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An *in vitro* antimicrobial activity of *Beauveria bassiana* secondary metabolites

Adil Aksoy[1*], Saif M. Dmour[2], Haitham Qaralleh[3], Waleed S. Alwaneen[4], Reem M. Farsi[5], Roaa M. Alreemi[6], Mahmoud W. El Hindi[7], Farida N. Mosleh[7], Sabreen El Mazloom[7], Dalia Sukmawati[8] and Almira M. Priskaningrum

[1]Eskil Vocational High School, Laboratory Veterinary Science, Aksaray University, Aksaray, Turkey; [2]Department of Medical Analysis, Princess Aisha Bent Al-Hussein College of Nursing and Health Sciences, Al Hussein bin Talal University, Jordan; [3]Department of Medical Laboratory Sciences, Faculty of Science, Mutah University, Mutah, Karak, 61710, Jordan, [4]Advanced Agriculture & Food Technologies Institute, King Abdulaziz City for Science and Technology (KACST), Riyadh, Saudi Arabia; [5]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia; [6]Department of Biochemistry, College of Science, University of Jeddah, Jeddah, Saudi Arabia; [7]Department of Biotechnology, Faculty of Science, Islamic University of Gaza, Gaza, Palestine;[8] Department of Biology, Faculty of Mathematics and Natural Science, Universitas Negeri Jakarta, Jakarta, Indonesia
*Corresponding author's e-mail: adilaksoy@aksaray.edu.tr

Fungi are organisms that provide various environmental services. *Beauveria (B.) bassiana* (Order: Hypocreales, Family: Cordycipitaceae) is an example of an entomopathogenic fungus developed and used for many agricultural, biomedical, and medicinal purposes. A crude extract of *B. bassiana* was made using a culture broth to test its antimicrobial effects against several animal pathogenic bacteria, comprising *Klebsiella (K.) pneumoniae*, *Escherichia (E.) coli*, and *Staphylococcus (S.) aureus* and fungi (*Candida (C.) albicans*). In 250 mL Erlenmeyer flasks, a liquid median (100 ml) was infected with 1 ml of an aqueous conidial suspension (10^7 conidia/ml). The flasks were incubated at 27.2°C on a rotary shaker (180 rpm) for seven days. The fermented broth was filtered to remove the biomass and obtain a crude extract of *B. bassiana* at various concentrations, which was later used for the disk diffusion method. Results demonstrated that the crude extracts effectively against the tested microorganisms and fungi. The *B. bassiana* extract showed an effect against *K. pneumoniae, C. albicans, S. aureus*, and *E. coli* with inhibition zones measuring 22, 23, 25, and 27 mm in diameter, respectively. The regression analysis indicated that each concentrate increase in *B. bassiana* culture filtrate showed a significant increase in the inhibition zone by 0.2342, 0.2330, 0.1909, and 0.2010 mm for the groups of *S. aureus, E. coli, K. pneumoniae*, and *C. albicans*, respectively. The amplification of the BbbsIS gene produced a single fragment of an approximate length of 156-190 bp. This study confirmed that the antimicrobial drug could be produced from *B. bassiana* extracts to prevent many diseases associated with microbial and fungal infections. It also developed an approach constructed by absolute PCR quantification for the expression of genes encoding the synthetase enzymes of the secondary metabolites *viz.*, bassianolide (*BbbslS*), beauvericin (*BbbeaS*), and B-tubulin during the initial times of contagion.

Keywords: Beauveria bassiana, antimicrobial activity, secondary metabolites, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus.

INTRODUCTION

Fungi are a major group of living organisms critical to the ecosystem. Moreover, those microorganisms have a significant impact (negatively or positively) on humans, animals, plants and others due to their secreting many secondary metabolites used in different economic values (Brakhage, 2013; Seyedsayamdost, 2019). Secondary metabolites of fungi have a lower molecular weight with multifunction in medicine, biopharmaceutical, biopesticides, plant growth-promoting agents, and other molecules/drugs significant to animal health (Singh *et al.*, 2017).

Beauveria (B.) bassiana is an entomopathogenic fungus (EF) that is widely applied for several agricultural purposes. Moreover, *B. bassiana* is an endophytic fungus that can live in plant materials via synergistic effects without triggering impairment for plants (Alagawany *et al.*, 2014; Saeed *et al.*, 2017, 2018; Abdel-Moneim *et al.*, 2020; Abd El-Hack *et al.*, 2021; Ávila-Hernández *et al.*, 2022). The secondary metabolites produced by *B. bassiana* act as potential biopesticide agents due to their effective antimicrobial

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actions. The main active molecules like bassianolide, oosporein, oxalic acid, beauvericin, bassianin, and tenellin are produced by *B. bassiana* (Molnar *et al.*, 2010). Based on the unique contents presented in *B. bassiana*, it has been widely used in fighting insect pests with significant insecticidal activity (Srivani and Jalaja, 2022). These modules can provide several biological activities for *B. bassiana*, such as antibacterial, anti-inflammatory, and antifungal properties in infected plants and animals (Tsoupras *et al.*, 2022). At the same time, studies on *B. bassiana* have focused on animal pathogenic bacteria (mainly *K. pneumoniae*, *E. coli* and *S. aureus*), which are generally restricted and need explorations (Liu *et al.*, 2022).

Animal pathogens cause main diseases in wild and domestic animals, producing huge economic losses. In invasive biology, the word pathogen indicates infectious microorganisms such as *C. albicans, K. pneumoniae, S. aureus* and *E. coli* and eliminates nonliving agents such as toxicants and toxins. Introducing novel pathogens into districts occupied by vulnerable host species menaces local wildlife, perturbs animal feeding ecosystems, and endangers animal health (Swartz, 2002). Among these, the bioactive molecules isolated from *B. bassiana viz.* beauvericin, bassianolide, enniatins, and isarolides display insecticidal activity (Srivani and Jalaja, 2022). Thus, performing biological control using *B. bassiana* is essential to accurately identify the isolates used (Coates *et al.*, 2002). Identification is required to measure the effectiveness of the fungal isolates and the detection of multiple infections in the host.

In genetic resource management, several molecular techniques are used to study genetic variability as a complementary tool to more conventional approaches (Keller, 2015). Scientists developed a method based on PCR quantification, which demonstrates the induction of genes that encode the synthetase enzymes of bassianolide (BbbslS), beauvericin (BbbeaS) and β-tubulin (Baldiviezo *et al.*, 2020). The β-tubulin gene encodes tubulins, which are structural components of microtubules, perhaps to be used as virulence factors, and then in moribund insects and/or cadavers to protect them from competitive microorganisms. Microtubules are straight hollow tubular structures with a wall of 13 columns of globular tubulins. Tubulin molecules are composed of two similar polypeptide subunits. Microtubules provide the molecular basis for developing and maintaining cell form, chromosomal segregation, cell division, flagellar cell motility, and intracellular transport. Tubulins are among the best-conserved proteins in eukaryotes (Wade, 2007). This work aimed to explore the antifungal and antibacterial abilities of *B. bassiana* secondary metabolites against selected bacterial pathogens (*C. albicans, E. coli, S. aureus* and *K. pneumoniae*).

MATERIALS AND METHODS

***Chemicals*:** All substances applied in this research, including Mueller Hinton agar (MHA), potato dextrose broth (PDB), Sabouraud dextrose agar (SDA), Mueller Hinton broth (MHB), Sabouraud dextrose yeast broth (SDYB), potato dextrose agar (PDA), and dimethylsulphoxide (DMSO), RPMI 1640 medium and distilled water were purchased from Liofilchem Com. (Roseto degli Abruzzi, Italy).

***Microorganisms*:** The bacterial and fungal microorganisms used in the susceptibility investigation (*E. coli*, *K. pneumoniae*, *S. aureus*, *B. bassiana*, and *C. albicans*) were acquired from Microbiology Laboratory, Islamic University, Gaza, Palestine.

***Preparation of B. bassiana metabolites*:** After being scraped, fourteen days-old PDA dish was immersed in a Tween-80 solution (0.05%) to obtain *B. bassiana* conidia. After vigorous agitation, mycelial debris was discarded via the filtration method through the cotton membrane. Once the conidia were counted, fifty microliters of the conidial suspension (1×10^7 conidia/ml) were inoculated into SDYB (20 ml) in an Erlenmeyer flask (100 ml) and cultured at 25-28°C. After a 10 min period, centrifugation was performed for 20 min at 13 X 10^3 rpm. Following removing the pellet, the supernatant was passed through a membrane filter (0.45 µm, ADVACTEC No. 2) to detach the crude extract from the spores and mycelia. Until being used for antibacterial testing, the fungal cell-free culture filtrates were maintained at -70°C (Shin *et al.*, 2013).

The tests of antimicrobial activity

***The agar disk diffusion (ADD) technique*:** The antimicrobial action of the fungal extract (2000-1.9) µg/ml was detected using the ADD procedure. For this purpose, pathogens were suspended with MHB and SDA. After solidification, 6-mm sterile filter paper disks saturated with 200 mg/ml of the B. bassiana crude extract were placed on the appearance of an agar dish. Following incubation duration of 24 h at 37°C for bacterial growth and 24-48 h at 37°C for fungal growth, the antimicrobial activity of the fungal extract was evaluated by assessing the inhibition zones surrounding the disks (Avila-Hernandez et al., 2022).

***Minimum inhibitory concentrations (MICs)*:** The antimicrobial activity of the fungal extract contra the selected animal pathogenic bacteria were determined using the broth microdilution method and MICs (minimum inhibitory concentrations). A range of different concentrations of the fungal extract was prepared with a sterile diluent (MHB) (2000-1.9) µg/ml. Next, the wells of the 96 well-plates, excluding those of the positive control, were added 10 µl of bacterial suspension, and the mixture was incubated at 37°C for 24 h for complete growth. Eighteen hours later, as an indicator, 50 µL of TTC (2,3,5 triphenyl tetrazolium chloride, 0.01%) was added. Following the addition of TTC, the plates were preserved for one hour in wells with bacterial growth

Table 1. **The primer sequences used for *BbbeaS, BbbslS* and β-tubulin analyses.**

Primer	Sequences 5'...3'	Amplified region	Amplicon (bp)	Reference
BbbeaS1	5'-GTTCTTCCTCCGCATTCCGTTC-3'	BbbeaS	97	Baldiviezo *et al.*
BbbeaS2	5'-TAGAGCGCAACGTCTTTCGGTC-3'			(2020)
BbbslS1	5'-CAATCGACTGAGACGCCATTCC-3'	BbbslS	156-190	
BbbslS2	5'-TTTGACCTGCGAATCCATACGG-3'			
Bt2a	5'-GGTAACCAAATCGGTGCTGCTTTC-3'	β-tubulin	500	Glass and
Bt2b	5'-ACCCTCAGTGTAGTGACCCTTGGC-3'			Donaldson (1995)

based on the method of Adwan *et al.* (2009) .The reduction of TCC by bacteria resulted in red color, while no color change in the wells after 1 h of incubation with TTC implies inhibited bacterial development (Tsoupras *et al.*, 2022).

The antifungal assay: Excluding those assigned to the positive control, each well was strengthened with 100 µl of a *C. albicans* suspension, and the plate was kept at 37°C for 24 h for biofilm creation. After ending the incubation duration, the wells were washed with PBS (phosphate buffer saline) to detach the median. A range of different concentrations of the fungal extract (2000-1.9) µg/ml was obtained by dilution with a sterile RPMI 1640 medium. Once these concentrations were fortified into the wells, the dishes were put for 48 h at 37°C. As described by the procedure of Pierce *et al*. (2010), the lowest levels that suppressed the visible growth of *C. albicans* were determined as the MICs.

***Molecular identification of fungi*:** The extraction of genomic DNA from *B. bassiana* was performed using the adjusted chemical analysis technique. A portion (fifty micrograms) of crushed mycelia was applied for genomic DNA isolation. The remainder of the specimens were kept at –20°C. Fungal DNA was excerpted via the DNeasy Plant Mini Kit (QIAGEN), as recommended by the manufacturer. Until being used as a PCR template, the isolated DNA was kept at –20°C (Wang *et al.*, 2022). In addition, primers design and PCR conditions for *B. bassiana*, the secondary metabolites bassianolide (BbbslS), beauvericin (BbbeaS), and β-tubulin were amplified by the PCR (polymerase chain reaction). Moreover, the primers used are presented in Table 1.

The PCR amplification of the β-tubulin gene was performed by a thermocycler, applying the subsequent circumstances and starting denaturation at 94°C for 3 min; 35 thermocyclers, each involving firstly 1 min at 94°C and then 1 min at 57°C for annealing, 2 min at 72°C for extension, and 5 min at 72°C for a final extension as described by Wang *et al.* (2022). The PCR amplification of the BbbeaS and BbbslS genes was performed in a thermocycler as follows: denaturation at 95°C for 10 min and 40 cycles of a three-segment amplification (firstly 30 s at 95°C for denaturation, secondly 30 s at 55°C for annealing, and thirdly 30 s at 72°C for extension).

Visualization of PCR products. The PCR products were visualized by gel electrophoresis on 1.5-2% agarose gels stained with ethidium bromide. For this purpose, the gel was run at 80 V in 1X TAE buffer (45 mM Tris base, 45 mM acetic acid, 1 mM EDTA). The size of the PCR products was estimated using a 100-bp DNA standard ladder (Invitrogen, CA, USA). A photo-documentation device took gel images.

Data analysis. Data were edited in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The relationship between the inhibition zone and the different concentrations of the *B. bassiana* culture filtrate was fitted by regression analysis (Proc REG; SAS, 2012). Figures were fitted using Graph-Pad Prism software 5.0 (Graph Pad, USA). Statistical significance was set at a p-value less than 0.05.

RESULTS

***The bioactivity of the crude extract by the ADD method*:** The consequences presented in Table 2 show the antimicrobial action of the crude extract against the tested microorganisms, as evaluated by the ADD method. The *B. bassiana* extract showed an effect against *K. pneumoniae*, *C. albicans*, *S. aureus*, and *E. coli*, with inhibition zones measuring 22 mm, 23 mm, 25 mm, and 27 mm in diameter, respectively.

The bioactivity of the crude extract by the micro-dilution method: TFmlhe MIC data shows that the entire selected

Table 2. Testing the antibacterial activity of the *B. bassiana* culture filtrate against some selected pathogens (zone of inhibition values in mm).

Conc. (µg/ml) / Test Organisms	10	20	30	40	50	60	70	80	90	100	150	P-G +	Strp -
S. aureus	-	-	-	-	-	-	14	17	20	22	25	15	-
E. coli	-	-	-	-	11	13	14	15.5	17	25	27	-	25
K. pneumoniae	-	-	-	-	7	9	11.5	12	15.5	19	22	-	20
C. albicans	-	-	-	-	-	-	-	15	16	18	23	-	-

– The concentration of secondary metabolites of *B. bassiana*: 10-150 µg/mL.
– Positive control of bacteria: Penicillin G (P-G) and Negative control of bacteria: Streptomycin.
– Control of fungi: Clotrimazole (100 µg/ml)

concentrations of the crude extract of *B. bassiana* displayed antimicrobial activity against *S. aureus, K. pneumoniae*, *C. albicans*, and *E. coli*, with MIC values extending between 62.5 and 125 µg mL^{-1}. The MIC of the *B. bassiana* crude extract was 62.5 µg mL^{-1} for *E. coli* and *K. pneumoniae*, and 125 µg mL^{-1} for *S. aureus* and *C. albicans.*

Molecular Characterization of B. bassiana

PCR amplification of beauvericin (BbbeaS): As presented in the current research, the PCR technique was used for detecting the successful amplification of Bbbeas gene for *B. Bassiana* (Plate 1, A). Depending on the primer-isolate combination, the *BbbeaS* gene band was scored as 96 bp, which agrees with previous results (Baldiviezo *et al.*, 2020). This gene was absent in *Aspergillus niger*, which was used as the negative control strain.

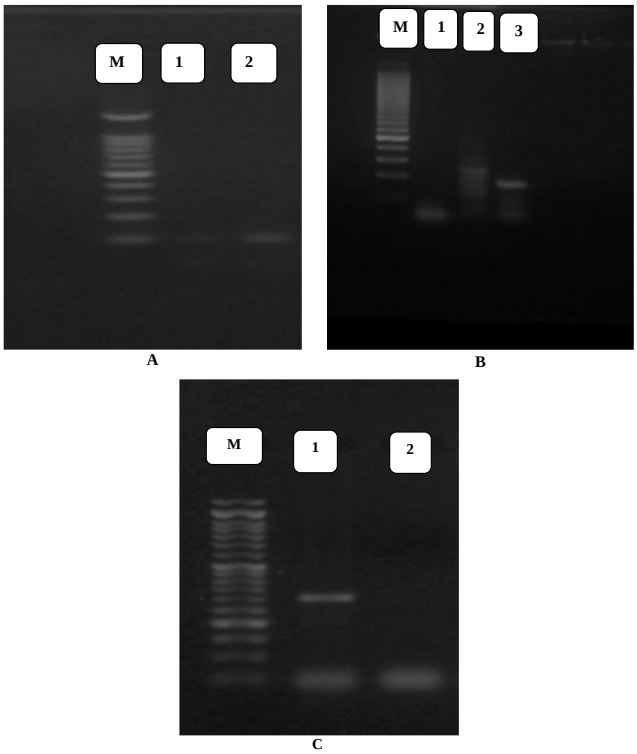

Figure 1. Fragment sizes of the PCR-amplified BbbeaS gene, as obtained by gel electrophoresis. A. In the image, products as compared to bands from a 100 bp DNA ladder (M) are shown. L1: Negative control *Aspergillus niger*, L2: BbbeaS gene for *B. bassiana* 96 bp. B. In the image, products as compared to bands from a 100 bp DNA ladder (M) are shown. L1: Negative control *Aspergillus niger*. L2 and L3: BbbsIS gene for *B. bassiana* 156-190 bp. C. In the image, products as compared to bands from a 100 bp DNA ladder (M) are shown. L1: Bt gene for *B. bassiana* 570 bp & L2: Bt gene for *Aspergillus niger* as a negative control.

PCR amplification of bassianolide (BbbslS): The amplification of the *BbbsIS* gene produced a single fragment of an approximate length of 156-190 bp, as shown in Plate 1, B. These marks correspond with previous research (Baldiviezo *et al.*, 2020). Regarding the β-tubulin gene, results signalized that it was successfully amplified for two fungus species. This amplified region in the *B. bassiana* isolate measured 570 bp in length and is shown in Plate 1, C.

DISCUSSION

B. bassiana is a filamentous fungus with significant impacts through its high source of natural molecules. The wide array of biologically active compounds (Pedrini *et al.*, 2022), including those with antimicrobial properties, are used in medicine, such as bassiatin, beauvericin, and tubulin, as described in the present work. Moreover, efforts to produce novel antibiotics have been ongoing for the past three decades; antimicrobial resistance has increased over the years (Dymarska *et al.*, 2018; Wang *et al.*, 2022; Tsoupras *et al.*, 2022). Thus, natural remedies have started to attract more attention. The medicinal mushroom *B. bassiana*, a source of several biologically active substances for treating diseases, offers an alternative that is not harmful to animal health.

In line with our results, Yin *et al.* (2020) observed a potent antimicrobial activity of beauvericin molecules isolated from *B. bassiana.* To enhance the efficacy of antimicrobial activity, the synergistic effects of *B. bassiana* and AgNPs were performed (Tyagi *et al.*, 2019). As reported by the last investigators, *B. bassiana* showed significant effects contra some animal pathogens bacteria such as *E. coli* and *S. aureus.* These natural molecules produced by *B. bassiana* are eco-friendly tool for combating animal pathogenic bacteria. Thus, they can be applied in various sectors where safety is the primary defense for sustaining animal health (McKinnon *et al.*, 2017; Pedrini *et al.*, 2022).

Previous reports have laid more ability to effectively suppress gram-negative bacteria (GNB) and gram-positive bacteria (GPB) (Sahab, 2012) and exhibited antifungal activity (80 and 150 µg/ml) against *C. albicans.* Parine *et al.* (2010) stated that the antibacterial properties of the crude extract of *B. bassiana* might be associated with its significant contents of several active compounds such as bassianolide, oosporein, oxalic acid, beauvericin, bassianin, and tenellin. The molecule of beauvericin, is a well-known as insecticidal virulence element, which also has considerable antimicrobial action against many pathogenic bacteria (Patocka, 2016; Yin *et al.*, 2022).

Hence, it is a potential candidate for medicinal and pesticide development (Vega, 2018). Our *in vitro* results suggested that the crude extract shows an acceptable level of efficacy in controlling the growth of several pathogens, but this needs to be confirmed by *in vivo* experiments. This study provides data on the genetic variability of *B. bassiana*, an entomo-pathogenic fungi (EPF) strain from the Gaza Strip in Palestine. Multiple molecular markers have been used to assess the genetic variability of *B. bassiana* and identify its distinct isolates. Studies showed that the BbbeaS gene is

responsible for the biosynthesis of beauvericin in *B. bassiana* (Pedrini *et al.*, 2022). At the same time, the down-regulation of this gene could produce a bassiatin molecule (Yu *et al.*, 2017). The PCR amplification of different regions of rDNA has greatly facilitated the classification of fungi. The taxonomic identity of *B. bassiana* has been confirmed based on molecular analyses (Baldiviezo *et al.*, 2020).

The virulence of EPF depends on the genetic composition of strains, which determines their adaptability to different ecological and geographical conditions and the expression of bioactive compounds. This study verified the production of the secondary metabolites bassianolide (*BbbslS*), beauvericin (*BbbeaS*), and β-tubulin by *B. bassiana* isolate on a medium supplemented with their respective substrates against bacteria. This wide variety of metabolites could be associated with the genetic variations and adaptive properties of the genes encoding bioactivity in *B. bassiana* (Aynalem *et al.*, 2021). The differential antifungal activity of the crude extract of *B. bassiana* may depend on the specific bioactive compounds this fungus produces. Hence, the present study suggests testing against more organisms to identify the antibacterial compounds produced by *B. bassiana* (Parine *et al.*, 2010). Yin *et al.* (2022) suggested that the most active compounds presented by *B. bassiana* are involved by the BbbeaS, and BbbslS genes as discovered via Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway screening. Following this hypothesis, previous reports indicated that insect mortality was significantly decreased when its biosynthetic gene BbBeas was disrupted in *B. bassiana*, presenting their significant role in the production of active compounds.

Conclusion: This study proved the efficiency of the *B. bassiana* crude extracts against some animal pathogenic bacteria. Moreover, the results confirmed that the antimicrobial drug could be produced from *B. bassiana* extracts to prevent many diseases associated with microbial infections. The same study may develop a methodology based on absolute PCR quantification for the expression of genes coding the synthesis enzymes of the derivative metabolites *viz* BbbeaS (beauvericin), BbbslS (bassianolide) and B- tubulin during the first times of infection, because of the possibility of these metabolites being used as virulence factors by pathogenic bacteria.

***Conflict of interest*:** The researchers declare no conflict of interest.

***Author's contributions statement*:** The authors declare that they have contributed to the article at a similar rate.

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