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Original article

The effects of clove oil on the enzyme activity of *Varroa destructor* Anderson and Trueman (Arachnida: Acari: Varroidae)

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ABSTRACT

Varroa destructor, a key biotic threat to the Western honey bee, has played a major role in colony losses over the past few years worldwide. Overuse of traditional acaricides, such as tau-fluvalinate and flumethrin, on *V. destructor* has only increased its tolerance to them. Therefore, the application of essential oils in place of traditional pesticides is an attractive alternative, as demonstrated by its high efficiency, lack of residue and tolerance resistance. To study the acaricidal activity of essential oils, we used clove oil (*Syzygium aromaticum* L.), a typical essential oil with a wide range of field applications, and examined its effects on the enzyme activities of Ca²⁺-Mg²⁺-ATPase, glutathione-S-transferase (GST) and superoxide dismutase (SOD) and its effects on the water-soluble protein content of *V. destructor* body extracts after exposure to 0.1 μl and 1.0 μl of clove oil for 30 min. Our results showed that the water-soluble protein content significantly decreased after the treatments, indicating that the metabolism of the mites was adversely affected. The bioactivity of GSTs increased significantly after a low dosage (0.1 μl) exposure but decreased at a higher dosage (1.0 μl), while the activities of SOD and Ca²⁺-Mg²⁺-ATPase were significantly elevated after treatments. These results suggest that the protective enzyme SOD and detoxifying enzymes Ca²⁺-Mg²⁺-ATPase and GST contributed to the stress reaction of *V. destructor* to the essential oils and that the detoxification ability of *V. destructor* via GST was inhibited at higher dosages. Our findings are conducive to understanding the physiological reactions of *V. destructor* to treatment with essential oils and the underlying mechanisms behind the acaricidal activities of these natural products.

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1. Introduction

The currently declining health status of the Western honey bee (*Apis mellifera*) has caused great concern globally over the past few years (Neumann and Carreck, 2010). *Varroa destructor* Anderson and Trueman (Arachnida: Acari: Varroidae), an ectoparasite mite of honey bees, is considered the most severe threat to colony health (Dietemann et al., 2012). *V. destructor* feeds on the haemolymph of honey bees, causing several detrimental effects to its

host, including attenuated lifespan, disorientation and decreased immunity (Amdam et al., 2004; Kralj et al., 2007; Rosenkranz et al., 2010; Nazzi et al., 2012). More grave is that these adverse effects of *V. destructor* are compounded by bee viruses, such as DWV and IAPV, thus proving a fatal role in colony collapse (Oldroyd, 2007; Martin et al., 2012). Previous research has shown that untreated varroa-infested colonies usually die within six months to two years (Le Conte et al., 2010). To maintain control of the *V. destructor* population within domesticated *A. mellifera* colonies, various acaricides have been applied in the past: organophosphate coumaphos; pyrethroids, such as tau-fluvalinate and flumethrin; and formamidin amitraz (Milani and Barbattini, 1988; Milani and Iob, 1998). While these acaricides were effective for a time, *V. destructor* mites have rapidly grown resistant to these drugs due to their overuse and the single active composition of traditional miticides (Elzen et al., 1999; Thompson et al., 2002). Furthermore, wide attention has been drawn to the fact that traces of these pesticides have been found

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in bee products, causing additional risk to human health (Bogdanov et al., 1998; Wallner, 1999).

Essential oils are secondary metabolism products that are produced during the course of plant growth (Wallace, 2004). They facilitate the primary metabolism of plants and provide certain defences against herbivores, pests and pathogens to keep the plants healthy (Isman, 2000). Previous research has shown that over 150 of these essential oils have demonstrated varied efficacies in terms of controlling *V. destructor* (Lindberg et al., 2000; Gashout and Guzmán-Novoa, 2009; Su et al., 2012). As an alternative to traditional acaricides, essential oil products present certain advantages, such as high toxicity to mites, low toxicity to bees, few residues in bee products and no drug resistance (Rosenkranz et al., 2010).

The efficacy of clove *Syzygium aromaticum* (L.) merr. et perry (Myrtaceae) oil as a treatment for *V. destructor* mites has been previously demonstrated both in the laboratory and in the field. In a 20-ml glass-vial residual test, 0.75 mg of clove oil caused a 96% mortality rate in *V. destructor* (Gashout and Guzmán-Novoa, 2009). A 2012 study by Su et al. showed that clove oil caused a 60% mortality rate in *V. destructor* mites at a dosage of 1.0 μl for 48 h, a level that is considered safe for bees. Additionally, eugenol, the principal constituent of clove oil, was discovered in beeswax during a two-week period under semi-field conditions, which supports the idea that medicinal treatment with clove oil is both stable and sustainable (Girisgin et al., 2014). Mahmood et al. (2014) also demonstrated the effectiveness of clove oil and regarded it as a promising alternative to treat *V. destructor* in the field. Despite the promising results of prior studies, the underlying acaricidal mechanisms of treatment with essential oils have yet to be elucidated. In this study, we determined the effects of clove oil on the enzyme activities of Ca^{2+} - Mg^{2+} -ATPase, glutathione-S-transferase (GST) and superoxide dismutase (SOD), as well as on the total protein content in the body extracts of *V. destructor* mites. Our aim is to understand the physiological reactions of *Varroa* mites to essential oils and the associated underlying mechanisms behind its acaricidal effects.

2. Materials and methods

2.1. Extraction of clove essential oil

Clove leaves were purchased from Tongrentang Chinese Medicine-Since. Essential oil was extracted according to steam distillation methods described in Chinese Pharmacopoeia (edition 2010). Briefly, 500 g of air-dried clove leaf powder and 5 L of distilled water were combined in a round-bottom distillation flask. The flask was connected to a condenser tube, and heated to a steady boil for five hours. The extracted essential oil was collected into Eppendorf tubes (1.5 ml) and stored at 4 °C until use. According to our previous study, this essential oil consists of 62.28% eugenol, 20.79% caryophyllene, 2.48% α -caryophyllene and 6.03% phenol-2-methoxy-4-(2-propenyl)-acetate (Su et al., 2012).

2.2. Mites collection

A. mellifera colonies that were highly infested with *V. destructor* mites and had been untreated for approximately six months prior served as the *V. destructor* donor colonies. Adult female mites from capped worker or drone brood combs were collected with a small soft brush, placed on wet tissue paper and fed five fifth instar worker bee larvae. Treatments on these mites began within one hour after feeding.

2.3. Exposure to clove oil

A group of *V. destructor* mites (100 mg, ≈ 350 mites) was gathered and placed into a petri dish ($\varnothing = 60$ mm) and fed five fifth instar worker bee larvae (Fig. 1A, B). Wet tissue papers were laid at the bottom of the petri dishes to maintain humidity. A piece of gauze was placed inside the cover of the petri dish. We then applied some clove oil to a small piece of filter paper and secured it between the gauze and the cover (Fig. 1C). According to Su et al. (2012), 1.0 μl clove oil is the maximum dosage that does not cause

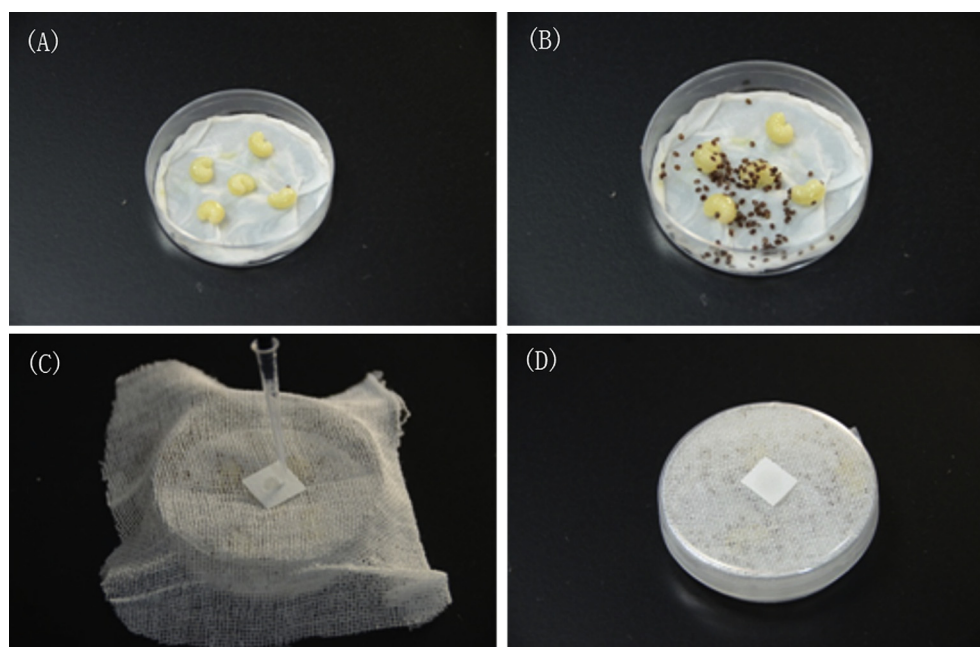


Fig. 1. Experimental setup of the study. (A) The varroa mites were fed five fifth instar worker bee larvae in a petri dish. (B) A group of *V. destructor* mites (100 mg, ≈ 350 mites) were collected and placed in a petri dish. (C) Clove oil was dropped on a small piece of filter paper and secured between the gauze and the cover of the petri dish. (D) The petri dishes containing varroa mites were sealed with a membrane to prevent the volatilization of essential oils.

acute toxicity to the honey bee; however, 20% of *V. destructor* mites died when exposed to this amount for 4 h. Therefore, this dosage was selected for our study. A dosage of 0.1 μl was added to the study to investigate the effects of a lower dosage. The negative control consisted of a test group that was not exposed to the clove oil. The petri dishes were sealed with a membrane to avoid the volatilization of essential oils (Fig. 1D). Three replicates were included in each group; therefore, approximately 1050 mites were used. The petri dishes containing mites and clove oil were incubated (34.5 °C, 70%RH) for 30 minutes. The petri dishes were then frozen (−80 °C) immediately after removal from the incubator.

2.4. Determination of protein content and enzyme activities

Treated mites were washed three times with normal saline (0.9% w/v of NaCl solution). Samples were homogenized in 900 μl of normal saline and centrifuged at 2500 r/min (4 °C) for 10 min. The supernatant was used to determine the water-soluble protein concentration, using a total protein quantification assay kit (A045-3, Nanjing Jiancheng Bioengineering Institute, China). Briefly, the proteins reduced cupric ion to cuprous ion, which was further adducted with bicinchoninic acid (BCA). The product has a maximum absorbance at 562 nm. The protein content was calculated based on the absorbance of the solution.

The enzyme bioactivities of GST, SOD and $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ were evaluated using a GST assay kit (catalog no. A004, Nanjing Jiancheng Bioengineering Institute, China), an SOD assay kit (catalog no. A001-3, Nanjing Jiancheng Bioengineering Institute, China) and a $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ assay kit (catalog no. A070-3, Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

2.5. Statistical analysis

The total protein content and the enzyme bioactivities of the different groups were analysed using a one-way ANOVA in SPSS (version 17.0).

3. Results

The water-soluble protein content of the *V. destructor* mites decreased significantly with exposure to clove oil: control group (690.84 \pm 8.95 $\mu\text{g/ml}$), 0.1 μl clove oil (479.43 \pm 51.35 $\mu\text{g/ml}$) and 1.0 μl clove oil (513.52 \pm 25.63 $\mu\text{g/ml}$) (df = 2, $F = 37.303$, $p_{0.1 \text{ vs control}} < 0.05$, $p_{1.0 \text{ vs control}} < 0.05$, Fig. 2A). However, no significant difference was observed between the two treatment groups ($p_{0.1 \text{ vs } 1.0} = 0.466$). As for the GST bioactivity of mites, it significantly increased at a dosage of 0.1 μl (78.42 \pm 9.84 U/mg protein) compared with that of the control group (44.59 \pm 2.15 U/mg protein, $p_{0.1 \text{ vs control}} < 0.05$); however, when treated with 1.0 μl of clove oil, the activity of this enzyme decreased (37.71 \pm 3.21 U/mg protein), but the difference was not significant compared to the control group ($p_{1.0 \text{ vs control}} = 0.424$, Fig. 2B). The bioactivity of $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ was significantly elevated when treated with 0.1 μl (5.19 \pm 0.47 U/mg protein) and 1.0 μl (5.25 \pm 0.31 U/mg protein) compared with the control group (3.62 \pm 0.145 U/mg protein). There was no significant difference between the two treatment groups (df = 2, $F = 22.271$, $p_{0.1 \text{ vs control}} < 0.05$, $p_{1.0 \text{ vs control}} < 0.05$, $p_{0.1 \text{ vs } 1.0} = 0.981$, Fig. 2C). A similar trend was observed for the enzyme activity of SOD, which was significantly elevated at 0.1 μl (40.11 \pm 5.23 U/mg protein) and 1.0 μl (37.10 \pm 1.46 U/mg protein) compared with that of the control group (28.52 \pm 0.65 U/mg protein, df = 2, $F = 15.924$, $p_{0.1 \text{ vs control}} < 0.05$, $p_{1.0 \text{ vs control}} < 0.05$, $p_{0.1 \text{ vs } 1.0} = 0.393$, Fig. 2D).

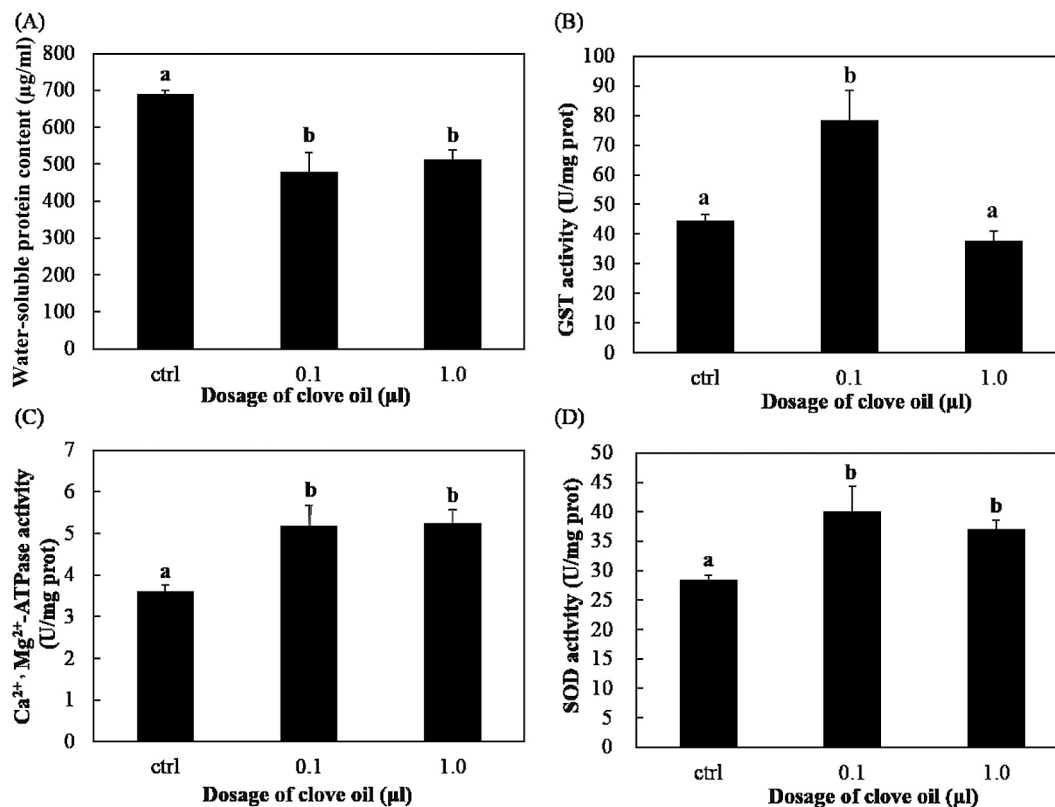


Fig. 2. Water-soluble protein content and enzyme bioactivities of *V. destructor* with and without clove oil treatment. Mean values \pm S.D. are shown. Different letters above the columns indicate significant differences among the groups ($p < 0.05$).

4. Discussion

In some countries, clove oil has been used to treat *V. destructor* in the field. However, an explanation of the biochemical mechanisms behind its acaricidal effects is lacking thus far. By determining the water-soluble protein content and enzyme activities of *V. destructor* when exposed to different amounts of clove oil, we explored its physiological effects on *V. destructor* mites.

The *V. destructor* mite feeds on the haemolymph of honey bees. Lacking proteinase, the mite makes use of the host's undigested haemolymph proteins to promote oogenesis. As the major tissue type of the open circulatory system of lower arthropods (Wyatt, 1961), haemolymph is the site of metabolic processes and the exchange and storage of substances, such as proteins (Gudderra et al., 2002), and it plays a vital role in immunity and defence mechanisms (Theopold et al., 2004). Because proteins are important functional components of living organs, our study examined the water-soluble protein content of *V. destructor*. Our results revealed that it significantly decreased after clove oil treatment, indicating that the metabolism and the immunity system of the mites were possibly affected. This may have been caused by a decrease in feeding activity or an increase in the digestion of proteins by the mites.

In oxidative metabolism, reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^\cdot) contribute to (Livingstone, 2001) oxidative stress, leading to amino acid and DNA damage (Imlay, 2003; Stadtman and Levine, 2003). Antioxidant enzymes, such as superoxide dismutase (SOD) (Dubovskiy et al., 2008), help prevent ROS-induced damage by removing oxygen free radicals; therefore, they play an important role in the antioxidant defence system (Park et al., 2009). In our study, SOD activity significantly increased after clove oil treatments, suggesting that the antioxidant reaction of *V. destructor* is triggered by essential oils.

Calcium-magnesium ATPase (Ca^{2+} - Mg^{2+} -ATPase), which is universally considered an indicator of cell health (Haya and Waiwood, 1983; Atli and Canli, 2013; Eroglu and Canli, 2013), acts as a membrane-bound enzyme and is responsible for the maintenance of calcium homeostasis in cells (Carafoli, 1991; Nadukuru and Yallapragada, 2015). Changes in ATPase activity, caused by a disruption in transmembrane cation transport, are identified as key factors in cellular dysfunction (Stephenson, 1985). While a temporary increase in intracellular calcium ion concentrations is necessary to activate physiological functions of tissues, a sustained increase will lead to cell death (Orrenius et al., 1989). Therefore, it is critical to protect calcium homeostasis against interference from any extraneous toxins. The bioactivities of Ca^{2+} - Mg^{2+} -ATPase were significantly elevated at both clove oil dosages, suggesting that clove oil may influence the cell function of *V. destructor* by inducing an excessive intracellular calcium ion concentration.

The glutathione-S-transferase enzymes are a major family of enzymes that detoxify cytosol (Ranson et al., 1997) by protecting against endogenous and exogenous toxic chemicals (Sheehan et al., 2001). GSTs protect other endogenous substances, such as proteins and nucleic acids, by catalysing the conjugation of activated xenobiotics and endogenous glutathione (GSH) to a water-soluble substrate. Elevated levels of GST activity are associated with insecticide resistance, such as DDT resistance in *Anopheles gambiae* (Ranson et al., 1997). In this study, the bioactivity of GSTs increased significantly after treatment with 0.1 μ l of clove oil, indicating that the self-protection mechanism of *V. destructor* mites was triggered by exposure to a low dosage of essential oil. In contrast, it was interesting to see that GST bioactivity decreased significantly when the mites were treated with a relatively high dosage. This indicates that the self-protection mechanism of *V. destructor*

mites may not work when the mites are exposed to a high dosage of clove oil. Moreover, these inverse trends of GST activity indicate that the amount of clove oil used could affect its acaricidal effects.

5. Conclusion

In this study, we determined the effects of clove oils on the water-soluble protein content and enzyme bioactivities of *V. destructor* mites. Our results show that water-soluble protein content and the activities of enzymes related with detoxification/protection were influenced. The significant change of these parameters after the treatment of 0.1 μ l clove oil suggested that the related physiological process were affected/activated even no acute toxicity occurred to mites. The decrease of GST activity after treatment with 1.0 μ l clove oil indicated dysfunction of this detoxification enzyme may be related to the acute toxicity caused by clove oil.

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