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Characterization and pathogenicity of *Beauveria bassiana* strains isolated from *Galleria mellonella* L. (Lepidoptera: Pyralidae) in Turkey

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Abstract

Background: The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), is among the most important wax pests economically. In the larval stage, the pest feeds on honeycomb wax and seriously damages the combs that were left unattended by the bees. Recently, the interest in ecologically safer alternatives to chemical insecticides has increased due to the significant success achieved with entomopathogenic microorganisms in the control of several lepidopteran pest larvae with minimum or no harm to the bees, natural rivals, animals, and humans. The current study was conducted to investigate the pathogenicity of two entomopathogenic fungus isolates (*Beauveria bassiana* G-A and G-B) isolated from dead *G. mellonella* larvae and their efficacy in pest control under laboratory conditions.

Results: Morphological and molecular identification revealed that the two isolates corresponded to *B. bassiana* species G-A and G-B strains. The response to the inoculation with the two fungal strains was conducted on *G. mellonella* larvae at $1 \times 10^{5-9}$ conidia/ml concentrations. The concentrations led to 96.54 and 89.66% mortality in G-A and G-B *B. bassiana* isolates at the highest concentration on day 10, respectively. LC_{50} was calculated between 0.2×10^6 (0.03 – 1.6) and 0.6×10^6 (0.07 – 6.1) conidia/ml.

Conclusion: The present study findings demonstrated that these isolates had the potential for *G. mellonella* control and *B. bassiana* isolates were a safe alternative to chemical control and could be recommended for use to protect stored wax products.

Keywords: *Galleria mellonella*, *Beauveria bassiana*, Biological control, Potential

Background

Several organisms such as the greater wax moth (GWM), *Galleria mellonella* (Fabricius, 1798, Lepidoptera: Pyralidae) (Słowińska et al. 2019), could harm the honeybees. It is the most severe pest that could infest honeybee hives globally. It attacks bee colonies and stored wax combs, leading to significant damage. Female adult moths deposit their eggs in honeycomb's wood cracks in beehives or in warehouses where beekeepers store the honeycombs (Firacative et al. 2020).

In the past, beekeepers in several countries employed chemicals to control this pest in honeycombs (Karazafiris et al. 2011). However, these chemicals could be hazardous to humans due to the chemical residues in honey and beeswax (Dubey et al. 2014). Various agents have been used in the biological control of *G. mellonella*. Currently, these agents include certain commercial bacteria and parasitic wasps (Kwadha et al. 2017). Data are available on other natural predators of GWM in the literature

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(Adly and Marzouk 2019). Certain biological control agents such as entomopathogens (viruses, bacteria, nematodes, and fungi) have been tested on this pest (El Husseini 2020).

Entomopathogenic fungi (EPFs) are one of highly specialized microorganisms that could infect and grow on arthropods (Vega 2018). EPFs such as *Beauveria bassiana* and *Metarhizium anisopliae* are widely used in the control of several insects (Butt et al. 2001). *Beauveria bassiana* (Balsamo) (Ascomycota: Hypocreales: Clavicipitaceae) is a common fungus with effects on a wide variety of insect species and is used as a biological pesticide (Xiong et al. 2013). Several studies have been published on the efficacy of EPF against *G. mellonella* (Abou-Shaara 2020).

The aim of the present study was to characterize and analyze the pathogenicity of two EPFs isolated from the dead *G. mellonella* larvae collected from various beehives in Turkey.

Method

Dead *G. mellonella* larvae were collected from the beeswax directly from beekeeper hives and transferred to the laboratory. Fungi were isolated from dead larvae. *G. mellonella* larvae used in bioassays were collected from laboratory cultures.

Isolation of the fungi

Fungi were isolated from *G. mellonella* larvae mycoses that were collected from the beehives and transferred to potato dextrose agar medium with 1% yeast extract (PDAY medium, Merck, Darmstadt, Germany) that included 40 µg/ml chloramphenicol to prevent bacteria growth. After the fungi were purified in the PDAY medium, the spore suspensions that were prepared with a single colony were stocked and stored at − 80 °C.

Morphological identification

The initial identification was conducted by observing the fungal infection in larvae and colony morphology in the medium. Fungal isolates that were isolated from insect samples were morphologically characterized based on the cultures in artificial media (Sabouraud CAF Agar, Liofilchem s.r.l., Italy). The monosporic pure cultures were characterized based on the appearance (shape, color, mycelium type, striations, and colony height) of the colony. Reproductive structures were examined with phase contrast microscopy. A microscopic examination was conducted to determine the shape and length of the conidia. The identification was conducted based on the procedure proposed by Humber (1997).

Molecular identification

Partial sequences of the internal transcribed spacers for ITS1-5.8S-ITS2 gene regions between the 18 S and 28 S rRNA subunits were conducted to identify the fungal isolates. Pure culture fungal spores were spread on the Sabouraud CAF Agar medium (Liofilchem s.r.l., Italy) and incubated for a week at 28 ℃. Then, the growing fungi were harvested with a sterile spatula and transferred into tubes. Genomic DNA was isolated with the ZR Fungal/Bacterial DNA MiniPrep (50, ZYMO RESEARCH) kit procedure and about 50 mg fungus. PCR reaction was prepared with the primers to amplify the ITS1-5 8S-ITS2 region of the two fungal isolates. ITS5 (5′-GGAAGT AAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′) were employed as the forward and reverse primers, respectively (White et al. 1990). Reaction buffer, each dNTP, primers, DNA polymerase, $MgCl_2$, and 50 ng genomic DNA were added to the 50 µl PCR reaction. PCR conditions were set as described in the PCR kit. PCR samples were loaded on 1.0% agarose gel with ethidium bromide and viewed under UV light. Then, the samples were sent to ETKA Biotechnology (Samsun, Turkey) for sequencing. The sequences were compiled and compared to those in the GenBank databases with BLAST (Benson et al. 2012). Multiple sequence alignment was conducted with ClustalW2 in BioEdit v.7.2.5 software (Hall 1999). Phylogenetic trees were constructed with neighbor-joining (NJ) algorithm and Molecular Evolutionary Genetics Analysis v.11.0 (MEGA11) software (Tamura et al. 2013), and phylogenetic analysis was conducted to compare these with similar species (Benson et al. 2012). The ITS1-5.8-ITS2 gene sequences of all isolates were uploaded to the NCBI data library with accession numbers.

Biological activity

Two *B. bassiana* isolates (G-A and G-B), isolated from dead *G. mellonella* larvae, were employed in the concentration–response experiments against the 3rd instar *G. mellonella* larvae. To obtain fresh spores from the fungus isolates, each isolate was inoculated with a Sabouraud CAF Agar medium in flasks and incubated at 25 ℃ for approximately 3 weeks. The fungus isolate spores were then harvested with a 0.1% Tween 80 (AppliChem) and counted under the microscope with a Neubauer hemocytometer to determine the concentrations. Conidial fungal isolate suspensions were adjusted to $1 \times 10^{5-8}$ conidia/ml. Fungal suspensions of all concentrations were determined with a sterile sprayer. Thirty healthy *G. mellonella* larvae were employed in each replicate, and all experiments were repeated three times on different days. Sterile 0.1% Tween 80 was administered to the control group.

All experiments were recorded daily to identify the dead insects for 10 days after exposure. The mortality (based on mycosis individuals) data were corrected with Schneider–Orelli's formula (Püntener 1981). The LC_{50} and LT_{50} were determined with probit analysis on MS Excel (Finney 1971).

Results

Two fungal isolates were classified as pathogens when mycelial growth was evident outside the cadavers. These isolates were obtained from *G. mellonella* larvae that were naturally infected by fungi and collected from beehives. Morphological observation of the two fungal isolates exhibited morphological and cultural properties similar to *Beauveria* species. Two isolates in the parasitic fungi genera Cordycipitaceae were obtained from the *G. mellonella* larvae. G-A and G-B isolates were determined as *Beauveria* spp. These isolates formed white, smooth, rounded colonies with a fluffy-to-powdery appearance. Colonies were round, lightly raised with a white powdery surface, a little downy with circular rings. The spores of these isolates were round, and the conidia-producing cells characteristically formed a globular or bottle-like, mostly zigzagged, structure. Several small microconidia could form clusters around conidiophores. Macroscopic and microscopic images of fungal isolates are presented in Fig. 1.

Total DNA, isolated from the two fungal isolates, was PCR-amplified in ITS1-5.8S-ITS2 gene region. DNA fragments of about 532 and 537 bp were amplified and visualized in agarose gel. The partial ITS1-5.8S-ITS2 sequences were employed to construct phylogenetic trees. Based on the phylogenetic analysis, two isolates (G-A and G-B) were identical to *B. bassiana* (Fig. 2). Their GenBank database entries were recorded under the accession numbers ON063911 (G-A) and ON063912 (G-B).

In concentration–response experiments, *B. bassiana* isolates G-A and G-B exhibited the *G. mellonella* larvae mortality rate within 10 days after the treatment with the $1 \times 10^{5-9}$ conidia/ml conidia suspensions (Figs. 3 and 4). The LC_{50} of the isolates was estimated as 0.2×10^6 (0.03 – 1.6) and 0.6×10^6 (0.07 – 6.1) conidia/ml (Table 1). Based on the LT_{50}, it was determined that the fungal isolates (G-A and G-B) killed 50% of the *G. mellonella* larvae within 2.68 and 2.43 days, respectively. All

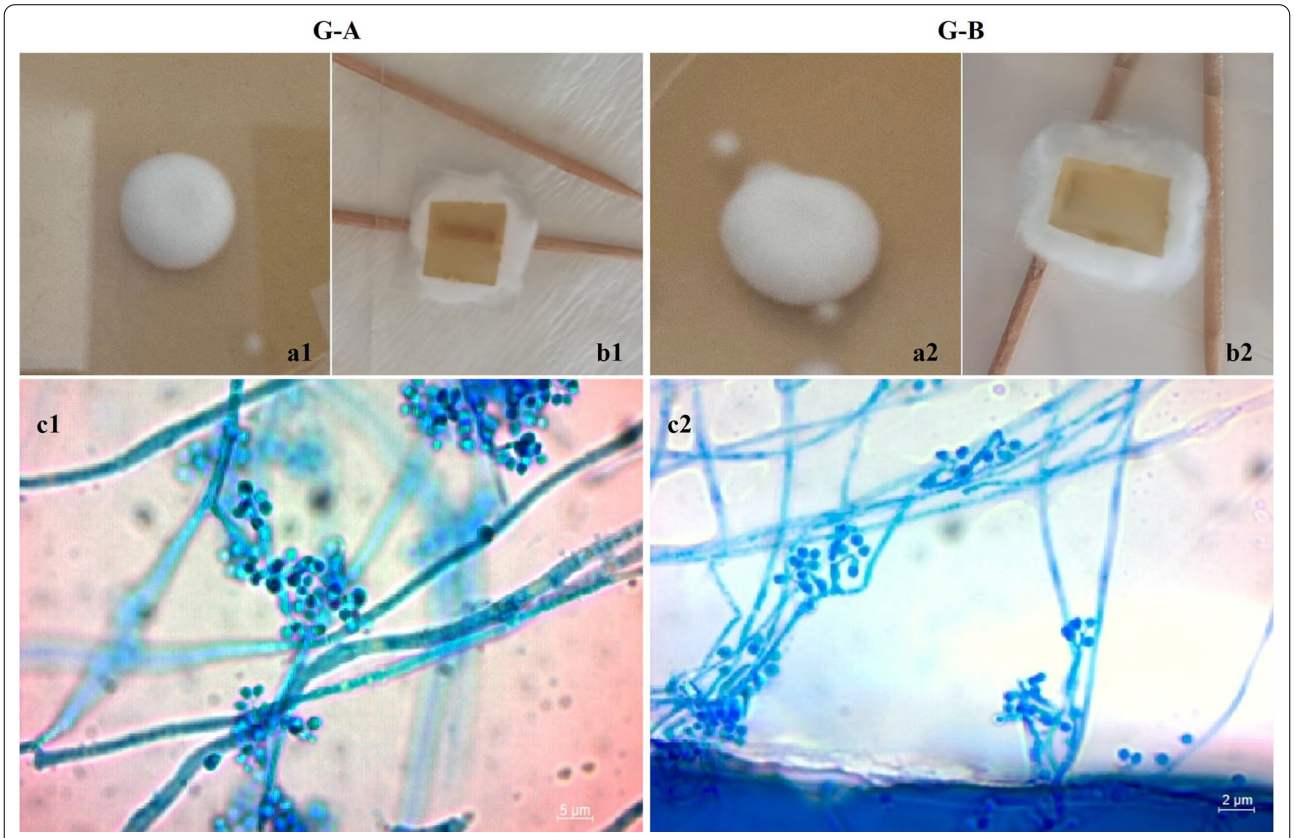

Fig. 1 Morphological observation of fungal isolates: **a1** and **a2**: colony morphology on Sabouraud CAF agar: **b1** and **b2**; block cultures of fungal isolates: **c1** and **c2**, phase contrast microscopic image of the isolates (Magnification: 100X) (1 is the *G*-a isolate, 2 is the G-B isolate)

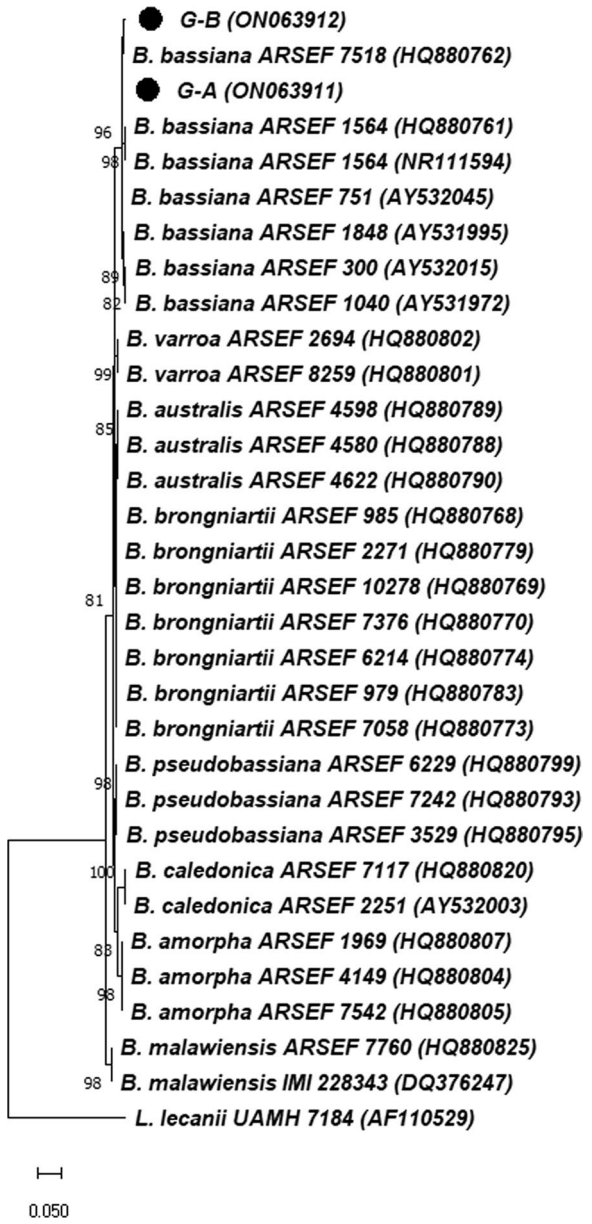

Fig. 2 Taxonomic map of the Beauveria bassiana G-A and G-B in the Beauveria genus based on the combined ITS gene region data. Bootstrap tests were conducted with 1000 replicates and neighbor-joining analysis

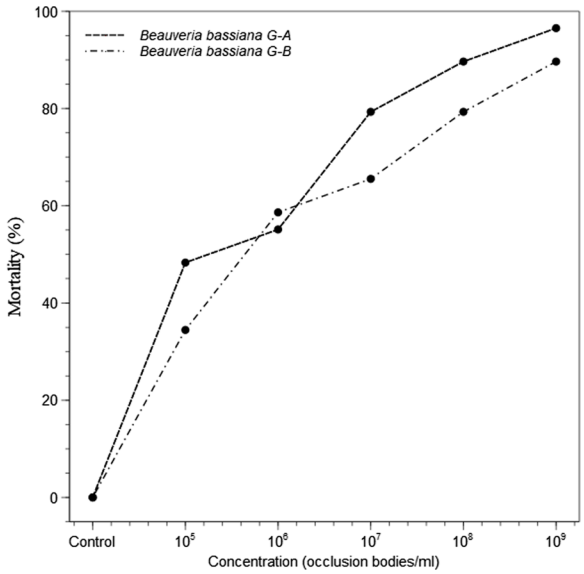

Fig. 3 Galleria mellonella larvae mortality after the administration of five spore concentration doses of G-A and G-B Beauveria bassiana isolates

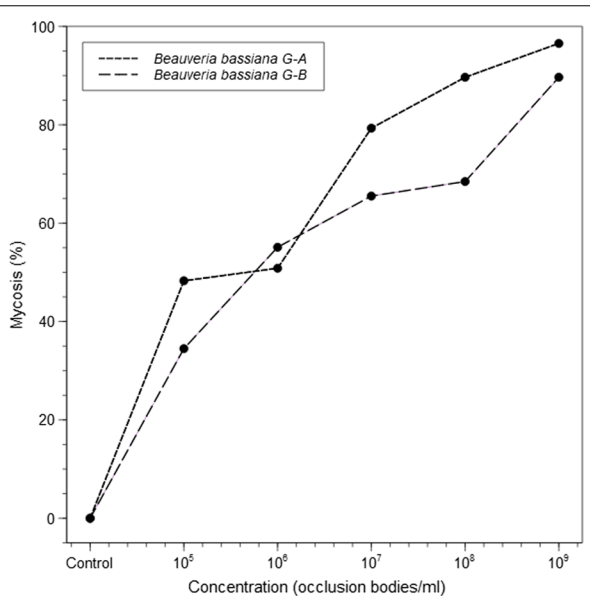

Fig. 4 Galleria mellonella larvae mycosis after the administration of five spore concentration of G-A and G-B Beauveria bassiana isolates

concentrations led to significantly higher mortality when compared to the control. In the control group, no mortality was observed.

Discussion

The chemicals employed to control GWM are hazardous for bees and humans. Identification of natural EPFs isolates that are compatible with the local environment and

Table 1 Lethal G-A and G-B *Beauveria bassiana* isolate concentrations on *Hyphantria cunea* larvae

Isolate	LC_{50} (conidia/ml)	Slope ± SE	*df*	X^2	LT_{50} (day)*
G-A	0.2×10^6 (0.03 – 1.6)	0.485 ± 0.432	3	0.881	2.68 (1.96–3.66)
G-B	0.6×10^6 (0.07 – 6.1)	0.396 ± 0.489	3	0.927	2.43 (1.56 – 3.78)

* 1×10^9 conidia/ml

more efficient in pest control is desirable as an alternative to common chemical pesticides.

In the present study, two native EPF isolates of *G. mellonella* larvae were isolated and identified and their biological activities were determined. The infected individuals were identified based on the mycelial growth outside the cadaver (Greenfield et al. 2016). Also, isolates were identified morphologically based on conidial morphology, shape, and size according to the current identification key (Humber 1997). The appearance of the fungal isolates in the medium was round, had a white cottony surface, and was slightly downy with circular rings. Fungal mycelium exhibited radial growth in the medium. The characterization based on morphological properties could not identify the species accurately.

Genetic diversity of *Beauveria* species could usually be determined based on ITS gene regions (Chen et al. 2018). In the current study, the nuclear ITS1-5.8S-ITS2 region was employed to obtain molecular sequences adequate for the development of fungal phylogenies (Zare and Gams 2001). The virulence of the *B. bassiana* isolates identified in different regions and that of the host insect varied (Takatsuka 2007), and *B. bassiana* was the most identified fungus in several fields (Meyling and Eilenberg 2007). The recovered *Beauveria* G-A and G-B isolate clusters were closely correlated with various *B. bassiana* isolates. No record of EPF isolation from naturally infected *G. mellonella* larvae exists in beehives. However, two entomopathogens, a nucleopolyhedrovirus (NPV) and a bacterium, were identified in *G. mellonella* (Biswas et al. 2003).

The use of chemicals is not recommended in hives since they contaminate bees, beeswax, and other bee products (Bogdanov 2006). Thus, biological control agents are the ideal and most recommended alternative method. Honeybee colonies are affected by several parasites and pests (Kwadha et al. 2017). Furthermore, *B. bassiana* has no harmful effects on honeybees (Ahmed and Abd-Elhady 2013). The potential of this fungus to control *G. mellonella* moths has not been studied. Since wax moth is harmful to the bees, rapid larval death or inhibition of larval growth could support the acceptance of *B. bassiana* as a potential control agent (Abou-Shaara 2020).

It was demonstrated in several laboratory studies that EPFs were effective in *G. mellonella* control (Fergani and Yehia 2020). Only a few studies analyzed the efficacy of EPFs in *G. mellonella* control (Shoukry et al. 2019). Although several studies have been conducted on the effects of EPFs on *G. mellonella*, there are no reports of natural isolation. The present study demonstrated that significant two native *B. bassiana* isolates exhibited significant pathogenic activity against *G. mellonella*. The fungal G-A and G-B isolates successively controlled the *G. mellonella* larvae. In general, this finding was consistent with previous empirical studies on larval exposure to this fungus (Abou-Shaara 2020).

Abou-Shaara (2020) investigated *B. bassiana* infection in *G. mellonella* and reported that it was not possible to expose *G. mellonella* larvae to a high fungal concentration under hive conditions. However, *B. bassiana* G-A and G-B isolates exhibited an over 80% mortality rate and LT_{50} calculations demonstrated that these isolates could terminate 50% of the pest within approximately 2.5 days. In another study, the experiments demonstrated that the *B. bassiana* isolated from the soil led to a mortality rate between 75 and 98.33% within 5 days in 4th instar *G. mellonella* larvae (Fergani and Yehia 2020).

Shoukry et al. (2019) studied the impact of three EPFs, *B. bassiana*, *M. anisopliae*, and *Trichoderma album*, on *G. mellonella* larvae. They reported that the highest mean larval mortality rate was obtained with *T. album*, *M. anisopliae*, and *B. bassiana* (47.61, 41.52, and 39.67%, respectively). Another similar study analyzed the pathogenicity of *B. bassiana* and *Paecilomyces lilacinus* against GWM (Ibrahim et al., 2016). The findings demonstrated that the mortality rate of the *B. bassiana* and *P. lilacinus* isolates was 98 and 87.5%, and LT_{50} values were 1.7 and 2.2 days, respectively. *B. bassiana* was more effective when compared to *P. lilacinus*. In the present study, it was observed that *B. bassiana* isolates had a similarly high impact on *G. mellonella*. Mansour et al. (2003) reported that the mortality rate increased with *B. bassiana* spore concentration, and the mortality rate was 82–96% at the end of 20 days under laboratory conditions. The present study data were consistent with the findings reported by Saleh et al. (2016) that the virulence of *B. bassiana* was effective *G. mellonella* larvae in the tested fungal isolates. *B. bassiana* was reported to exhibit the highest larval mortality when compared to other fungal isolates (Fathy et al. 2018). In the current study, the mortality rate of the *B. bassiana* G-A and G-B isolates was 89.66 and 96.54%, respectively. These findings were consistent with the findings reported by Klingen et al. (2002), where it was reported that *M. anisopliae* and *B. bassiana* fungi led to high mortality in *G. mellonella* larvae (100%).

Conclusion

The current study findings demonstrated that the biological control agent potential of the *B. bassiana* G-A and G-B isolates was high in *G. mellonella* larvae. However, the commercial viability of the G-A and G-B isolates should be determined. Field conditions include external factors such as UV, humidity, and temperature variations; thus, the isolates should also be investigated under field conditions. The suitability of the fungus for mass production and the stability of a formulated or

unformulated product that includes the fungus should also be warranted under various conditions.

Abbreviations
UV: Ultraviolet; PDAY: Potato dextrose agar medium with 1% yeast extract; ITS: Internal transcribed spacers; DNA: Deoxyribonucleic acid; PCR: Polymerase chain reaction; $MgCl_2$: Magnesium chloride; LC_{50}: Lethal concentration 50; LT_{50}: Lethal time 50.

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Author contributions
ZB and DG conducted the experiments, collected the data, analyzed the data, collected literature, and wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

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Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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