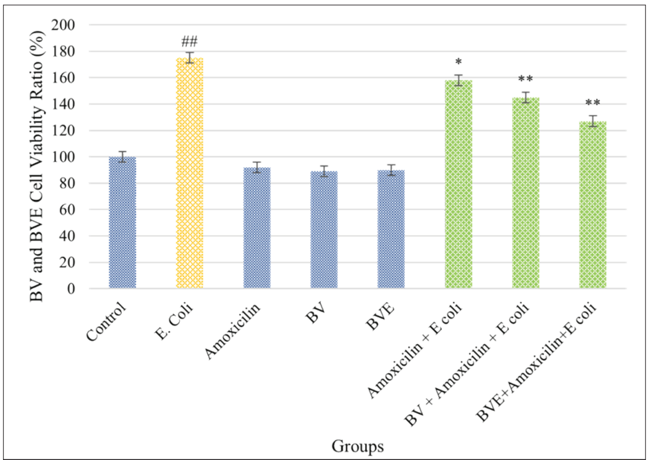

Fig 2. Cell viability rate of L929 cell after 24 hours. Control (cell medium only), *E. coli*, Amoxicillin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE (Bee venom exosome) 1.95 µg/mL, Amoxicillin 4 mg/mL + *E. coli*, BV 62.5 µg Viability rates of /mL + Amoxicillin 4 mg/mL + *E. coli*, BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups are shown. Control (cell medium only), *E. coli*, Amoxicillin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE (Bee venom exosome) 1.95 µg/mL compared with the control group (## P<0.001). The viability rates of Amoxicillin 4 mg/mL + *E. coli*, BV 62.5 µg/mL + Amoxicillin 4 mg/mL + *E. coli*, BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups were compared with the *E. coli* group (*P<0.05, ** P<0.001)

Immunofluorescence Results

Data of immunofluorescent staining results and statistical analysis results are also presented in *Table 5* and *Fig. 3*. Our results were in line with cell culture results.

Discussion

Bee venom (BV) antimicrobial and antibiofilm activity may be due to the presence of various peptides such as melittin, melectin, apamin, adolapin, mast cell degranulating peptides, enzymes, biologically active amines, and non-peptide components [23-25]. There are many studies on the antimicrobial and antibiofilm activity of bee products [26-30]. In this study, we tried to determine the antibacterial and antibiofilm activity of bee venom and exosome, which is a

Table 5. *Data and statistical analysis results of immunofluorescent staining results*

Groups	**8-OHdG**
Control	18.19±2.79[a]
E. coli	71.73±4.5[c]
Amoxi (Amoxicillin)	26.44±1.96[a]
B (BV)	28.55±3[a]
Bexo (BVE)	27.56±2.08[a]
Amoxi (Amoxicillin) + *E. coli*	58.13±2.76[d]
BV (B) + Amoxi (Amoxicilin) + *E. coli*	41.12±4.94[b]
Bexo (BVE) + Amoxi (Amoxicilin) + *E. coli*	29.18±4.46[b]

[a,b,c,d] *different letters in the same column are considered statistically significant difference (P<0.05)*

bee product, against *E. coli* K99 (F5) strain. Keles et al.[31], in their study investigated the etiology and predisposing factors of diarrheal calves from Kayseri province and surrounding provinces between January 2016 and September 2019. 270 newborn calves from diarrhea included in this study. It was determined that 15.6% (42) caused by *E. coli* K99 strain. Alternative treatments are important in diarrhea cases due to the antibiotic resistance of *E. coli* strains. Increasing antibiotic resistance has led to an increase in the search for bee products and alternative treatments [32-36]. Studies have shown that *E. coli* strains show high resistance to antibiotics. Karacan Sever et al.[37] in their study, 99 *E. coli* strains were isolated from poultry. High antibiotic resistance in isolated *E. coli* strains and serotyped *E. coli*. It was determined that O78 was the dominant serotype in strains. Cujova et al.[17] reported that honey BV contains melittin, which is more active against gram-positive bacteria than gram-negative bacteria. In our results, antimicrobial and antibiofilm effects of bee venom and bee venom-derived exosome were determined. The antimicrobial activity of the exosome was 1.95 µg/mL, and the FIC concentration created by the antibiotic showed a synergistic effect of 0.44. In a study, the MIC values of bee venom against *S. salyarius*, *S. sobrinus*, *S. mutans, S. mitis, S. sanguinis, L. casei* and *E. faecalis* were found to be between 20 and 40 µg/mL. Melittin, one of the main components of this poison, showed MIC values ranging from 4 to 40 µg/mL, while the MIC value of PLA2 was found to be over 400 µg/mL [26]. FIC values of bee venom combined with traditionally administered drugs yielded fractional inhibitory concentration (FIC) indices ranging from 0.24 to 0.5. [27] BV and melittin are a potent antimicrobial against Methicillin-resistant *Staphylococcus aureus* (MRSA) at MIC values of 6-800 µg/mL showed activity [28]. In another study, it was determined that both melittin and bee venom had a bactericidal effect on MRSA ATCC 33591 strain [29]. Previously, honey and honey-derived defensin-1 have reported antibiofilm activity on

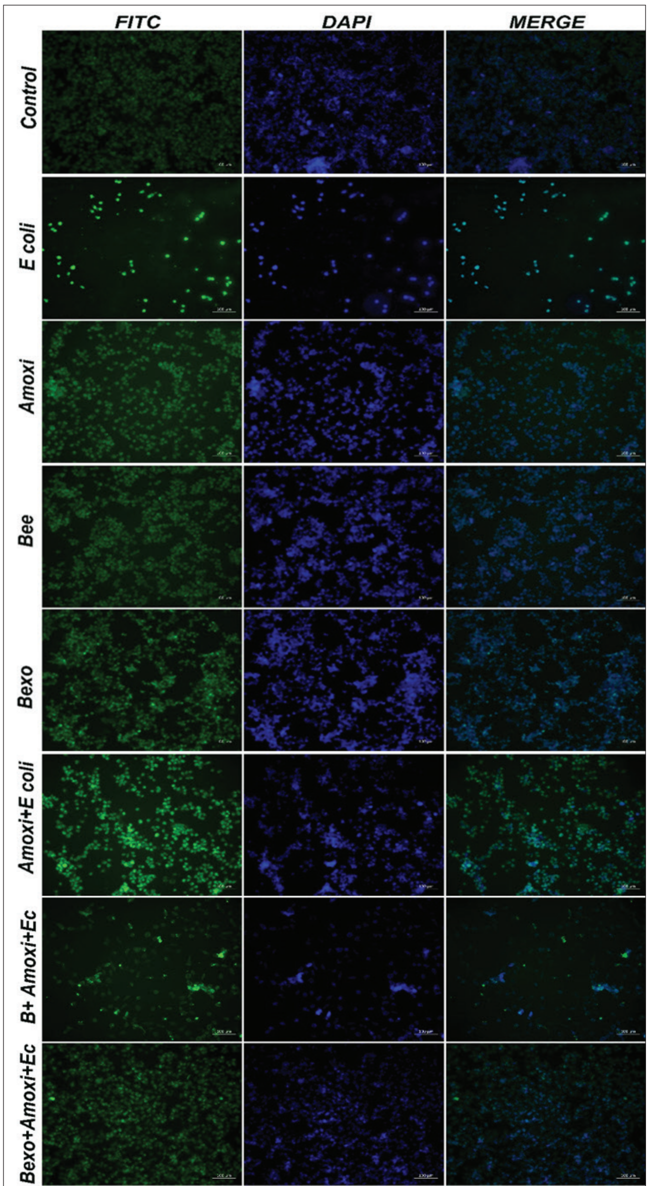

Fig 3. Control, *E. coli*, Amoxi (Amoxicillin) 4 mg/mL, B (BV) 62.5 µg/mL, Bexo (BVE) 1.95 µg/mL, Amoxi (Amoxicillin) 4 mg/mL + *E. coli*, B (BV) 62.5 µg/mL + Amoxi (Amoxicillin) 4 mg/mL + *E. coli*, Bexo (BVE) 1.95 µg/mL + Amoxi (Amoxicillin) 4 mg/mL + *E. coli* groups, 8-OHdG expressions in L929 cells are shown. (FITC), IF Bar:100 µm

wound pathogens [30,32]. In a study by Arteaga et al.[33], a MIC value of 512 µg/mL was determined against *S. enterica* isolated from poultry, and their potential to inhibit biofilm formation was found to be up to 68%. In a study by Elsayed et al.[34], when the antimicrobial activities of *Apis mellifera* venom were examined, it was reported that a MIC value of 15.65 µg/mL was detected in *E. coli* ATCC 8739 strain. In our study, the MIC value of bee venom was determined as 62.5 µg/mL. The MIC value obtained from the exosome of bee venom was determined as 1.95 µg/mL. Considering the damage of *E. coli*, which is the causative agent of calf diarrhea. In addition, the synergistic effect of antibiotic and bee venom-derived exosome adds originality to our study. It is understood that exosomes, which act as a cargo element, which is a bioactive molecule, are effective especially at low concentrations and will be considered as an alternative in the search for new antimicrobials. In the study of Lima et al.[35], *in vitro* and *in vivo* antibacterial and anti-biofilm activities of melittin, a peptide derived from honey bee venom, against uropathogenic *E. coli* were examined and the MIC values were found to be 0.5 to 8 µM. It has also been reported that it degrades the biofilm layer by 39.58%. In a study by Picoli et al.[36], melittin had 40-42.5 µg/mL (~13 µM) MIC and 64-128 µg/mL (~20-40 µM) MBC's against *E. coli* ATCC 8739. In a study by Han et al.[38], it was reported that the MIC of melittin purified from honey bee venom against *E. coli* ATCC 25922 was 0.125 µg/mL (~0.04 µM). The cytotoxicity test with melectin, a component of bee venom, was evaluated using normal human fibroblast cells and it was determined that melectin at 32 µM showed low cytotoxicity, such as 10%, at concentrations below 16 µM. In our study, cytotoxicity was very evident in the toxicity model made with fibroblast cells [39]. Although not, it has been shown to significantly and significantly reduce the bacterial population in co-cultures. Similar results are shown in immunohistochemistry analyses.

Although studies on bee venom and its peptides are presented in the literature, there are no studies on the antibacterial and antibiofilm activity of exosomes obtained from bee venom. It has been determined that bee venom exosome has a synergistic effect when used in combination with antibiotics. It is important that more studies should be done on the cytotoxic effect, which is not seen in studies on cells. We see that bee venom and exosome will shed light on further studies and as a bioactive antimicrobial candidate against increasing antibiotic resistance.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (D. Celebi) on reasonable request.

Funding Support

The authors declared that this study has received no financial support.

Competing Interests

The authors have no conflicts of interest to declare.

Author Contributions

D.C., O.C., S.B. and A.T.: Concept, Design, Supervision, Resources, Materials Data, Collection and/or Processing, Analysis and/or Interpretation, Literature Search, Writing and Critical Reviews

References

1. Rehman K, Fiayyaz F, Khurshid M, Sabir S: Antibiotics and antimicrobial resistance: Temporal and global trends in the environment. **In,** Antibiotics and Antimicrobial Resistance Genes in the Environment. Vol. 1. 7-27, Elsevier, 2020. DOI: 10.1016/B978-0-12-818882-8.00002-4

2. Memariani H, Memariani M, Shahidi-Dadras M, Nasiri S, Akhavan MM, Moravvej H: Melittin: From honeybees to superbugs. *Appl Microbiol Biotechnol,* 103, 3265-3276, 2019. DOI: 10.1007/s00253-019-09698-y

3. Elmonir W, Shalaan S, Tahoun A, Mahmoud SF, Remela EMA, Eissa R, El-Sharkawy H, Shukry M, Zahran RN: Prevalence, antimicrobial resistance, and genotyping of shiga toxin-producing *Escherichia coli* in foods of cattle origin, diarrheic cattle, and diarrheic humans in Egypt. *Gut Pathog,* 13:8, 2021. DOI: 10.1186/s13099-021-00402-y

4. de Verdier K, Nyman A, Greko C, Bengtsson B: Antimicrobial resistance and virulence factors in *Escherichia coli* from Swedish dairy calves. *Acta Vet Scand,* 54 (1):2, 2012. DOI: 10.1186/1751-0147-54-2

5. Han SM, Kim JM, Hong IP, Woo SO, Kim SG, Jang HR, Pak SC: Antibacterial activity and antibiotic-enhancing effects of honeybee venom against methicillin-resistant *Staphylococcus aureus*. *Molecules,* 21 (1):79, 2016. DOI: 10.3390/molecules21010079

6. Shin S, Ye M, Choi S, Park K: The effects of melittin and apamin on airborne fungi-induced chemical mediator and extracellular matrix production from nasal polyp fibroblasts. *Toxins*, 9 (11):384, 2017. DOI: 10.3390/toxins9110348

7. Sobral F, Sampaio A, Falcão S, Queiroz MJR, Calhelha RC, Vilas-Boas M, Ferreira ICFR: Chemical characterization, antioxidant, anti-inflammatory and cytotoxic properties of bee venom collected in Northeast Portugal. *Food Chem Toxicol,* 94, 172-177, 2016. DOI: 10.1016/j.fct.2016.06.008

8. Son DJ, Lee JW, Lee YH, Song HS, Lee CK, Hong JT: Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds. *Pharmacol Ther,* 115, 246-270, 2007. DOI: 10.1016/j.pharmthera.2007.04.004

9. El-Seedi H, El-Wahed A, Yosri N, Musharraf SG, Chen L, Moustafa M, Zou X, Al-Mousawi S, Guo Z, Khatib A: Antimicrobial properties of *Apis mellifera*'s bee venom. *Toxins,* 12 (7):451, 2020. DOI: 10.3390/toxins12070451

10. Mata ÉCGd, Mourão CBF, Rangel M, Schwartz EFJJoVA, Diseases TiT: Antiviral activity of animal venom peptides and related compounds. *J Venom Anim Toxins Incl Trop Dis,* 23:3, 2017. DOI: 10.3390/molecules25102402

11. Somwongin S, Chantawannakul P, Chaiyana W: Antioxidant activity and irritation property of venoms from Apis species. *Toxicon,* 145, 32-39, 2018. DOI: 10.1016/j.toxicon.2018.02.049

12. Al-Ani I, Zimmermann S, Reichling J, Wink M: Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens. *Phytomedicine,* 22, 245-255, 2015. DOI: 10.1016/j.phymed.2014.11.019

13. Han S, Yeo J, Baek H, Lin SM, Meyer S, Molan P: Postantibiotic effect of purified melittin from honeybee (*Apis mellifera*) venom against *Escherichia coli* and *Staphylococcus aureus*. *J Asian Nat Prod Res,* 11, 796-804, 2009. DOI: 10.1080/10286020903164277

14. Jamasbi E, Batinovic S, Sharples RA, Sani MA, Robins-Browne RM, Wade JD, Separovic F, Hossain MA: Melittin peptides exhibit different activity on different cells and model membranes. *Amino Acids,* 46, 2759-2766, 2014. DOI: 10.1007/s00726-014-1833-9

15. Raposo G, Stoorvogel W: Extracellular vesicles: Exosomes, microvesicles, and friends. *J Cell Biol,* 200 (4): 373-383, 2013. DOI: 10.1083/jcb.201211138

16. Cocucci E, Meldolesi J: Ectosomes and exosomes: Shedding the confusion between extracellular vesicles. *Trends Cell Biol,* 25 (6): 364-372, 2015. DOI: 10.1016/j.tcb.2015.01.004

17. Takahashi A, Okada R, Nagao K, Kawamata Y, Hanyu A, Yoshimoto S, Takasugi M, Watanabe S, Kanemaki MT, Obuse C, Hara E: Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. *Nat Commun,* 8:15287, 2017. DOI: 10.1038/s41467-018-06613-3

18. Maia J, Caja S, Strano Moraes MC, Couto N, Costa-Silva B: Exosome-based cell-cell communication in the tumor microenvironment. *Front Cell Dev Biol,* 6:18, 2018. DOI: 10.3389/fcell.2018.00018

19. Zhang Q, Higginbotham JN, Jeppesen DK, Yang YP, Li W, McKinley ET, Graves-Deal R, Ping J, Britain CM, Dorsett KA, Hartman CL, Ford DA, Allen RM, Vickers KC, Liu Q, Franklin JL, Bellis SL, Coffey RJ:

Transfer of functional cargo in exomeres. *Cell Rep,* 27 (3): 940-954, 2019. DOI: 10.1016/j.celrep.2019.01.009

20. Kalluri R: The biology and function of exosomes in cancer. *J Clin Invest*, 126 (4): 1208-1215, 2016. DOI: 10.1172/JCI81135

21. Celebi D, Taghizadehghalehjoughi A, Baser S, Genc S, Yilmaz A, Yeni Y, Yesilyurt F, Yildirim S, Bolat I, Kordali S, Yilmaz F, Hacimuftuoglu A, Celebi O, Margina D, Nitulescu GM, Spandidos DA, Tsatsakis A: Effects of boric acid and potassium metaborate on cytokine levels and redox stress parameters in a wound model infected with methicillin-resistant *Staphylococcus aureus*. *Mol Med Rep*, 26 (3):294, 2022. DOI: 10.3892/mmr.2022.12809

22. Niu C, Gilbert ES: Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. *Appl Environ Microbiol,* 70 (12): 6951-6956, 2004. DOI: 10.1128/AEM.70.12.6951-6956.2004

23. Park JW, Jeon JH, Yoon J, Jung TY, Kwon KR, Cho CK, Lee YW, Sagar S, Wong R, Yoo HS: Effects of sweet bee venom pharmacopuncture treatment for chemotherapy-induced peripheral neuropathy: A case series. *Integr Cancer Ther*, 11 (2): 166-171, 2012. DOI: 10.1177/1534735411413265

24. Kim HW, Kwon YB, Ham TW, Roh DH, Yoon SY, Lee HJ, Han HJ, Yang IS, Beitz AJ, Lee JH: Acupoint stimulation using bee venom attenuates formalin-induced pain behavior and spinal cord fos expression in rats. *J Vet Med Sci,* 65 (3): 349-355, 2003. DOI: 10.1292/jvms.65.349

25. Zolfagharian H, Mohajeri M, Babaie M: Bee venom *(Apis Mellifera)* an effective potential alternative to gentamicin for specific bacteria strains: Bee venom an effective potential for bacteria. *J Pharmacopuncture*, 19 (3): 225-230, 2016. DOI: 10.3831/KPI.2016.19.023

26. Han SM, Lee KG, Yeo JH, Baek HJ, Park K: Antibacterial and anti-inflammatory effects of honeybee *(Apis mellifera)* venom against acne-inducing bacteria. *J Med Plants Res,* 4, 459-464, 2010. DOI: 10.5487/TR.2012.28.1.001

27. Leandro LF, Mendes CA, Casemiro LA: Antimicrobial activity of apitoxin, melittin and phospholipase A2 of honey bee *(Apis mellifera)* venom against oral pathogens. *An Acad Bras Cienc,* 87, 147-155, 2015. DOI: 10.1590/0001-3765201520130511

28. AL-Ani I, Zimmermann S, Reichling J, Wink M: Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens. *Phytomedicine,* 22, 245-255, 2015. DOI: 10.1016/j.phymed.2014.11.019

29. Flávia A, Pereira M, Albano M, Cristina F, Alves B, Fernanda B, Teles M, Furlanetto A, Mores VL: Influence of apitoxin and melittin from *Apis mellifera* bee on *Staphylococcus aureus* strains. *Microb Pathog*, 141:104011, 2020. DOI: 10.1016/j.micpath.2020.104011

30. Sojka M, Valachova I, Bucekova M, Majtan J: Antibiofilm efficacy of honey and bee- derived defensin-1 on multispecies wound biofilm. *J Med Microbiol,* 65, 337-344, 2016. DOI: 10.1099/jmm.0.000227

31. Keleş İ, Ekinci G, Tüfekçi E, Çitil M, Güneş V, Aslan Ö, Onmaz AC, Karaca Bekdik İ, Varol K, Deniz: Etiological and predisposing factors in calves with neonatal diarrhea: A clinical study in 270 case series. *Kafkas Univ Vet Fak Derg*, 28 (3): 315-326, 2022. DOI: 10.9775/kvfd.2021.26981

32. Majtan J, Bohova J, Horniackova M, Klaudiny J, Majtan V: Anti-biofilm effects of honey against wound pathogens *Proteus mirabilis* and *Enterobacter cloacae. Phytother Res*, 28, 69-75, 2014. DOI: 10.1002/ptr.4957

33. Arteaga V, Lamas A, Regal P, Vázquez B, Miranda JM, Cepeda A, Franco CM: Antimicrobial activity of apitoxin from *Apis mellifera* in *Salmonella enterica* strains isolated from poultry and its effects on motility, biofilm formation and gene expression. *Microb Pathog,* 137:103771, 2019. DOI: 10.1016/j.micpath.2019.103771

34. Elsayed K, Bakhiet Hosni AM, Elshehaby HM: *Apis mellifera* venom inhibits bacterial and fungal pathogens *in vitro*. *Pak J Biol Sci*, 25, 875-884, 2022. DOI: 10.3923/pjbs.2022.875.884

35. Lima WG, Batista Filho FL, Lima IP, Simião DC, Brito JCM, da Cruz Nizer WS, Cardoso VN, Fernandes SOA: Antibacterial, anti-biofilm, and anti-adhesive activities of melittin, a honeybee venom-derived peptide, against quinolone-resistant uropathogenic *Escherichia coli* (UPEC). *Nat Prod Res,* 36 (24): 6381-6388, 2022. DOI: 10.1080/14786419.2022.2032047

36. Picoli T, Peter CM, Zani JL, Waller SB, Lopes MG, Boesche KN, Vargas GDÁ, Hübner SO, Fischer G: Melittin and its potential in the destruction and inhibition of the biofilm formation by *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* isolated from bovine milk. *Microb Pathog,* 112, 57-62, 2017. DOI: 10.1016/j.micpath.2017.09.046

37. Karacan Sever N, Şahan Yapıcıer Ö, Akan M: Distribution of serotypes and antibiotic resistance of avian pathogenic *Escherichia coli* strains isolated from chickens. *Kafkas Univ Vet Fak Derg*, 28 (6): 767-772, 2022. DOI: 10.9775/kvfd.2022.28271

38. Han S, Yeo J, Baek H, Lin SM, Meyer S, Molan P: Postantibiotic effect of purified melittin from honeybee *(Apis mellifera)* venom against *Escherichia coli* and *Staphylococcus aureus*. *J Asian Nat Prod Res*, 11 (9): 796-804, 2009. DOI: 10.1080/10286020903164277

39. Ko SJ, Park E, Asandei A. Choi JY, Lee SC, Seo CH, Luchian T, Park Y: Bee venom-derived antimicrobial peptide melectin has broad-spectrum potency, cell selectivity, and salt-resistant properties. *Sci Rep,* 10:10145, 2020. DOI: 10.1038/s41598-020-66995-7