AGRICULTURAL SCIENCE AND TECHNOLOGY, VOL. 13, No 4, pp 404-408, 2021
Published by Faculty of Agriculture, Trakia University, Bulgaria
ISSN 1313-8820 (print)
ISSN 1314-412X (online)
http://www.agriscitech.eu
DOI: 10.15547/ast.2021.04.065

Evaluation of the antimicrobial and anti-*Varroa destructor* L. activity of the essential oil of clove (*Syzygium aromaticum* L. Myrtaceae)

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(Manuscript received 11 March 2021; accepted for publication 5 September 2021)

Abstract. *Antimicrobial and anti-Varroa destructor L. activity of the essential oil (EO) of clove (Syzygium aromaticum L. Myrtaceae) was evaluated in this study. Antimicrobial activity concerned 9 bacterial strains (Bacillus cereus*, *Escherichia coli* ATCC 25911, *Staphylococcus aureus* 29213 ATCC, *Proteus mirabilis*, *Serratia marcescens*, *Enterobacter sakazakii, Pseudomonas aeruginosa* ATCC, *Escherichia coli and Acinetobacter* sp.*), a fungal strain (Penicillium sp.) and a yeast species (Candida albicans). The extraction of the EO was carried out by the method of hydrodistillation. Results showed that EO has no toxicity on bees Apis mellifera. EO demonstrated effective and stable anti-V. destructor activity, indicating the absence of possible resistance, in contrast to what was observed for Amitraz. The inhibitory activity of EO revealed an inhibition zone of diameter varying between 20 mm and 42 mm for bacteria. However, no inhibition zones were observed for Pseudomonas aeruginosa ATCC. The inhibitory activity of EO on Penicillium sp and Candida albicans revealed an inhibition zone of 39 mm in diameter. This study shows that the EO of cloves constitutes a simple and natural treatment, without inconveniences, with a high activity antimicrobial and anti-Varroa destructor which merits it to be proposed as a means to fight against varroosis and the tested pathogens.*

Keywords: Algeria, antimicrobial activity, essential oil, *Syzygium aromaticum* L. Myrtaceae, *Varroa destructor*

Introduction

The problems of toxic residues and antimicrobial resistance of most antimicrobial drugs severely compromise the effectiveness of their curative and protective effects (Han and Parker, 2017). The use of more effective molecules is vital to prevent these inconveniences. Natural products are an ideal solution because of their low toxicity for the environment and the absence of residues in food products (Bogdanov, 2006). Among these natural products are essential oils (Damiani et al., 2009; Kloucek et al., 2012).

Essential oils are the concentration of a hydrophobic liquid containing multiple volatile aromatic components present in the glands located in different parts of aromatic plants: flowers, leaves, seeds, fruits, bark and roots (Bayala et al., 2014). Essential oils have antimicrobial, antiparasitic, antiviral and antifungal properties (Hyldgaard et al., 2012; Turek and Stintzing, 2013).

Syzygium aromaticum L. Myrtaceae, also known as clove, is a dried flower bud member of the Myrtaceae family (Bhuiyan et al., 2012; Cortés-Rojas et al., 2014). It contains various bioactive compounds such as volatile oil (15-20%) and phenolic propanoids (60-90%) (Chaieb et al., 2007). All known oils worldwide have shown variable antimicrobial activity. Clove, besides its use as perfume and food flavor, has culinary and medical properties by its antiseptic, anti-inflammatory, antioxidant, antifungal, antiviral and antiparasitic actions (Alitonou et al., 2012; Mohamed and Badri, 2017). The antimicrobial activity of clove is due to eugenol, oleic acids and lipids found in its essential oils (Hammer et al., 1999).

Varroa destructor is an external parasitic mite of honey bees that can cause significant losses of honey bee colonies worldwide. They destroy bee colonies during larval and pupae development by feeding on hemolymph, resulting in decreased body weight and reduced bee life span (Rosenkranz et al., 2010). Many synthetic drugs are used to control varroosis but the use of these drugs is limited for reasons such as the development of resistance and contamination of bee products, particularly honey and beeswax, which led to the idea of finding new and safer ways to control these mites (Damiani et al., 2009). In addition, the use of antibiotics in the treatment of bee diseases has been banned in some countries (Genersch, 2010).

The use of essential oils in the treatment of bee mites is a recent method (Turek and Stintzing, 2013). They have a repulsive and toxic effect on arthropods through fumigation, topical use or ingestion (Umpiérrez et al., 2011). It has been reported that the use in bees of Clove oil has considerable acaricidal properties against varroa mites (Mahmood et al., 2014; Li et al., 2017).

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The main objective of the study is, for the first time in Algeria, to evaluate the antimicrobial and anti-*Varroa destructor* L. activity of the essential oil of clove (*S. aromaticus* L.).

Material and methods

Extraction of the essential oil (EO)

The extraction of the EO was carried out on 900 g of clove flower buds, *S. aromaticum* L., by the method of hydrodistillation using Clevenger's apparatus where the plant material to be extracted was placed in direct contact with boiling water, the water vapor produced carries with it the essences of the plant (Baser and Buchbauer, 2010). Briefly, 150 g of dried and crushed cloves were placed in a round bottom flask containing 500 ml of distilled water, connected to the hydrodistillation system and heated for 30 minutes. Then, the resulting mixture containing the oil mixed with water was collected and refrigerated for 24 hours at 4 °C to separate the EO. The EO was dried with sodium sulfate and stored in dark bottles in the refrigerator until use.

Evaluation of EO activity against the parasite Varroa destructor

The study was carried out in the Blida region and involved three hives infested with *V. destructor*. Two doses of the EO were prepared as follows:

- Dose 1 (D1) = 0.25 mg of the essential oil + 99.75 ml of Tween 80 at 5%;
- Dose 2 (D2) = 0.15 mg of the essential oil + 99.85 ml of Tween 80 at 5%.

Strips of concentrated plastic polymer were each impregnated with the different dilutions (D1, D2) and with Amitraz, which is known for its better efficacy in the control of *V. destructor* (Semkiw et al., 2013). The first lot of beehives was treated with D1 (0.25% EO), the second lot of beehives was treated with D2 (0.15% EO) and the third lot was treated with Amitraz. Two strips were placed per beehive. Metal plates, impregnated with fat as an adhesive, were placed under each beehive to trap varroa mites. Parasite samples were taken once a week for 6 weeks.

EO toxicity test for the bee Apis mellifera

For this test, we put 10 *Apis mellifera* bees in Petri dishes each with one drop (1 ml) of EO at 0.15% and 0.25% concentration, and then the Petri dishes were covered with a compress.

Evaluation of the antimicrobial activity of EO

The evaluation of the antibacterial activity was performed according to the disc diffusion method or aromatogram as described by Houle (2004). A total of 9 bacterial strains (*Bacillus cereus*, *Escherichia coli* ATCC 25911, *Staphylococcus aureus* 29213 ATCC, *Proteus mirabilis*, *Serratia marcescens*, *Enterobacter sakazakii*, *Pseudomonas aeruginosa* ATCC, *Escherichia coli* and *Acinetobacter* sp.), a fungal strain (*Penicillium* sp.) and a yeast species (*Candida albicans*) were used to test the antimicrobial and antifungal efficacy of Clove EO. These strains were provided by the reference hygiene laboratory, Blida.

Replicating the strains

Bacteria and fungi were subcultured, respectively, on Mueller Hinton and Sabouroud agar with chloramphenicol 0.5 mg/ml.

Preparation and deposit of antibiotic discs

Blank sterile antibiotic discs of 9 mm diameter were impregnated with 10 µl of the EO of *Syzygium aromaticum* L. Myrtaceae and deposited in the center of the petri dishes containing cultures of the previous bacterial strains, the fungus *Penicillium* sp or the yeast *Candida albicans*. For each pathogen, two Petri dishes were tested. The Petri dishes were incubated at 37°C for 24 hours for the bacteria, at 30°C for 72 hours for *Penicillium* sp and at 37°C for 48 hours for *Candida albicans*.

Measurement of the inhibition zones

The estimation of the antimicrobial activity of the EO has been classified into 5 classes, according to the diameter of the microbial growth inhibition zone (Ela, 1996):

- >28 mm: Very strongly inhibitory;
- 20-28 mm: Strongly inhibitory;
- 16-19 mm: Moderately inhibitory;
- 10-15 mm: Mildly inhibitory;
- <10 mm: Non-inhibitory.

A positive control was used by testing the sensitivity of the strains used in this study by the antibiogram to two antibiotics, Josacin and Cefalexin.

Results and discussion

In the present study, we tested the anti-varroa and antimicrobial activity of the EO of the flower buds of the plant *Syzygium aromaticum* L. Myrtaceae. The results showed a very effective activity compared to the positive controls with no toxicity to bees. Amitraz showed high efficacy during the first week, which gradually decreased, suggesting varroa resistance. The extraction by hydrodistillation of 900 g of clove flower buds gave a quantity of 19 ml of EO, of which we have calculated the yield which is 2.11%.

Essential oils are beginning to have a very promising interest as a potential source of natural bioactive molecules. They are being studied for their possible use as an alternative for insecticide, acaricide, bactericide, nematicide and fungicide treatments (Raveau et al., 2020).

The use of essential oils products in bees has several advantages including their low toxicity to bees and the absence of any resistance to these drugs (Rosenkranz et al., 2010). In the present study, after 10 min, 20 min up to 30 min of observation of the petri dishes, we noticed that the bees did not present any physical or behavioral anomaly; they flew away at the opening of the dish.

The number of *Varroa destructor* parasites collected (Figure 1), in this study, during the first week post-treatment was 80 and 70 for lots treated with EO at 0.25% and 0.15%, respectively. The number of parasites sampled after each week remained almost stable with a slight increase during the fourth (S4) and fifth (S5) sampling for the first and second lot, respectively

(Figure 2). For the lot treated with Amitraz, the number of parasites sampled after the first week was very high (312), but this number decreased suddenly to 102 during the second week and to 58 during the last sampling (S6).

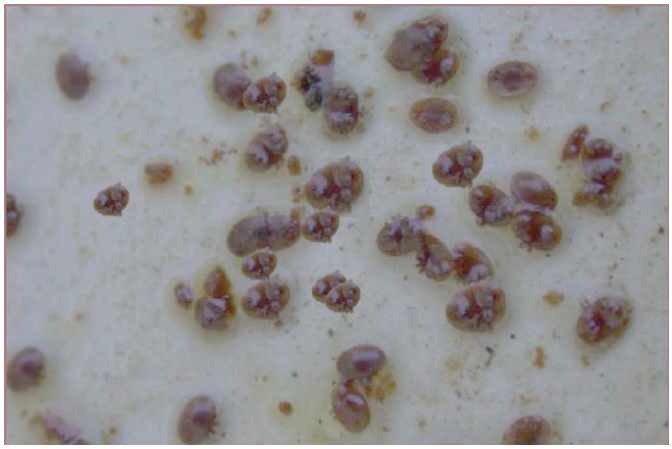

Figure 1. Metal plate containing *Varroa destructor* mites stuck on the fat

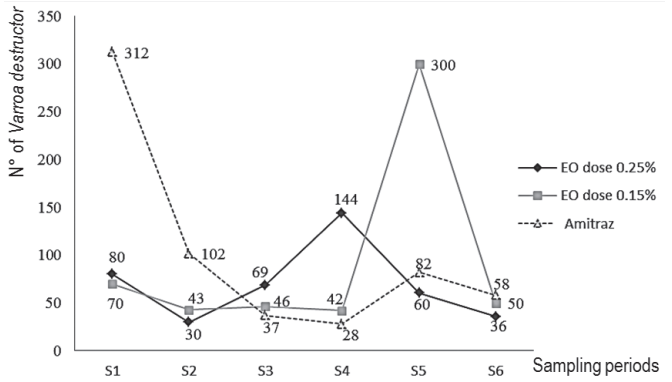

Figure 2. Number of *Varroa destructor* collected during the six sampling periods (S1-S6) after treatment with EO (dose 0.25% and 0.15%) and Amitraz

Varroosis is considered one of the most important diseases in *Apis mellifera* bees (Martin et al., 2012). The anti-varroa activity of *Syzygium aromaticum* (L.) clove oil has been previously demonstrated. The mortality rate can reach 60-96% (Su et al., 2012; Mahmood et al., 2014). Eugenol is the main constituent of this EO, which was recovered in beeswax over a two-week period, supporting the idea that medicinal treatment with clove oil is both stable and long-lasting (Mahmood et al., 2014). Eugenol has different biological properties: bactericidal, antifungal, antiparasitic, antioxidant, anti-inflammatory (Rana et al., 2011).

In our study, we have observed that the oil has no toxicity towards bees and that its use to control *Varroa destructor* has given effective and stable results, which indicates the absence of any possible resistance, contrary to what was observed for Amitraz.

Various acaricides have been applied to control Varroas mites, particularly amitraz (Milani and Iob, 1998). On the other hand, Varroas mites have rapidly developed resistance to this drug (Thompson et al., 2002) which confirms our finding in this study.

Several studies have demonstrated the antimicrobial efficacy of clove oil against different fungal and bacterial strains. Cloves can destroy the cell walls of pathogenic microorganisms and penetrate cells to inhibit normal DNA and protein synthesis (Xu et al., 2016). The study by Dorman and Deans (2000) demonstrated the antibacterial efficacy of clove oil against 25 strains of gram-negative and gram-positive bacteria.

In this study, the inhibitory activity of EO revealed an inhibition zone of diameter equal to 22 mm for *Escherichia coli* ATCC 25911 (Figure 3A), 20 mm for *Escherichia coli*, 40 mm for *Bacillus cereus*, 42 mm for *Staphylococcus aureus* 29213 ATCC, 20 mm for *Proteus mirabilis*, 22 mm for *Serratia marcescens*, 21 mm for *Enterobacter sakazakii* and 29 mm for *Acinetobacter* sp. However, no inhibition zones were observed for *Pseudomonas aeruginosa* ATCC. The inhibitory activity of EO on *Penicillium* sp. and *Candida albicans* revealed an inhibition zone of 39 mm in diameter (Figure 3B,C).

Figure 3. Inhibition zone of microbial growth due to EO effect: A- *Escherichia coli* ATCC 25911; B-*Penicillium* sp.; C- *Candida albicans*

For the positive control results, the inhibitory activity of Cefalexin was 24 mm, 23 mm, 21 mm, 23 mm, 23 mm, 21 mm, 30 mm, and 25 mm for *Escherichia coli* ATCC 25911, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* 29213 ATCC, *Proteus mirabilis*, *Serratia marcescens Enterobacter sakazakii*, and *Acinetobacter* sp., respectively. However, no inhibition zones were observed for *Pseudomonas aeruginosa* ATCC and *Acinetobacter* sp. Josacin inhibitory activity was observed only in two bacteria *Bacillus cereus*, and *Staphylococcus aureus* 29213 ATCC (35 mm and 20 mm, respectively). Also, no inhibition zones were observed for the other bacteria.

The antimicrobial efficacy of clove oil has been demonstrated against *Serratia marcescens*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus vulgaris*, *E. coli Enterobacter* sp., *Acinitobacter* sp. by many authors (Jirovetz et al., 2006; Fu et al., 2007; Liu et al., 2012; Abdullah et al., 2015). In our study, the inhibitory activity of EO revealed zones of inhibition varying in diameter from 21 mm to 42 mm for the bacteria tested with the exception of *Pseudomonas aeruginosa* ATCC where no zone of inhibition was observed. Oulkheir et al. (2017) found that the clove EO produced an inhibition zone against *E. coli* of 16 mm and a higher inhibitory zone (20 mm) against *Salmonella* species.

The antifungal efficacy of clove oil has been documented by Rana et al. (2011). Eugenol is the main component of clove responsible for its fungicidal characteristics against *Candida albicans* (Manohar et al., 2001). Clove EO has been proven to be effective against *Penicillium* sp. (Anjum and Akhtar, 2012; Wang et al., 2017).

The oil of *S. aromaticum* showed high activity (22 to

28 mm) against *B. subtilis* and *S. aureus* and 26 to 21 mm against *E. coli* and *P. aeruginosa* though the sensitivities of the microorganisms varied (Fagere and Al Magboul, 2016).

Conclusion

For the first time in Algeria, to our knowledge, this study revealed that the essential oil (EO) of cloves (*Syzygium aromaticum* L. Myrtaceae) has no toxic effect on bees and presented a high activity anti-*Varroa destructor*. It therefore presents the best means of controlling this mite in beehives because no resistance was observed unlike Amitraz. EO of cloves also showed high antibacterial activity against *Escherichia coli* ATCC 25911, *Escherichia coli, Bacillus cereus, Staphylococcus aureus* 29213 ATCC, *Proteus mirabilis*, *Serratia marcescens*, *Enterobacter sakazakii*, and *Acinetobacter* sp. No activities were observed only for *Pseudomonas aeruginosa* ATCC. EO of cloves also presented high fungicidal characteristics against *Penicillium* sp. and *Candida albicans*. This EO constitutes a simple and natural treatment, without disadvantages, which merits it to be proposed as a means of control against *Varroa destructor*; the tested bacteria, *Penicillium* sp. and *Candida albicans*.

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