Chemical composition, insecticidal and biochemical effects of *Melaleuca alternifolia* essential oil on the *Helicoverpa armigera*

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**Abstract**
Pesticide resistance has developed as a result of long-term and extensive use of chemical pesticides. Essential oils from aromatic plants may provide a new and safe alternative to conventional insecticides. In this study, the insecticidal activities of the essential oil of *Melaleuca alternifolia* and their chemical constituents against *Helicoverpa armigera* Hubner were investigated, and the underlying mechanisms were studied. The essential oil showed distinct antifeedant (AFC₅₀ = 8.93 mg/ml) and good contact (LD₅₀ = 50.28 µg/larva) activities against *H. armigera* at 24 hr. Ten chemical components were identified using a gas chromatograph/mass spectrometer, and mainly included terpinen-4-ol (40.09%), γ-terpinene (21.85%), α-terpinene (11.34%), α-terpineneol (6.91%), α-pinene (5.86%), terpinolene (3.24%) and 1,8-cineole (1.83%). Among them, five components were determined and results showed that these constituents possessed obvious antifeedant activities. The activities of acetylcholinesterase and glutathione S-transferase were notably inhibited by the essential oil, as compared with the control, with strong dose- and time-dependent effects. The results provide a basis for their development and utilization in the control of insects in the future.

**KEYWORDS**
acetylcholinesterase, antifeedant, glutathione S-transferase, *Helicoverpa armigera* Hubner, *Melaleuca alternifolia*

1 | INTRODUCTION

Cotton is an important economic crop worldwide (Alpermann, 2010; Baffes, 2004). The larva of the moth *Helicoverpa armigera* Hubner is one of the most serious pests of cotton crops worldwide (Kranthi et al., 2005). For example, a nationwide outbreak of *H. armigera* in the early 1990s in China directly caused 10 billion yuan loss. Currently, the prevention and control of *H. armigera* is heavily dependent on chemical pesticides (Ali, Kumar, & Kumar, 2011). However, extensive long-term use of chemical pesticides can result in pesticide resistance, damage to the environment and non-target organisms, disruption of ecological balance and threaten to human health (Bird & Akhurst, 2007; Neupane & Shrestha, 2015).

In order to effectively prevent and control *H. armigera*, China introduced *Bacillus thuringiensis* toxin Cry1Ac (Bt-Cry1Ac) cotton from the United States in 1997 (Liu et al., 2009; Peferoen, 1997); the widespread cultivation of Bt-Cry1Ac cotton has benefited cotton production in China. However, *H. armigera* resistance to Bt-Cry1Ac cotton in field populations has been developed, thus seriously damaged cotton crops in recent years (Huang, Chen, Qiao, & Wu, 2015; Huang, Hu, Fan, Pray, & Rozelle, 2002). Therefore, it is crucial to find a new, safe and highly effective alternative insecticide.

Many plants contain various bioactive secondary metabolites, such as terpenes (Chen et al., 2015), flavonoids (Sharififar et al., 2016) and alkaloids (Jarmusch et al., 2016), which are natural pesticides source. To cope with bioactive secondary metabolites, the insects can...
decrease the sensitivity of the target site of pesticides, such as the nerve conduction enzyme acetylcholinesterase (AChE) (Bezeria da Silva et al., 2016). Or, the insects can utilize a variety of detoxifying enzymes, including glutathione S-transferase (GST) (Li et al., 2013). Thus, the elucidation of insecticidal mechanisms by determining the activity of detoxifying enzymes and nerve conduction enzymes is currently main aspect to study the insecticidal toxicity of essential oils (Mohamed et al., 2016; Potter & Wadkins, 2006).

Increased attention has been given to botanical pesticides due to their natural origin, the wide range of potential material resources, as well as many secondary plant metabolites that are degradable, harmless and non-resistant (Kedia, Prakash, Mishra, Singh, & Dubey, 2013; Valmas & Ebert, 2006). The essential oil is derived from a native Australian plant, Melaleuca alternifolia (Baldissera, Da Silva, Oliveira, Santos, et al., 2014). This species is widely cultivated to use as fragrances, and flavours in the perfume and food industries, as well as in aromatherapy (Ponce, del Valle, & Roura, 2004). Previous studies on M. alternifolia essential oil mainly focused on its antimicrobial and anti-inflammatory effects (Baldissera, Da Silva, Oliveira, Vaucher, et al., 2014; Ireland et al., 2012; Thomsen, Hammer, Riley, Van Belkum, & Carson, 2013). Few studies have demonstrated that the essential oil of M. alternifolia is biologically active as an antifeedant or for contact against H. armigera.

Plant essential oils have been mainly explored as potential natural fumigants for control stored product insects. The oils are rarely applied in the prevention and control of the Lepidoptera. Therefore, this study was carried out to evaluate the insecticidal activity of essential oil by studying the mode of action of essential oil against Lepidoptera. In this study, the antifeedant activity and contact toxicity of the M. alternifolia essential oil, and its constituents against H. armigera, as well as its effects on the activities of two important enzymes (acetylcholinesterase and glutathione S-transferase) in H. armigera, were determined to assess the value of applying M. alternifolia essential oil to control H. armigera.

2 | MATERIALS AND METHODS

2.1 | Insect culture

H. armigera larvae and their artificial diets were obtained from Keyun Bioinsecticide Research and Development Center of the Chinese Academy of Sciences Institute of Zoology (Henan, China) and were maintained in the insectarium at the Plant Protection College of Anhui Agricultural University for more than four generations (Hefei, China). The insects were reared in a laboratory on artificial diets, and all tests conducted in this study were performed in incubators in a controlled environment with temperature (26 ± 1°C) and relative humidity (75% ± 5%) being held constant, and a light–dark cycle of 14:10 hr.

2.2 | Plant essential oil and chemical compositions analysed by gas chromatography–mass spectrometry

M. alternifolia essential oil was purchased from Fujian Senmeida Biotechnology Co., Ltd (Fujian, China). The essential oil was diluted in acetone, and 1 μl oil with three independent samples was injected. Gas chromatography (Agilent 7890B) and mass spectrometer (Agilent 5977A, Agilent Technologies Inc., State of California, USA) analyses were used to measure and identify chemical compositions. GC was equipped with a flame ionization detector and capillary column with HP-5MS (30 m x 25 μm x 2.5 μm film thickness). The GC settings were as follows: The initial oven temperature was held at 40°C for 2 min, and ramped at 10°C/min to 280°C for 5 min. The injector temperature was maintained at 250°C. The carrier gas was helium. The GC/MS system was operated in the EI mode at 70 Ev, the device was equipped with a splitless injector. The interface temperature was maintained at 280°C, the ion source temperature was maintained at 230°C, and the quadrupole temperature was maintained at 150°C. The solvent was delayed for 3.0 min. Spectra were scanned from 5 to 550 m/z at 2 scans s⁻¹ (Boligon et al., 2013; Homer et al., 2000).

2.3 | Estimation of antifeedant activity

The antifeedant activity of M. alternifolia essential oil and the major compounds of the oil sample were tested by using a leaf disc no-choice method (Isman, Koul, Luczynski, & Kamiński, 1990). Fresh cotton leaf discs of 1.5 cm in diameter were punched using a cork borer and were dipped in the essential oil of M. alternifolia and their main constituents at different doses. Leaf discs treated with acetone were used as a negative control, and water was used as a blank control. In each petri dish, wet filter paper was placed to avoid early drying of the leaf discs and third-instar H. armigera larvae were introduced into each petri dish. Three replicates were maintained for each treatment, with 20 larvae per replicate. Progressive consumption of the leaf disc area by larvae in all treatments was recorded after 24 hr using the leaf area metre method. Leaf area, eaten by larvae in each treatment group, was corrected by area eaten in the negative control. Meanwhile, the antifeedant rate and antifeedant concentration (AFC₅₀) were calculated. The antifeedant activity was measured with the coordinate grid method (Isman et al., 1990).

2.4 | Estimation of contact larval toxicity

Contact toxicity of M. alternifolia essential oil was tested by using the topical application method (Zhu, Zhao, Chu, & Liu, 2012). Serial dilutions of the essential oil (5.0, 10.0, 20.0, 30.0 and 40.0 mg/ml) were prepared with acetone. Aliquots of 2 μl of solution were dispensed by an Automatic Micro-applicator (Burkard 900-X, Burkard Scientific Co., Ltd, USA) and applied to the dorsal thorax of individual third-instar H. armigera larvae. Acetone was applied to larvae designated as negative control, and water to larvae as blank control. Both treated and control larvae were then transferred to a foster box. Three replicates were maintained for each treatment, with 20 larvae per replicate. Mortality of H. armigera was observed at 24, 48 and 72 hr post-treatment.

2.5 | Determination of enzyme activity

Based on the results from the contact toxicity test, the LD₅₀ value of the essential oil after treatment was 25.14 mg/ml. Another set of test
insects were treated by a topical application method, with doses of 5.00, 10.00, 20.00, 30.00 and 40.00 mg/ml, respectively, and sampled at 24 hr after treatment. The test insects were weighed accurately and washed two or three times with pre-cooled saline. Excess fluid was removed using filter paper, and the insects were mechanically disrupted in liquid nitrogen using a mortar and pestle. The resultant powder was transferred to a centrifuge tube, and approximately 0.9 ml of saline was added to obtain a 10% tissue homogenate. The extract was centrifuged (Allegra X-30R, Beckman Coulter, Inc. USA) at 4°C, under 4,200 g for 10 min. The supernatant was used as an enzyme solution for assessing GST and AChE activities and was stored at −20°C. The entire extraction process was performed in an ice bath (Kang, Moon, Lee, & Park, 2013).

The total protein content was analysed by using Coomassie Brilliant Blue staining, in accordance with the instructions of total protein quantitative assay kits, and the activities of AChE and GST were measured according to the instructions of the acetyl cholinesterase and glutathione S-transferase assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. Three replicates were maintained for each treatment, and each replicate was performed three times.

2.6 | Statistical analyses

Percentage of contact activities was calculated using the following modified Abbot’s formula (Abbott, 1925). The percentage of insect mortality was converted into arcsine-square-root values for the analysis of variance (ANOVA) using the software IBM SPSS Statistics 22.0 (SPSS, USA). The mean value of corrected mortality or antifeedant rate (calculated from three independent experiments) was compared and separated using Scheffe’s test with a p value <.05. The mean ± SE were presented from the untransformed data. The LD_{50} values were subjected using the Probit analysis (Fong, Kim, Chen, & DeSarbo, 2016). μg/larva was transformed from the product of LD_{50} values and application of solution volume. The effects of M. alternifolia essential oil on the activities of the AChE and GST enzymes were analysed using Origin Pro 9.0 (OriginLab Corporation, USA).

3 | RESULTS

3.1 | Antifeedant activity of M. alternifolia essential oil

The antifeedant activities of M. alternifolia essential oil on the third-instar larvae of H. armigera, at 24 hr, are shown in Table 1. The results showed that the essential oil had a pronounced antifeedant effect, and a 97.8% antifeedant rate at a concentration of 40.00 mg/ml, but it had a 17.1% weak antifeedant activity at 2.5 mg/ml. Treated larvae consumed less food than untreated larvae, and close to a third of the treated larvae stopped feeding at 10.00 mg/ml. The AFC_{50} value of the essential oil is 8.93 mg/ml at 24 hr after treatment.

3.2 | Contact toxicity of M. alternifolia essential oil

The contact toxicity of M. alternifolia essential oil against the third-instar larvae of H. armigera, exposed by direct contact, is presented in Table 2. The contact toxicity of the essential oil was also distinctly enhanced by increasing doses, and the largest dose of 40.00 mg/ml caused mortality rates of 73.33, 90.00 and 93.33%, in the third-instar larvae of H. armigera at 24–72 hr. The results revealed that the increasing effects of contact toxicity were more evident when the exposure time was prolonged from 24 to 48 hr at same high dose, compared with the difference between 48 and 72 hr of exposure. The significant treatment effects were separated using Scheffe’s test with a p value <.05.

Chi-square value.

### TABLE 1 Antifeedant activity of M. alternifolia essential oil against the third-instar larvae of H. armigera (mean ± SE)

| Concentration (mg/ml) | Feeding area (cm²) | Antifeedant rate (%) ± SE |
|-----------------------|--------------------|---------------------------|
| 2.50                  | 1.30               | 17.07 ± 1.86b             |
| 5.00                  | 1.13               | 28.05 ± 2.82d             |
| 10.00                 | 0.86               | 45.12 ± 3.23c             |
| 20.00                 | 0.40               | 74.39 ± 2.54b             |
| 40.00                 | 0.04               | 97.80 ± 2.88a             |
| Control               | 1.57               | —                         |

*Thin column, mean ± SE followed by the same letter do not differ significantly using Tukey’s test, p ≤ .05.

### TABLE 2 Contact toxicity of M. alternifolia essential oil against the third-instar larvae of H. armigera (mean ± SE)

| Concentration (mg/ml) | Corrected mortality (%) |
|-----------------------|-------------------------|
|                       | 24 hr | 48 hr | 72 hr |
| 5.00                  | 6.67 ± 1.67d^a          | 6.67 ± 1.67e          | 11.67 ± 1.67e |
| 10.00                 | 8.33 ± 1.67d^a          | 18.33 ± 1.67d         | 21.67 ± 4.29d |
| 20.00                 | 31.67 ± 1.67c           | 56.67 ± 1.67c         | 73.33 ± 1.67c |
| 30.00                 | 61.67 ± 1.67b           | 73.33 ± 3.33b         | 81.67 ± 1.67b |
| 40.00                 | 73.33 ± 3.33a           | 90.00 ± 0.00a         | 93.33 ± 1.67a |

95% CI^b = 23.46–26.71 95% CI = 16.29–18.55 95% CI = 11.42–16.34

χ²^c = 3.51 χ² = 2.10 χ² = 6.93

*Thin column, mean ± SE followed by the same letter do not differ significantly using Tukey’s test, p ≤ .05.

^b95% confidence limits (mg/L).

^cChi-square value.
3.3 | Chemical analysis of *M. alternifolia* essential oil

The results of the components present in the *M. alternifolia* essential oil, determined using GC/MS analyses, are shown in Table 3. The main components of the essential oil are terpinen-4-ol (40.09%), followed by γ-terpinene (21.85%), α-terpinene (11.34%), α-pinene (5.86%), terpinolene (3.24%) and 1,8-cineole (1.83%), which all conform to the requirements of the International Organization for Standardization (ISO), standard number 4730 (Baldissera et al., 2014). Studies have reported the components of terpinen-4-ol and 1,8-cineole in many essential oils from aromatic plants (Baldissera et al., 2016; Nogueira, Aquino, Rossa, & Spolidorio, 2014; Raina & Abraham, 2015).

3.4 | Antifeedant toxicities of constituents identified from the essential oil of *M. alternifolia*

Based on the GC/MS results of the essential oil, and the reported components, five constituents in the essential oil produced antifeedant activity against *H. armigera*, and the results are shown in Table 4. The results demonstrate that the constituents of terpinen-4-ol, γ-terpinene, α-terpineol, α-terpineol and 1,8-cineole distinctly deterred the feeding activity of *H. armigera*. In particular, terpinen-4-ol demonstrated the most potent biological activity with an AFC$_{50}$ value of 4.62 mg/ml, followed by α-terpineol and 1,8-cineole, with the AFC$_{50}$ values of 5.64 and 6.23 mg/ml, respectively, at 24 hr after treatment. As a whole, the constituents of terpinen-4-ol, α-terpineol and 1,8-cineole had a higher toxicity with AFC$_{50}$ than γ-terpinene and α-terpinene, revealing that oxygen-containing compounds could lead to a remarkable change in bioactivity.

3.5 | Inhibitory effect of the essential oil on enzymes activity

To determine the AChE activity in third-instar larvae of *H. armigera* in vivo, changes in treatment time and dose of *M. alternifolia* essential
oil were evaluated, based on contact toxicity after topical application of the essential oil. Figure 1a showed the activity of AChE in third-instar larvae of *H. armigera*, contacted with doses of 5.00, 10.00, 20.00, 30.00 and 40.00 mg/ml, respectively, at 24 hr after treatment. The results showed that the AChE activity levels of the contacted insects were moderately lower than those of the control, but the oil invoked the weak inhibitory effect at 5.00 mg/ml. Figure 1c shows the activity of AChE in third-instar larvae of contacted *H. armigera*, tested at 12, 24, 48, 60 and 72 hr, respectively. The results showed that the AChE of *H. armigera* in vivo was inhibited as a whole, and showed a significant time effect at 12–48 hr after treatment. Moreover, the effects of the essential oil on AChE activity were initially inhibited in treated larvae at 12–48 hr after treatment, but were slightly restored after further exposure at 48–72 hr, which suggests that the highest inhibition ratio was observed at 48 hr after treatment with the LD$_{50}$ dose.

The activity of GST in third-instar larvae of *H. armigera*, measured at 24 hr after treatment, in five concentrations, is shown in Figure 1b. The results indicated that the essential oil also had a moderate inhibitory effect and that the inhibition rate of GST activity was as high as 26.91% at the maximum dose, 24 hr after treatment. However, the activity of GST was inhibited as a whole, and with increasing concentration of treatment, inhibition of enzyme activity was enhanced. The activity of GST in *H. armigera* treated at LD$_{50}$ doses, tested at five different times, is shown in Figure 1d. The GST activity in *H. armigera* after treatment for 12–24 hr was clearly inhibited, compared with the control, and the inhibition of GST activity peaked at 18.1% at 24 hr after treatment. The GST activity recovered to a certain extent with an extension of time, probably because *H. armigera*'s self-defence regulation against *M. alternifolia* essential oil caused the enzyme activity to recover gradually.

**FIGURE 1** Effects of *M. alternifolia* essential oil at different concentrations on acetylcholinesterase (AChE) (a), glutathione S-transferase (GST) (c), and at different times with sublethal concentration (LD$_{50}$) of oil (25.140 mg/ml) on AChE (b) and GST (d) in third-instar larvae of *H. armigera* in vivo. CK represents the control groups. Results are reported as mean ± SE (calculated from three independent experiments). Different lowercase letters at the top of the columns mean significant differences of essential oil at a *p* value of .05. [Colour figure can be viewed at wileyonlinelibrary.com]
DISCUSSION

Essential oils from aromatic plants, and their major constituents, are considered to be an alternative to conventional pesticides for controlling many insect pests, due to several advantages over synthetic pesticides, including rapid degradation, low residual and not easily generated cross-resistance (McDonnell et al., 2016; Werdin González, Stefanazzi, Murray, Ferrero, & Fernández Band, 2015; Wollinger et al., 2016). Moreover, plant essential oils are high volatile and a rich source of bioactive chemicals (Kim, Lee, Jang, Jung, & Park, 2016). Acting on the nervous system of insects is one of the important modes of action of high volatility, which lead antifeedant and repellent effect to insects (Abdullah et al., 2015; El-Wakeil, 2013).

Currently, there are no commercially viable insect antifeedants that have been developed. Essential oils possess aromatic properties and make insects disgusted by food, reducing or stopped feeding (Arasu et al., 2013). In this study, the constituents of essential oil were analysed using GS/MS. The results showed that the most abundant compound was terpinen-4-ol (40.09%), revealing that the essential oil was a chemotype of high terpinen-4-ol oil. In the antifeedant assay, the essential oil had a pronounced antifeedant effect, but exhibited lower level of antifeedant compared with other essential oils in previous studies (Abdullah et al., 2015; Quesada-Moraga, Carrasco-Díaz, & Santiago-Alvarez, 2006; Ribeiro et al., 2015). Notably, the constituents of terpinen-4-ol had distinctly deterred feeding activity on H. armigera, which is well worth further research and development. Furthermore, the structure–activity relationships of the essential oil constituents indicated that the constituents with hydroxyl or aldehyde groups are stronger than constituents belonging to hydrocarbons, which is consistent with the report by Choi, Kim, Shin, and Park (2007) and Seo et al. (2009) (Choi et al., 2007; Seo et al., 2009). It is helpful for the future synthesis of a new type of insecticide or insect antifeedants.

The percentages of constituents in essential oils differ, due to geographical location, environmental conditions, nutritional status, etc. (Ogendo et al., 2008), which causes M. alternifolia to have six different chemotypes, varying in relative levels of 1,8-cineole, terpinen-4-ol and terpinolene (Wheeler, 2006). In our study, the constituents of terpinen-4-ol demonstrated the most potent biological activity and the content of which accounted for 40.09%. We deduced that the terpinen-4-ol chemotype is the main insecticidal active components. The amount of terpinen-4-ol directly affects the insecticidal activity of the essential oil, in accordance with the results of antifeedant toxicities of constituents identified from the essential oil. Thus, we suggest that the chemotype of high terpinen-4-ol has potential for further investigation.

In the contact assay, the essential oil also had a moderate toxic effect compared with other essential oils in previous studies, for example essential oil of Syzygium aromaticum (LD_{50} = 47.8 μg/larva; Jiang, Akhtar, Zhang, Bradbury, & Isman, 2012) and trans-anethole (LD_{50} = 71.2 μg/larva; Wilson & Isman, 2006). However, the essential oil was relatively weak compared with the antifeedant activity at the same dose. The contact toxicity of the essential oil was distinctly enhanced by increasing doses and was moderately increased by increasing times. These findings indicated that the assay method had an effect on the insecticidal activity of the essential oil against H. armigera.

Elucidation of insecticidal mechanisms, by determining the activity of detoxifying enzymes and target enzymes, is currently the main aspect needed to study the insecticide toxicology of essential oils (Mohamed et al., 2016; Potter & Wadkins, 2006). AChE is known as a target enzyme for insect control chemicals, which plays an important role in the maintenance of normal transmission of neural impulses in synaptic clefts, by catalysing the hydrolysis of the neurotransmitter acetylcholine (Bezerra da Silva et al., 2016; Kim, Issa, Cooper, & Zhu, 2015). Similarly, GST is an important detoxifying enzyme against pesticides in the body, which can convert the lipid metabolites induced by insecticidal material, or combine with insecticidal molecules via chelation, to protect tissues from oxidative damage (Korkina, 2016). To determine the mode of action of the essential oil, we studied its ability to inhibit AChE and GST, and the oil caused a pronounced inhibition of AChE and GST, with moderate dose effects, and the overall trend reflected a time-based effect. Furthermore, AChE was more sensitive to essential oils than GST, suggesting that AChE may be the main target of the essential oil, and perhaps, the decline of the AChE enzyme activity is one of the main reasons that caused the death of the insects. Meanwhile, the essential oil pronounced that inhibition of AChE and GST may be due to the presence of several active ingredients that operate via several modes of action.

CONCLUSIONS

In conclusion, the essential oil of M. alternifolia had potent antifeedant activity and contact toxicity on H. armigera. Among the identified constituents from the essential oil, terpinen-4-ol showed the strongest antifeedant activity against H. armigera. In addition, the insecticidal activity of the essential oil might be due to its inhibitory effects on AChE and GST activities. Therefore, we suggest that M. alternifolia essential oil should be explored as potential natural insecticide, or be involved in the future synthesis of a new type of insecticide, based on the active constituents of M. alternifolia essential oil.

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AUTHOR CONTRIBUTION

H-QC, ML and J-JX conceived and designed the study. ML, J-JX and L-JZ performed the experiments. XY and FT analysed data. J-JX wrote the manuscript. H-QC, R-MH and X-WH edited and revised the manuscript. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.
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