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Original article

Antimicrobial activity of camphor tree silver nano-particles against foulbrood diseases and finding out new strain of *Serratia marcescens* ***via DGGE-PCR,*** as a secondary infection on honeybee larvae

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ABSTRACT

American foulbrood (AFB) and European foulbrood (EFB) are the two major bacterial diseases affecting honeybees, leading to a decrease in viability of the hive, decreasing honey production, and resulting in significant economic losses to beekeepers. Due to the inefficiency and/or low efficacy of some antibiotics, researches with nanotechnology represent, possibly, new therapeutic strategies. Nanostructure drugs have presented some advantagesover the conventional medicines, such as slow, gradual and controlled release, increased bioavailability, and reduced side-effects. In this study, different infected larvae were collected from two apiaries; the combs that had symptoms of American and European foulbrood were isolated. *In vitro* antimicrobial activity of camphor tree silver nano-particles against foulbrood diseases were characterized using UV–Vis spectrophotometry and scanning electron microscope (SEM) that proves the formation of silver nanoparticles with size range 160–660 nm. The antimicrobial activity of the silver nanoparticles was tested using agar diffusion assay and proved their ability to effectively cease the pathogenic bacterial growth in both AFB and EFB. DGGE-PCR technique has been applied for the identification of un-common bacterial infections honeybees depending on 16S rRNA amplification from their total extracted DNA and has been identified as *Serratia marcescens* (TES), deposited in GenBank with a new accession number (MT240613). The results were confirmed strain has been detected by DGGE-PCR analysis causing uniquely infected brood that was attacked by the American Foulbrood It could be concluded that greenly synthesized silver nanoparticles is projected to be used as effective treatment for honeybee bacterial diseases. These material need more investigations under field conditions and study the possibility of its residues in honeybee products such as honey, and beeswax.

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1. Introduction

Honeybees, (*Apis mellifera*), perform an essential use as pollinators of horticultural and agricultural crops, and pollination by honeybees in agricultural sector production is estimated to be worth 15 billion dollars per year in the USA (Morse and Calderone, 2000; Klein et al., 2007; Potts et al., 2010). The healthy and activity of honeybees became a worldwide concern, as presented over the last decades by decline its populations (Lee et al., 2015; Traynor et al., 2016). Factors caused this decline include a combination of environmental conditions, infectious diseases, pesticides and lack of flowering plants and habitat as well (Le Conte and Navajas, 2008; Pârvu et al., 2013; Goulson et al., 2015; Masry and Abdelaal, 2016). This insect is menaced by numerous pathogens, including bacteria, fungi, viruses, and protozoa. American foulbrood (AFB) and European foulbrood (EFB) are severs dangerous honeybee infections caused by the Gram-positive bacterium *Paenibacillus larvae* and *Melissocccus plutonius*, respectively (Sabaté et al., 2009; Forsgren, 2010; Masry et al., 2014).

Bacterial diseases are the most serious pathogens agent affected honeybee colonies. The AFB (*Paenibacillus larvae*), is a highly virulent and harmful disease that attacks honeybee larvae and pupae stages causes death of these brood (Williams, 2000; Genersch et al., 2006). *Paenibacillus larvae* main characteristic is the formation of its spores which are endospores. These spores have high resistant properties and can germinate after about 35 years in scales (Lindström, 2008). Controlling of AFB is a critical practice for beekeepers and they faced many difficulties to find a good and affective treatment for these spores. Therefore, *P. larvae* caused huge economic losses of honeybee colonies and considered as one of the most important factors of colony collapse disorder. In the case of outbreak of the AFB, because of its destructive effects, beekeepers are mandated to burn their hives to stop the spread of the disease and sterilize their equipment's (Mutinelli, 2003).

To control AFB researchers started test many antibiotics from 1940s (Katznelson, 1950). The most effective antibiotic against *P. larvae* at this time was aureomycin (chlortetracycline), followed by penicillin, chloramphenicol, streptomycin and other. However, from 1950s oxytetracycline (OTC) was allowed to prevent and control AFB and EFB. Continued application of antibiotic resulted resistance strains of *P. larvae*. Moreover, heavy applications of OTC lead to a residue in bee products; honey, pollen and beeswax (Kochansky et al., 2001). For these reasons, recently, OTC had been forbidden in several countries (Mutinelli, 2003).

The random treatments of antibiotic resulted resistant to OTC and high cost losses for beekeepers. Therefore, scientists focused on the natural products and strategies for controlling *P. larvae* (Alonso-Salces et al., 2017). Essential oils, plant extracts, propolis and royal jelly were used as natural strategies for controlling infected colonies with *P. larvae* (Bachanová et al., 2002; Ruffinengo et al., 2006; Reyes et al., 2013; Wilson et al., 2015). Recently, lactic acid bacteria (LAB) are used against *P. larvae* spores. Furthermore, the LABs isolated from honeybees and its honey stomach plays an important role for protecting the adult bees and their larvae against pathogens and had an *in vitro* inhibitor effect on the causative agents of AFB and EFB (Killer et al., 2014; Olofsson and Vásquez, 2009). Moreover, El-Sohaimy et al., (2020) isolated twenty-five strains of lactic acid bacteria from the stomach and honey sac of different honeybee race and hybrids. From the 25 strains there were 6 new novel isolates which had a significant effect as antimicrobial activities against AFB and EFB. Recently, Khan et al. (2019) determined the potential of extract of medicinal plants and their derived nanoparticles against fungi (*Ascopharea apis*), bacterial (AFB and EFB), mites (*Varroa* spp. and *Tropilaelap s*sp) and microsporidia (*Nosema apis* and *N. cerana*) in honeybee colonies.

The bacteria *Serratia marcescens* that infected many plants and animals, is abundant investigated in the gut of honeybees, recently isolated from dead bees (Raymann et al., 2018). The first isolated of this bacterium from honeybees' larvae was recorded in 1987 (El Sanousi et al., 1987). Recently, Burritt et al., (2016) isolated a new novel stain of *S. marcescens* from honeybee adults and from the ectoparasitic mite *Varroa destructor*. Raymann et al., (2018) reported that the infected bees cannot fly and tendency to separate from uninfected mates. Also, they observed that the guards and housekeeping honeybees did not move the infected ones out of their hive. Moreover, authors declared that the cytometry experiment indicated septic bees' loss their cellular immunity.

Denaturing gradient gel electrophoresis (DGGE) is a type of polymerase chain reaction (PCR) that depending on the amplification of fingerprinting gene such as bacterial 16SrRNA that is obtained from a community of extracted DNA (Muyzer et al., 1993). It is a culture-independent genetically fingerprinting technique that has the ability to describe the structure of a microbial community and the diversity of environmental samples referring to their taxonomic level or even their genus level (de Oliveira et al., 2006; Qing et al., 2007). It has an advantage of allowing immediate determination of genetic diversity of the microbial community, making it better than cloning and subsequent sequencing. However, the latter technique is preferred to identify the key microorganisms (Rombaut et al., 2001). DGGE-PCR technique has been successfully applied for the detection of bacterial communities in different sample types such as river biofilms (Lyautey et al., 2005), micro-flora of farmed fish (Hovda, 2007), drinking water (Wu et al., 2006), diversity of Bacteroides, bifidobacteria and clostridium cluster IV in institutionalized elderly (Zwielehner et al., 2009), detection of cadmium resistant bacteria (Qing et al., 2007), diversity of bacteria in earthworm gut (Zhang et al., 2013), thermophilic bacteria in milk (Delgado et al., 2013), and food samples (Dufossé et al., 2013).

Hence, the aim of this work was to phenotypically and genotypically isolate and characterize bacterial pathogens correlated with the infected honeybees with AFB and to determine the potential of biosynthesized silver nanoparticle for controlling honeybee bacterial diseases. Moreover, the target was extended to genetically detect the bacterial strains that might exist in one foulbrood and absent from the other through the same environmental conditions using DGGE-PCR technique.

2. Materials and methods

2.1. Collections of samples

Two honeybee apiaries located at arid lands, North Coast, Alexandria, Egypt, 20 colonies each, were inspected for honeybee bacterial diseases during winter season 2018. The combs that had symptoms of American foulbrood (Fig. 1) and European foulbrood (Fig. 2) (Shimanuki and Knox, 1988) were isolated, marked and kept in sterilized plastic bags (one comb/bag). At the laboratory one brood comb of each apiary was selected and pieces of beeswax that contained dead and infected larvae in the brood cells were cut and kept at 4 °C until subject to the experiments and isolation (OIE, 2009; Masry et al., 2014).

2.2. Isolation of bacteria from infected honeybee brood with American and European foulbroods

The bacterial pathogens that responsible for infection of honeybee larvae were isolated from the infected honeybee brood cells

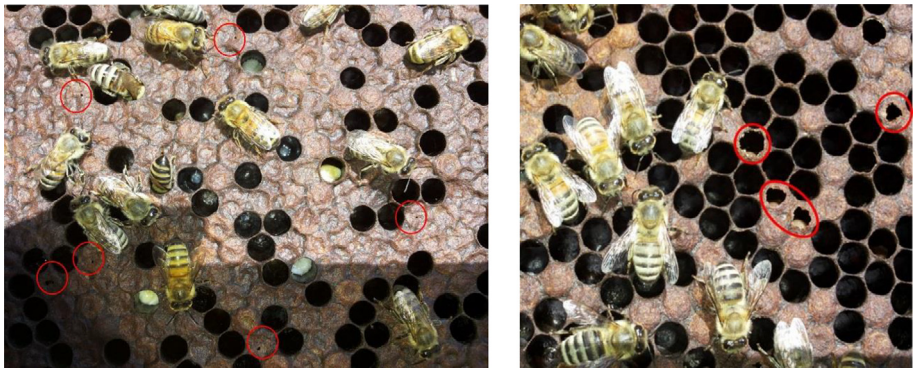

Fig. 1. Pattern of capped brood with sunken and punctured caps typifying American foulbrood (AFB) disease.

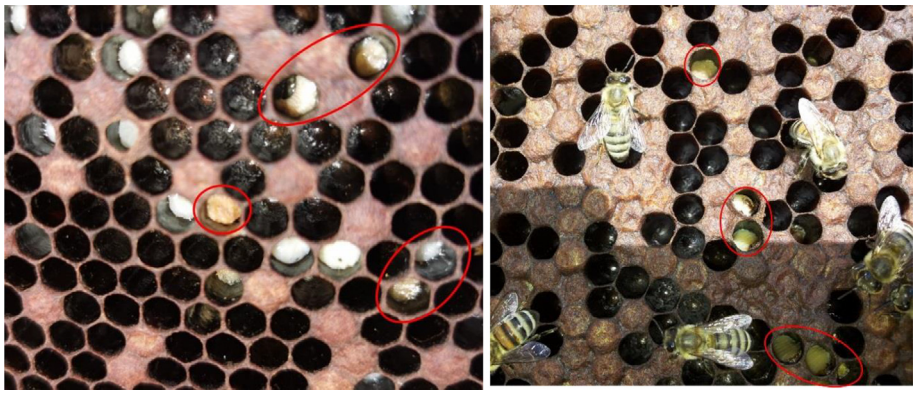

Fig. 2. Irregular pattern of brood typifying European foulbrood (EFB) disease. Isolation of bacteria from American and European foulbroods.

using sterile cotton swap. At first, total bacterial pathogens were collectively isolated through several touches of sterile cotton swaps with subsequent transfer into sterile Luria-Bertani (LB) broth. The inoculated tubes were incubated at 30 °C with shaking at 150 rpm for 24 h. One milliliter of each of the growing bacterial cultures were used for total DNA extraction and the rest of both cultures were serially diluted using sterile normal saline solution (0.9%). Fifty micro liters of 10^{-5} and 10^{-6}dilutions were spread over LB plates and were incubated at 30 °C for 24 h. The formed single colonies were transferred to new LB plates for purifications and were tested for bacterial resistance/sensitivity pattern using biosynthesizedsilver nanoparticles.

2.3. Preparation of plant extract

The leaves of camphor tree were collected from Borg El-Arab City, Alexandria. The collected leaves were washed using distilled water and air dried for 24 h followed by drying at 60 °C for 48 h. After drying, 10% of the leaves were boiled in distilled water for 10 min. The obtained mixture was filtered through whattman filter paper and the obtained filtrate was cooled down and kept at 4 °C till use.

2.4. Synthesis of silver nanoparticles

The prepared camphor extract was used for the preparation of silver nanoparticles using $AgNO_3$ as raw material. At first, 1 mM of $AgNO_3$ was added to 500 ml of camphor extract and incubated at 30oC and 150 rpm for three days. The solution was then centrifuged at 15000 rpm for 30 min in order to collect the synthesized silver nanoparticles. The formed pellet was washed twice using distilled water and once using absolute ethanol. The pellet was then dried at 50 °C and kept dry in a clean container till use.

2.5. Characterization of obtained silver nanoparticles

2.5.1. UV–Vis spectrophotometry

The obtained silver nanoparticles were primarily characterized usingUV–Vis spectrophotometry. This step was achieved through the suspension of 10 mg of silver nanoparticles in 1 ml distilled water followed by UV–Vis scanning from 200 to 800 nm. The formed peak that isindicating the formation of silver nanoparticles was investigated. In addition, the wave-scan of 10 mM of silver nitrate was also determined using the same wavelengths.

2.5.2. Scanning electron microscopy (SEM)

The micrographs and particle size of the formed nanoparticles were illustrated using scanning electron microscopy under different magnifications (JSM 6360 LA, JEOL, Japan).

2.5.3. Bacterial isolation and morphological identification

One gram of the infected larvae of each characterized disease colony was dissolved in PBS buffer (pH.7) and then 100 μl of each sample were inoculated into 100 ml LB. Ten μl of each culture was separated on Petri dishes contain LB medium and the obtained colonies of each culture were tested under light microscope and characterized as American and European foulbroods.

2.6. Antimicrobial activity

Based on the disease symptoms (Masry et al., 2014) and the morphological characterization obtained bacterial colonies were

differentiated. More differentiation was tested based on the isolate sensitivity against the biosynthesized particles. The antimicrobial activity of silver nanoparticles against the isolated bacteria of American and European foulbroodwas investigated using agar well diffusion method. At the beginning, LB agar media was prepared and sterilized by autoclaving at 120 °C and 15 psi for 20 min. The media was poured into Petri dishes and left at room temperature till solidification. Overnight LB broths of each bacterial isolate were diluted using normal saline till 0.5 McFarland standards. A sterile cotton swap of each bacterial isolate was spread over the prepared LB plates followed by the formation of a hole at the plate's center. Different concentrations (0, 1000, 2000, and 3000 μg/100 μl) of the synthesized silver nanoparticles were added to the holes and the plates were then kept at 4 °C for 1 h followed by incubation at 30 °C for 24 h. The formed clear zones were measured, recorded and photographed.

2.7. DNA extraction

In addition to the above methods DDG-PCR was used also to ensure the difference between the obtained bacterial isolates (*Paenibacillus larvae* and *Melissocccus plutonius*). Total genomic DNA of both of collected bacterial cultures of either American or European foulbrood was extracted using Gene JET Genomic DNA Purification Kit according to the instruction manual (Thermo Scientific, EU Lithuania).

2.7.1. Amplification of total 16S rRNA genes

The amplification of total 16S rRNA genes from the collected cultures has been achieved using universal primers with GC clamp. The sequence of the forward primer was 5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC −3′ and the sequence of the reverse primer was 5′-CGG TGT GTA CAA GGC CCG GGA ACG −3′. A total of 25 μl reaction was composed of: 2.5 μl of 10x Taq buffer containing $MgCl_2$, 10 mMdNTPs, 10 pm of each primer, 0.25 μl Taq polymerase (5 U) and 10 ng total genomic DNA. The PCR conditions were started with denaturation step at 95 °C for 10 min followed by 35 cycle of; 95 °C for 30 sec; 55 °C for 30 sec and 72 °C for 30 sec, with subsequent final extension step at 72 °C for 7 min. An aliquot of each PCR product was loaded to 1% agarose gel containing ethidium bromide,migrated using 120 V for 30 min, visualized using UV transilluminator (UVP Dual-Intensity Transilluminator) and photographed using gel documentation system (Syngene, UK).

2.8. ***DGGE-PCR analysis***

At first, some optimization experiments have been done followed by preparation of the required gel with the obtained optimum conditions. Perpendicular DGGE was performed using D-Code Universal Mutation Detection System (BioRad) as described by Lyautey, et al. 2005, with some modifications. The gradient polyacrylamide gel ranging from 50 to 70% was prepared using urea and deionized formamide as denaturants (100% denaturant is 7 M urea and 40% deionized formamide). After gel polymerization; 15 μl of each PCR sample was loaded and the DGGE was run at 50 V for 18 h at 60 °C.

After electrophoresis, the polyacrylamide gel was carefully transferred for 15 min to a container contains 300 ml of 1X TAE buffer with 30 μl of 10 mg/ml ethidium bromide for staining. The gel was then de-stained by transferring to 300 ml of 1X TAE buffer without stain. The stained gel was photographed using gel documentation system and the bands of interest were excised by sharp razor.

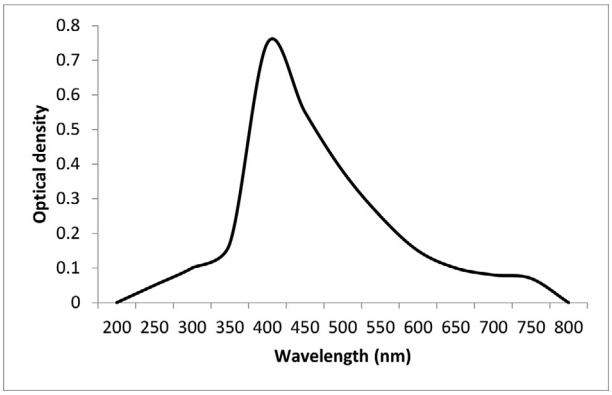

Fig. 3. UV–Vis spectrophotometry scanning of the biosynthesized silver nanoparticles.

2.8.1. Gene purification and sequencing

The selected bands of interest were subsequently excised form the DGGE gel and placed in sterile 1.5 ml eppendorf tubes containing 50 μl of sterile injection water. The tubes were submitted to three cycles of freeze-thawing (-20 °C/37 °C) for DNA elution. After that, 5 μl of that solution were used as template in a second PCR using the same previous conditions. The purity of the obtained products was investigated using 1.5% agarose gel. The genes were then submitted for sequencing and the obtained sequences were compared with similar sequences in GenBank. The phylogenetic tree of the obtained gene and other similar genes was constructed using MEGA 5.1 program.

3. Results

3.1. Characterization of silver nanoparticles

3.1.1. UV–Vis spectrophotometry

Scanning of the prepared silver nanoparticles using UV–Vis spectroscopy has been performed as a fast technique for characterization of silver nanoparticles. As shown in Fig. 3, the scanning pattern was in the range from 200 to 800 nm. The obtained results revealed that the maximum absorbance was observed between 400 and 450 nm.

The morphological appearance of the synthesized silver nanoparticles was investigated using scanning electron microscopy. The particles were in the form of spheres with rough surfaces. The particles showed a variable size in the range of 160–660 nm as shown in Fig. 4 (A-2). Some of the particles tended to aggregate forming bigger particle size that can reach 6.06 μm as shown in Fig. 4 (A-1).

3.2. Bioassay of biosynthesized silver nanoparticles against isolated bacteria

Multiple bacterial isolates with different colony shapes were isolated from honeybee brood cells that had symptoms of American foulbrood (AFB) and European foulbrood (EFB). All the isolates were submitted for successive purifications followed by antimicrobial testing. Different concentrations of the biosynthesized silver nanoparticles were tested against the isolated bacteria using agar will diffusion assay. As shown in Table 1, all the tested concentrations affected the isolated bacteria of each foulbrood category. The isolates of AFB were highly affected by the three tested nanoparticles concentrations and showed clear zones ranged from 2 to 3.2 cm. While the isolates of EFB showed slightly higher resistance to silver nanoparticles than the AFB. The nanoparticles affected the

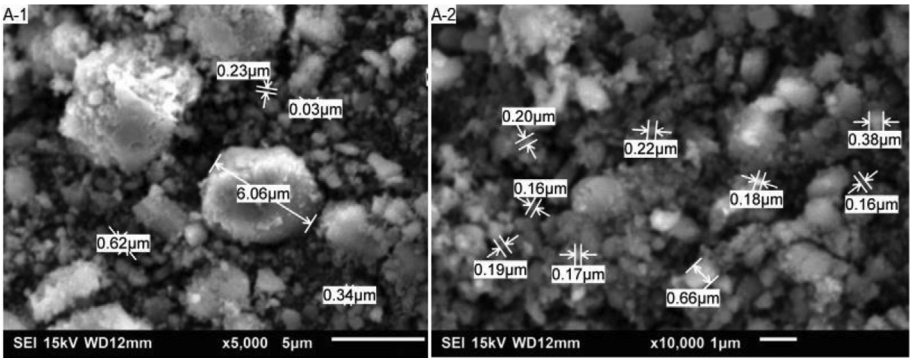

Fig. 4. SEM of the synthesized silver nanoparticles using different magnifications. (A-1): 5000x and (A-2): 10000x.

Table 1
The antibacterial activity of biosynthesized silver nanoparticles (AgNPs) against bacterial American and European foulbrood.

AgNPs concentration (µg/100 µl)	American foulbrood isolates Clear zone (cm)				European foulbrood isolates Clear zone (cm)			
	A1	A2	A3	Mean	E1	E2	E3	Mean
0 [I]	0	0	0	0	0	0	0	0
1000 [II]	2.3	2	2.2	2.1	2	1.5	1.9	1.8
2000 [III]	2.7	2.5	2.1	2.4	2.5	1.5	2.2	2
3000 [IIIV]	3.2	3	3.2	3.1	2.7	2.5	1.2	2.1

isolates with clear zones in the range of 1.2–2.7 cm. However, it could be concluded that the three tested nanoparticles concentrations significantly recorded antimicrobial activity against all isolated bacteria of AFB and EFB. Fig. 5 represents the shape of clear zones formed through the addition of silver nanoparticles to the central holes of agar plates inoculated with AFB and EFB bacterial isolates. In general, the green synthesized silver nanoparticles showed a potent antibacterial activity against multiple bacterial pathogens (Peiris et al., 2017; Soliman et al., 2018; Ramanathan et al., 2019).

3.3. DGGE-PCR analysis

The purpose of using DGGE-PCR analysis for the extracted genomic DNA of American and European foulbrood was the differentiations among the existed bacterial strains that could and/or couldn't be isolated and cultivated by conventional microbiological techniques. As shown in Fig. 6, the DGGE-PCR analysis of 16S rRNA of total extracted DNA for American and European foulbrood samples succeeded to amplify and separate 500 bp of multiple genes that share the same length but different sequences. These genes were alternatively migrated at the same horizontal lines in some locations and in different locations for the rest of the separated genes. The amplified and separated genes that can be seen in Fig. 6 indicate the presence of a total of six bands in the American foulbrood sample and four bands in the European foulbrood sample. However, two bands of the American foulbrood sample were unique and did not show identical genes in the European foulbrood sample. Both two bands were excised from the gel and submitted to purification and sequencing as mentioned in the methods section. It worth mentioning that, the unique genes were the most genes of interest in the current study to differentiate genetically

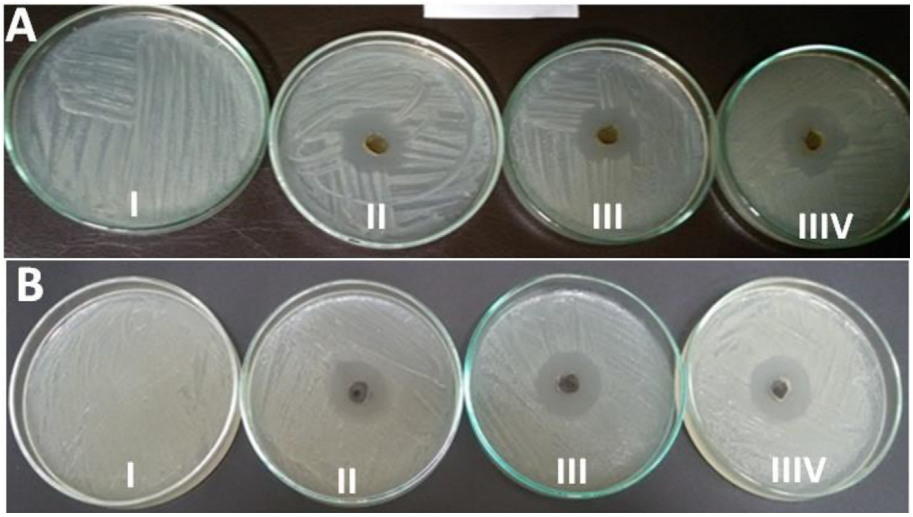

Fig. 5. Representative photos of the clear zones formed after the inoculation of bacterial plates with biosynthesized silver nanoparticles.

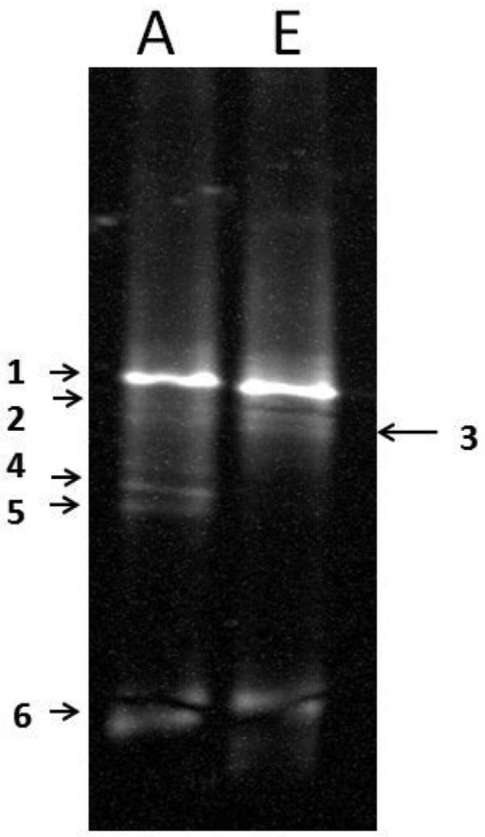

Fig. 6. DGGE-PCR polyacrylamide gel indicating the separated 16S rRNA bands of both American and European foulbroods. (A): American foulbrood and (E): European foulbrood. The numbers on the figure indicates the separated genes of both foulbroods.

among the microbes that can infect the larvae, which ensure that there are six different bacterial isolates belong to the American foulbrood and four different bacterial isolates belong to the European foulbrood. That is considered of what is called mixed infection.

3.3.1. Sequencing and phylogenetic analysis

The selected bands that were uniquely found in the American foulbrood infected larvae amplified ribosomal genes were excited from the gel, purified and submitted for sequencing. One of the bands failed to represent a matched sequence with GenBank deposited sequences. However, the other band showed a 100% similarity with deposited GenBank sequences and was highly matched with *Serratia marcescens* strains. The obtained sequence was subsequently deposited in GenBank using the name *Serratia marcescens* TES with a new accession number (MT240613). The phylogenetic tree of the obtained sequence and other similar sequences in GenBank was constructed using neighbor-joining tree method with 1000 bootstrap options of MEGA 5.1 program (Fig. 7).

4. Discussion

Scanning of the prepared silver nanoparticles using UV–Vis spectroscopy is a simple and fast technique for laboratory investigation of prepared nanoparticles (Kim et al., 2003; Kim et al., 2006). The obtained results revealed that the maximum absorbance was observed between 400 and 450 nm which is indicating the formation of silver nanoparticles as previously investigated (Aziz et al., 2017; Abu-Saied et al., 2020). The morphological structure of the synthesized silver nanoparticles showed that the aggregation may be attributed to the interaction forces such as van der Waals forces of attraction (Prathna et al., 2011). However, the scanned particles revealed the formation of non-structures in the nano size which confirm the formation of silver nanoparticles as would be matched with the UV–visible pattern investigation.

According the Bioassay of biosynthesized silver nanoparticles against isolated bacteria, the obtained results concluded that the three tested nanoparticles concentrations significantly recorded antimicrobial activity against the isolated bacteria of both American and European foulbroods. In general, the green synthesized

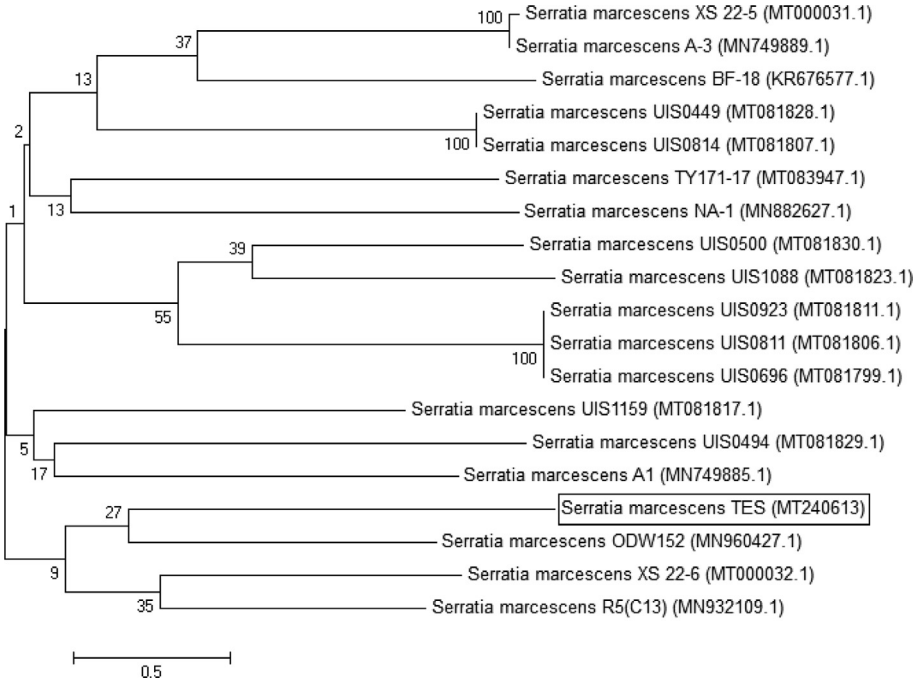

Fig. 7. Phylogenetic analysis of *Serratia marcescens* TES 16S rRNA gene and related sequences in GenBank.

silver nanoparticles showed a potent antibacterial activity against multiple bacterial pathogens (Peiris et al., 2017; Soliman et al., 2018; Ramanathan et al., 2019). The efficiency of the synthesized nanoparticles which in micron scale might showed high efficacy as mentioned by Yassin et al. (2016). The activity of the biosynthesized silver nanoparticles might due to the active ingredient presented in the camphor plant extract and coated the silver particles as previously reported by Leitão et al. (2018). Or the antibacterial activity might back to the bactericidal activity of silver particles itself (Clement and Jarrett, 1994).

Honeybee bacterial diseases AFB and EFB can be treated with many types of antibiotics such as Tetracyclines, oxytetracycline, Tylosin and others. However, the use of the antibiotics in honeybee hives is strictly regulated due to the antimicrobial resistance and its residues in honey and other honeybee products (Cox et al., 2005; Murray et al., 2007). Therefore, there is an urgent need to find alternative materials, drugs or new therapeutic strategies for controlling honeybee bacterial pathogens. Recently, nanostructure materials presented advantages and therapeutic properties and have used as conventional medicines (Cho et al., 2005; Pal et al., 2007). Because of benefits of nanomaterial, researchers paid attention to test it in the field of apiculture and evaluate *in vitro* against AFB and EFB. However, tee tree oil (TTO) and tee tree oil nanoparticles had evaluated against AFB and EFB. The use of TTO nanoparticles was the first time tested as natural alternative to antibiotics in *in vitro* against AFB and EFB with a high activity (Santos et al., 2014). Recently, the toxicity of gallic acid-conjugated silica nanoparticles against AFB and adult honeybee workers was studied (Domínguez et al., 2019). Accordingly, the biosynthesized nanosilver had a high potential antibacterial activity and they are safer for the adult honey bee and the larvae as well. This finding agree with scientists synthesized the silica nanoparticles with gallic acid which presented high attributes to be used in the field of beekeeping for controlling AFB disease, moreover, these nanoparticles had high potentially effective against AFB, safe and non-toxic for honeybee workers and larvae (Domínguez, et al. 2019). According to Khan et al. (2019), it can be concluded that the extracts of some medicinal plants and their synthesized nanosilver particles showed high antimicrobial activity against honey bee pathogen such as fungi (*Ascosphaera apis*), bacteria (*Melissococcus plutonius* and *Paenibacillus larvae*), and microsporidia (*Nosema apis* and *Nosema ceranae*).

According to the principle of DGGE-PCR technique, the genes that migrate at the same horizontal positions in different lanes have the same nucleotide sequence for the tested samples. While, the genes that migrate in different positions are definitely have different nucleotide sequences (Díez et al., 2001). It worth mentioning that, the amplified 500 bp from the infected larvae either with American foulbrood or European ones showed six and four different bands which ensure that there are six different bacterial isolates infect the honey bee larvae belong to the American foulbrood. On the other hand, only four genotypes of European bacterial infect the honey bee worker brood in Egypt. Peters et al. (2006), reported that they isolated different bacterial genotypes of the *Paenibacillus larvae* when they tested worker brood combs and honey samples of 54 apiaries. Genersch et al., (2006) used the REP-PCR for genotyping the pathogenic bacteria into RIC I, ERIC II, ERIC III and ERIC IV. It was reported that the American foulbrood may be include more that 12 genotypes (de Graaf et al. 2013). In case of the European foulbrood, Grossar et al. (2020) isolated 16 different bacterial isolated from infected honey bee larvae and grouped them into three main groups based on their virulence. Arai et al. (2012) succeeded to make diversity of *Melissococcus plutonius* from honeybee larvae in Japan and both the cultural and biochemical characterization revealed to 14 of typical *M. plutonius* and 19 *M. plutonius*-like strain isolates.

5. Conclusion

Both of the American and European foulbrood (AFB & EFB) are the two major bacterial that acquired some resistant against the chemical antibiotics, and researchers try to find out a good substitution such as nanotechnology. Silver nanoparticles have been prepared greenly from camphor extract and characterized through UV–Vis spectrophotometry and SEM analysis that proved their formation in the range 160–660 nm. All tested concentrations of the prepared nanoparticles proved their ability to positively control the isolated bacterial pathogens of both American and European foulbrood. The obtained results suggested that silver nanoparticles could be considered as a promising antibacterial treatment for controlling the two honeybee bacterial diseases. Genetic analysis of the pathogenic bacteria of using DGGE-PCR technique proved the existence of *Serratia marcescens* strain associated with the 6 different genotypes of American foulbrood rather than four different genotypes of the European foulbrood.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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