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In-vitro Evaluation of the Antibacterial Activity of *Micromeria barbata* Essential Oil Against *Paenibacillus larvae* Spores, Causative Agent of AFB

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Abstract

Paenibacillus larvae are the causative agent of American foulbrood, one of the most widespread and destructive honey bee brood diseases. The misuse and overuse of antibiotics in treating AFB can potentially promote antibiotic resistance and the residues of the antibiotics used may be found in honeybee products. Residues of antibiotics in honeybee products make them harmful and unsafe if consumed by humans, especially pregnant women and infants, until the age of eight. Using a natural product, such as plant essential oils, instead of synthetic antibiotics may aid in treating such diseases. In this study, the antibacterial activity of *Micromeria barbata* ssential oil was investigated against both forms of *Paenibacillus larvae* and the toxic effect of this essential oil on honeybees was also tested. The broth dilution method of the tested essential oil was used to determine the MIC of the oil against the vegetative form *Paenibacillus larvae* and two other methods were used for the spore form *Paenibcillus larvae*. The acute toxicity of *Micromeria barbata* essential was tested using different routes of administration. The results showed a difference in the action of the essential oil between the two forms of the tested bacteria. The tested essential oil appeared to be bactericidal against the vegetative form of the tested bacteria and sporicidal against the spore form one. Spraying and feeding the essential oil appeared safe and not toxic to honeybees. in summary, our findings indicate that the tested essential oil can be used as a natural alternative to synthetic antibiotics in treating American foulbrood disease.

Keywords: Essential oils, *Micromeria barbata*, antibacterial action, toxicity, American foulbrood, *Paenibacillus larvae*, Honeybee

1. Introduction

Honeybee (tribe Apini) is a group of insects in the family Apidae (order Hymenoptera) that broadly includes all bees that make honey. They are social creatures that enroll in a caste system to accomplish the tasks that ensure the colony's survival. Honeybee hive's inhabitants are generally divided into three types, the workers, the queen, and the drones and each one has its role. Honey bees play an essential role in the pollination and production of certain beneficial products such as propolis, royal jelly, and especially honey (Suwannapong et al., 2012). Products of honeybees are beneficial and have different biological activities including, antibacterial, anti-viral, anti-fungal, anti-protozoal, anti-inflammatory and antioxidant (Anjum et al., 2019; Pasupuleti et al., 2017; Vijay D. Wagh, n.d.).

Several diseases can affect honeybees, including Varroa mite infestation, Nosema, American foulbrood, European foulbrood, Chalkbrood, and Deformed wing virus. These diseases can weaken the bees, reduce their honey production, and even lead to colony collapse (Hedtke et al., 2011; Jończyk-Matysiak et al., 2020). *Paenibacillus larvae* is a gram-positive, spore-forming bacterium that causes American foulbrood (AFB) disease, which is a contagious, lethal bacterial disease of honeybee brood (*Apis mellifera*) and it has a global distribution. Alippi et al. (2007) stated that AFB was first discovered in Argentina in 1989 and has since spread extensively to all beekeeping areas, with the highest occurrence rate of over 50% in the Buenos Aires Province (Alippi et al., 2007). There are two forms of *Paenibacillus larvae,* the vegetative and spore forms. The vegetative form of *Paenibacillus larvae* is the active, growing form of the bacterium. In this form, the bacterium can move, metabolize nutrients, and replicate. The vegetative form is typically found in the midgut of infected larvae, where it feeds on the larvae's tissues and secretions. The spore form of *Paenibacillus*

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larvae is a dormant, resistant form of the bacterium that can survive in harsh environments. In the spore form, Paenibacillus larvae are able to survive for extended periods of time and can be spread between colonies on contaminated equipment or by drifting bees. Both the vegetative and spore forms of Paenibacillus larvae are important in the pathogenesis of American Foulbrood disease, with the vegetative form causing the initial infection and the spore form allowing the disease to persist and spread(Lindström et al., 2008). American foulbrood is characterized by brown, viscous larval remains forming a sticky thread when drawn out with a matchstick(Chemurot et al., 2016).

Locke et al. (2019) stated that the most feasible control method to prevent the spread is to burn the infected hive. Antibiotics are used as prophylactic treatment, and they are not considered a sustainable strategy since they only conceal the disease symptoms and do not eliminate the spores responsible for the disease's spread*(Locke et al., 2019)*. Antibiotic use has been banned in most European countries since they can potentially leave residues in honey, which is then consumed by humans (Locke et al., 2019; Yost et al., 2016). Only two antibiotics, oxytetracycline, and tylosin, are approved for preventing and controlling AFB in honeybee colonies. Nevertheless, certain areas of the United States, Canada, and Argentina have reported the presence of P. larvae isolates that are resistant to oxytetracycline(Alippi et al., 2007). Antibiotics are ineffective against spores, but they are used to stop the replication of vegetative bacteria(Meghan Milbrath, 2018). Minimal levels of tetracycline residues in honey might pose a health risk to consumers and contribute to the emergence of AMR (antimicrobial resistance) bacteria in humans (Sarkar et al., 2023). In addition, the use of tetracyclines during pregnancy is not recommended due to potential hepatotoxicity (liver damage) in the mother and the possibility of causing permanent discoloration (yellow or brown) of the fetus' teeth, as well as impeding the growth of fetal's long bones. In addition, plant essential oils have been used in the treatment of AFB disease and there are many in vitro research that show the antimicrobial activity of the essential oils extracted from many plants; each one has a different antimicrobial activity due to environmental and genetic factors as well as extraction method(Tutun et al., 2018).

Essential oils are highly concentrated plant extracts that are derived from various parts of plants, including buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood, and bark. These oils are extracted using different methods. Essential oils are known for their medicinal and therapeutic properties since they act as anti-bacterial, anti-fungal, anti-viral, analgesic, sedative, anti-inflammatory, spasmolytic, and locally anesthetic products. Products such as cosmetic products, perfumes, creams, soaps, food flavors, and preservatives have been incorporated with essential oils (Bakkali et al., 2008). Ramsey et al. (2020) stated that the emergence of antimicrobial resistance had encouraged other researchers to investigate more about the antimicrobial effects of essential oils. For example, *Thymus vulgaris essential oil inhibits the growth of S. aureus, V. parahaemolyticus, C. perfringens,* and *Origanum vulgare* essential oil also inhibits the growth of *Poliovirus, Adenovirus,* and *L. monocytogenes (Ramsey et al., 2020).* A study by *Alippi et al.(2008)* shows that lemon grass and thyme essential oils are effective inhibitors of *P.larvae* bacteria*(Alippi et al., 2008).*

Micromeria barbata is a plant that belongs to the genus *Micromeria*, is a member of *Labiatae* family and is widespread in Mediterranean regions (Bakkour et al., 2012). Traditionally, *Micromeria* has been used to cure inflammation, fever, asthma, skin diseases, cardiac problems, and digestive system disorders. Biological activities of *Micromeria* species, such as anti-fungal, antimicrobial, antioxidative, anticholinesterase, and anti-inflammatory activities, as well as the gastroprotective, hepatoprotective, and cytotoxic activities, have been confirmed in several studies (Küpeli Akkol et al., 2019). Studies showed that *Micromeria barbata* essential oil has antimicrobial activity against several microbes such as *Mycobacterium tuberculosis* (including MDR), *Mycobacterium kansasii*, and *Mycobacterium gordonae(El Omari et al., 2019)* and against certain gram positive and gram negative strains as well as *Candida albican* fungi (Alwan et al., 2016).

This study aims to evaluate the antibacterial activity of *Micromeria barbata* essential oil on both forms of *Paenibacillus larvae* bacteria and to check the acute toxicity of this oil on honeybees.

2. Materials and Methods

2.1 Study area

The study was conducted in Tripoli and Dinnieh, districts in North Lebanon from June 2022 till April 2023. Laboratory work was conducted in Quality Control Center (QCC) at the Chamber of Commerce, Industry, and Agriculture of Tripoli and North Lebanon and in Al Jinan University laboratories.

2.2 Plant material

The samples of *Micromeria barbata* plant were harvested from an area in Dinniyeh, North Lebanon, at 1350m height. The collected plant was dried using shade drying at room temperature for five days and then crushed for oil extraction*(Alwan et al., 2014).*

2.3 Essential oil extraction

Micromeria barbata's essential oil was extracted using hydro-distillation technique for 3 hours in an all-glass Clevenger type that separates oil from water*(Alwan et al., 2014)*. The procedure took about 3 hours. At the end of distillation, two phases were observed, the aromatic water and the essential oil. The essential oil was collected and stored in a dark bottle at 4°C.

2.4 Sample collection

An ATCC *Paenibacillus larvae* CMUL (Collection microbiologique-Université Libanaise) 146 was used. This strain was taken from Ecole Doctorale des Science et de Technologie at Lebanese University. Sheep blood agar was used for the cultivation and growth of tested microorganisms.

2.5 Vegetative form Paenibacillus larvae

2.5.1 Preparation of bacterial suspension

In a test tube containing 10ml buffer peptone water, we inoculated the tested microorganism and mixed it. We kept inoculating colonies of the tested microorganism until reaching 0.1-0.12 Absorbance on the spectrophotometer, i.e., 10^8 CFU/ml. After that, we diluted the prepared mixture until reaching 10^6 CFU/ml bacterial suspension.

2.5.2 Disk diffusion method

On a blood agar plate, we inoculated the tested microorganism from the prepared bacterial suspension. Two antibiotic discs against gram-positive bacteria (Streptomycin and Tetracycline) and one against gram-negative bacteria (Sulpha/Trimethoprim) were used. An additional filter paper disc was impregnated with 5μl *Micromeria barbata* essential oil and placed on the inoculated plate. The plate was then incubated at 37°C for 24 hours to measure the diameter of the zone of inhibition.

2.5.3 Determination of minimum inhibitory concentration

The *Micromeria barbata* essential oil was evaluated for its potential antibacterial activity by using the broth dilution method. In a conical tube, we mixed 500 μl of essential oil with 2-3 drops of Tween80 for the oil to be emulsified with the buffer peptone water during the serial dilution method. Then, a serial dilution of the essential oil (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048, 1/4096) was prepared in conical tubes containing buffer peptone water, 50μl of the prepared bacterial suspension was added to each of the 12 tubes, and they were incubated at 37°C for 24 hours. Two additional tubes were used as negative and positive controls, the positive control tube containing buffer peptone water and bacteria and the negative control tube containing buffer peptone water only.

2.5.4 Determination of minimum bactericidal concentration

The MBC is the lowest concentration of the compound at which no growth was observed in plates. After checking the MIC, we checked the turbidity to subculture the tubes starting from the last three non-turbid tubes to the first turbid one. Then, we inoculated the tubes on blood agar and incubated them for 24 hours at 37°C.

2.6 Spore form Paenibacillus larvae

2.6.1 Cultivation of spore form

We inoculated the colonies of vegetative form of the tested microorganism on blood agar and left it at room temperature for about two weeks.

2.6.2 Gram stain and carbol fuchsin stain

Gram staining method was performed to confirm and check the morphology of the spore form bacteria. Carbol fuchsin stain was also used by adding a few drops of carbol fuchsin to a heat-fixed bacteria and we left it for about 30 seconds and washed and blot dried the slide to be observed under the microscope.

2.6.3 Determination of minimum inhibitory concentration

- **Disk diffusion**

A serial dilution of the essential oil (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048, 1/4096) was prepared in conical tubes containing buffer peptone water. On ten blood agar plates, we inoculated colonies of *Paenibacillus larvae* spores. In each tube, we dipped a filter paper disc and placed it on each plate. Then, we left them at room temperature for 24 hours.

- **Incorporating essential oil with blood agar**

We prepared and autoclaved about 200ml blood agar mixtures. Next, we prepared a serial dilution of essential oil from tubes 1 to 12 using ½ dilution factor. Then, we took from each of the 12 essential oil mixture tubes about 5ml and we mixed them with 5ml of prepared blood agar. After that, we inoculated the spore form of the tested microorganism on each petri dish and left them at room temperature for 24 hours. An additional positive control (bacteria on regular blood agar) petri dish was inoculated too.

2.6.4 Determination of minimum bactericidal concentration

We sub-cultured all the plates that showed no growth of bacteria in nutrient broth and incubated them at 37°C for 24 hours. After incubation, we sub-cultured each tube on a blood agar plate and checked the growth after incubation for 24 hours at 37°C.

2.7 Acute toxicity of Micromeria barbata essential oil on honeybees

Special boxes made up of cork have been used to place the tested bees in them. These boxes have a design similar to that of the apiary but are smaller in size and have the same conditions as the regular apiary. Two methods have been used to check the acute toxicity of *Micromeria barbata* essential oil on honeybees through feeding and spraying.

- **Through feeding**

In each box, about 50 bees with a queen were placed. Using powdered sugar, water, essential oil and Tween 80, we formed a sugar paste to be placed in cork boxes. Two different concentrations resulting from the MIC tested against both forms of tested bacteria were used. The third box was a control box containing water and powdered sugar only. After that, the boxes were left in an open area for about four days.

- **Through spraying**

In a spraying bottle, we mixed the concentrations resulting from the MIC tested with water and Tween 80, sprayed the honeybees in the cork boxes with it, and left them for about 2 hours. An additional control box was used and it was sprayed with water only.

3. Results

3.1 Anti-bacterial activity of Micromeria barbata essential oil on the vegetative form P.larvae

3.1.1 Disk diffusion method

The selected three antibiotics with the tested essential oil have been screened for their antibacterial activity against *P.larvae* using the disk diffusion method. The results were represented as the diameter of the inhibition zone. *P.larvae* was susceptible to Streptomycin, Tetracycline, and *Micromeria barbata* essential oil with a diameter of 3.3cm, 3cm, and 2cm, respectively. The negative control antibiotic, Sulpha Trimethoprim, showed no inhibition against the bacteria.

3.1.2 Determination of MIC and MBC

Antibacterial activity of *Micromeria barbata* EO against vegetative form *P.larvae* was evaluated by determining the MIC and MBC. For the MIC, the results showed that tube 9 was the last tube with no turbidity with a 1/512 dilution factor. After inoculation of the last three non-turbid tubes (tubes 7,8,9) and the first turbid one (tube 10), the bacteria grew on plate 10 only (Table 1). This means that the essential oil remained 100% active against *Paenibacillus larvae* to a 1/512 dilution factor.

Table 1 Minimum inhibitory concentration (MIC), turbidity for different concentrations of Micromeria barbata essential oil after incubation

Dilution factor	Bacterial growth(turbidity)
1/2	Not turbid
1/4	Not turbid
1/8	Not turbid
1/16	Not turbid
1/32	Not turbid
1/64	Not turbid
1/128	Not turbid
1/256	Not turbid
1/512	Not turbid
1/1024	Turbid
1/2048	Turbid
1/4096	Turbid

3.2 Anti-bacterial activity of Micromeria barbata essential oil on the spore form P.larvae

3.2.1 Cultivation and detection of *P.larvae* spores

P.larvae spores were cultivated on sheep blood agar and were detected using the gram stain and the carbol fuchsin stain. The gram staining of the tested bacteria appeared to be bright rod-shaped bacteria and the carbol fuchsin staining appeared to be bright ellipsoidal bacteria.

3.2.2 Determination of MIC

Spores are more resistant to antibacterial products than the vegetative form bacteria. We performed two ways to detect if the *Micromeria barbata* essential oil has bacteriostatic or bactericidal activity against *Paenibacillus larvae* spores.

- **Disk diffusion method**

Zone of inhibition appeared around disks that are placed on plates 1,2,3 and 4. Plate 5 showed no zone of inhibition. This means that the MIC using disk diffusion method appeared on plate 4 with 1/16 dilution factor (Figure 1).

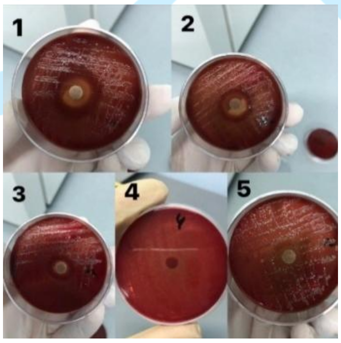

Fig. 1 A zone of inhibition shown in 4 different plates around the disk that are dipped in 4 different concentrations of *Micromeria barbata* essential oil

- **Incorporating essential oil with blood agar**

After incubation at room temperature, there was no growth of bacteria in the first four plates and the growth started to appear on plate 5. This means the MIC using this method appeared on plate 4 with a 1/16 dilution factor (Figure 2).

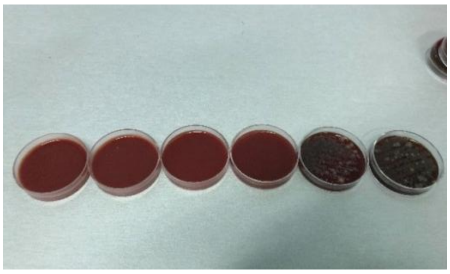

Fig. 2 No appearance of Paenibacillus larvae spores in the first four plates

3.2.3 Determination of MBC

MBC was determined by subculturing the plates that showed no growth of bacteria. The result showed that the bacteria grew on the four plates after incubation. Gram staining of bacteria was also done to confirm our results. This showed that the *Micromeria barbata* essential oil has a bacteriostatic and not a bactericidal effect on *Paenibacillus larvae* spores.

3.3 Acute toxicity of Micromeria barbata essential oil on honeybees

Exposing the bees to *Micromeria barbata* essential oil through feeding (oral route) and spraying (inhalation route) showed 0 mortality of bees (Figure 3). This means that *Micromeria barbata* essential oil is nontoxic to bees and it is safe to be used in treating bees through feeding or spraying.

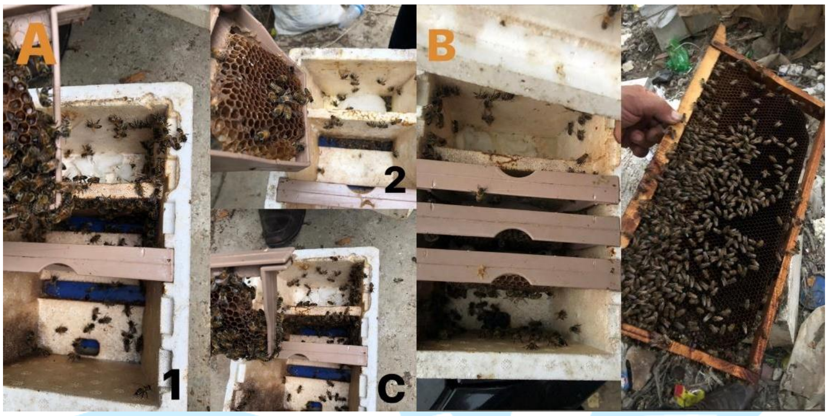

Fig. 3 (A) 0 Mortality of bees after feeding and **(B)** 0 Mortality of bees after spraying

4. Discussion

Antimicrobial resistance is a critical health issue that threatens human and animal health. It is primiraly due to the misuse and overuse of antibiotics. Extensive agricultural use of antibiotics can lead to the transfer of antibiotic resistance from treated animals to humans through the consumption of animals and their products*(Ventola, 2015).* Natural products, including essential oils, offer an alternative to synthetic antibiotics and may offer some benefits for preventing or treating certain infections. However, the effectiveness of natural products can vary, and they may not be as potent or effective as synthetic antibiotics for treating certain infections (Yap et al., 2014).

Honeybees are important pollinators for many crops and wild plants and pollination can increase crop yield and quality and reduce fruit drop unlike other pollinators*(Suwannapong et al., 2012)*. Honeybees are susceptible to a variety of diseases, including bacterial, fungal, viral and parasitic infections such as, American foulbrood (AFB), European foulbrood, Nosema, Chalkbrood, Varroa mites, Deformed wing virus (DWV) and Sacbrood virus (SBV). Bacterial infections of bees such as American foulbrood (AFB), European foulbrood (EFB) and Nosemosis have been treated and controlled using antibiotics ("Food and Agricultural Organization of the United Nations," 2018).

Our present study has primarily focused on the importance of honeybees and their products and how to use a natural product to treat the most contagious honeybee disease, American foulbrood instead of synthetic antibiotics. Several essential oils have been tested for their potential in treating honeybee diseases. *Tutun et al. (2018)* stated that essential oils of Clove, Thyme, Sage, Lemon grass, Mint and Rosemary have an antiparasitic activity against Varroa mites (Tutun et al., 2018). American foulbrood disease can also be treated by using essential oils such as Thymus vulagaris (thyme) and Origanum vulgare (oregano) (González and Marioli, 2010).

In this study, we checked the antibacterial activity of *Micromeria barbata* essential oil on both forms of *Paenibacillus larvae*. Both forms are important in the pathogenesis of American Foulbrood disease, with the vegetative form causing the initial infection and the spore form allowing the disease to persist and spread*(Lindström et al., 2008)*. Therefore, effective control of American Foulbrood disease requires strategies that target both forms. The disk diffusion results showed that the tested microorganism is susceptible to the *Micromeria barbata* essential oil with 2cm diameter of zone of inhibition. Certainly, the essential oil's minimum inhibitory concentration (MIC) cannot be determined by the disk diffusion method, so a serial dilution method and others was used. Several studies have demonstrated the antimicrobial effects of essential oils against vegetative bacteria. For example, tea tree oil and Ginger oil has been shown to have potent antibacterial activity against common pathogens such as *Staphylococcus aureus* and *Escherichia coli*

(Carson et al., 2006; Wang et al., 2020). In this study, checking the MIC and MBC of tested essential oil on the vegetative form bacteria showed that the essential oil remained 100% active against *Paenibacillus larvae* to a 1/512 dilution.

On the other hand, some essential oils have a sporicidal activity against spore-forming bacteria. A study published in the Journal of Microbiology and Biotechnology in 2009 found that the essential oils of cardamom, juniper leaf, and tea tree have a sporicidal activity against Bacillus subtilis spore forming bacteria*(Lawrence and Palombo, 2009)*. However, some essential oils are considered sporostatic, i.e., they can inhibit the germination of spores into vegetative form bacteria. A study done by *Chaibi et al. (1997)* found that EO of camomile and grapefruit were sporostatic against the spores of *Bacillus cereus spores* with (≥300 ppm)*(Chaibi et al., 1997)*. In our study, MIC and MBC showed that the EO of *Micromeria barbata* has a sporostatic and not a sporicidal effect on *Paenibacillus larvae* spores.

The toxicity of essential oils on honeybees can depend on various factors, including the type of essential oil, the oil concentration, and the application method. Different essential oils have different chemical compositions, which can affect their toxicity to bees. For example, Thymol, a major constituent of essential oils of thyme, negatively affects honeybees and has been found in wax, pollen and, honey. Similarly, carvacol, which is found in high concentrations in oregano essential oil, also harms honeybees (Glavan et al., 2020). Hýbl et al. (2021) stated that carrot, peppermint, litsea, and pelargonium essential oils are not toxic to honeybees (Hýbl et al., 2021). Alonso-Salces et al. (2017) stated that there are different routes for administering essential oils to honeybees, such as feeding, spraying and systemic administration*(Alonso-Salces et al., 2017)*. Dealing with honeybees is considered delicate, so cooperating with beekeepers was required. In our study, we contacted several beekeepers who trained us to apply the tested essential oil on honeybees through spraying and feeding and to read the results obtained. Our results proved that introducing *Micromeria barbata* essential oil through feeding and spraying honeybees showed 0 mortality of honeybees. We aimed to check the antibacterial action of the tested essential oil directly on diseased honeybees but it was difficult for us to find such disease and due to other circumstances that faced us while investigating.

5. Conclusion

In recent years, the phenomena of drug resistance are becoming more and more widespread. The use of essential oils as an alternative to synthetic antibiotics in treating honeybee diseases has been tested and confirmed in different studies. However, proper testing, standardization, and regulation of essential oils are essential to ensure their safety and efficacy as a potential alternative to synthetic antibiotics. In this study, the antibacterial activity of *Micromeria barbata* essential oil against *Paenibacillus larvae* has been confirmed and its use has been considered safe and nontoxic to honeybees. Further studies regarding the in vivo testing of the tested oil directly on diseased honeybees should be done to check if this essential oil has both preventive and treatment measures.

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Declaration of Conflict

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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