Exploration of Clove Bud (Syzygium aromaticum) Essential Oil as a Novel Attractant against Bactrocera dorsalis (Hendel) and Its Safety Evaluation

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Simple Summary: Bactrocera dorsalis is among the most economically harmful pests. The lure-and-kill approach is an environmentally friendly and innovative method that offers an opportunity for sustainable surveillance and control of B. dorsalis. However, such a strategy requires highly effective pest attractants. In the current study, we compared the attractive activity of twelve flower essential oils against B. dorsalis adults via the indoor trapping assay. Meanwhile, we studied the attractive features of clove bud essential oil (CBOE) for males under laboratory conditions. Further, we also investigated CBOE’s attractancy to their natural predator, the ladybirds, and its cytotoxicity against non-target organisms’ cells. In particular, sexually mature males were dramatically attracted to the CBOE. Furthermore, the CBOE exhibited no significant attractancy to ladybirds nor cytotoxicity against normal human and mouse cells. The results revealed that CBOE shows potential for development as an eco-friendly and novel attractant agent for the control of B. dorsalis.

Abstract: The oriental fruit fly Bactrocera dorsalis (Hendel) is a destructive polyphagous species that targets many economically important fruits and vegetables. The primary control of B. dorsalis relies mainly on the use of synthetic chemicals, and excessive use of these chemicals has adverse effects on both the environment and human health. Environmentally friendly management of pests involving plant essential oils is useful for controlling the populations of pests responsible for decreasing the yields and quality of crops. In the present study, we demonstrate that clove bud essential oil (CBOE) is strongly attractive to sexually mature males. Mature males responded to the CBOE differently throughout the day; the strongest response was elicited during the day and decreased at dusk. Virgin and mated mature males did not respond differently to CBOE. No obvious response behaviour to the CBOE was observed in two species of beneficial natural predator ladybirds. In addition, a cytotoxicity assessment demonstrated that CBOE is nontoxic to normal human and mouse cells. Based on our laboratory experiments, CBOE may serve as a promising, sustainable, and environmentally friendly attractant for B. dorsalis males; however, field experiments are needed to confirm this hypothesis.

Keywords: Bactrocera dorsalis; clove bud essential oil; olfactometer bioassay; attractancy; cytotoxicity
1. Introduction

*Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) is a highly destructive invasive pest throughout the world that is capable of causing extensive damage to a broad range of cultivated and wild fruit and vegetable products [1–3]. A serrated ovipositor allows adult females to pierce through the skin of intact ripe fruit and deposit eggs in the interior. The eggs hatch into larvae that tunnel deeper into the fruit as they feed on the fruit pulp, rendering it damaged and unmarketable [2,4]. The application of synthetic insecticides remains an effective method for rapidly suppressing *B. dorsalis* in the field, but it inevitably has detrimental impacts on the environment and human health [2,5–7]. Broad-spectrum insecticides may result in the development of insect resistance and deplete populations of natural enemies. In particular, a powerful phytocatalytic lure, methyl eugenol (ME), has been employed extensively for monitoring, detection, and management of *B. dorsalis* [8,9]. Despite its benefits, ME is associated with risks; it is carcinogenic to humans and is only attractive to male flies, limiting its application for prolonged periods [2,3,10,11]. Therefore, to reduce pesticide application and prevent negative consequences, a more ecologically friendly and novel attractant is urgently required to control *B. dorsalis*.

Plant essential oils (EOs) are natural secondary metabolites derived from aromatic plants and contain complex mixtures of active compounds [12]. In particular, EOs possess antimicrobial, antifungal, antiviral, insecticidal, and herbicidal properties [4,13–15]. Studies of the attractant, deterrent, antifeedant, and toxic properties of EOs have been chiefly conducted on numerous phytophagous insects [16–18]. For instance, an EO extract from the leaf of *Magnolia citrata* (Giói chanh) shows moderately strong attractive power towards males of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) [19]. Moreover, clove oil (*Eugenia caryophyllata* L. Myrtaceae) was studied as a source of attractant to *C. capitata* male flies [20]. *Bactrocera invadens* (Diptera: Tephritidae) males exhibited a significantly positive response to clove essential oils [21]. In addition, a study on olfactory behaviour responses found that clove oil at a 5% concentration is strongly attractive to *Bactrocera zonata* (Saunders) adults [22]. Moreover, Ali et al. [23] reported that clove essential oils exhibited insecticidal activity against *B. zonata* pupa. Thus, in conjunction with other sustainable pest management strategies, EOs can be an effective alternative to conventional plant protection products. However, to date, few studies have investigated the biological activity of EOs, especially clove EO, towards *B. dorsalis*.

A toxicological safety assessment of natural extracts provides scientific evidence of their potential applications. To develop agrochemicals, it is crucial to assess the potential risk of natural extracts for non-target organisms [24,25]. Notably, human or insect cultured cell lines provide a straightforward, ethically compliant, and powerful tool for investigating chemical toxicity [3,26,27]. In the current study, the attractant activity of twelve flower-derived EOs, including clove bud, *Michelia alba* (Magnoliaceae), peony, gardenia, peach blossom, jasmine, pear blossom, chrysanthemum, cerasus, *Rosa chinensis* (Yuejihua), *Osmanthus fragrans* Lou., and rose EOs, against mature *B. dorsalis* adults was assessed first. The responsiveness characteristics of *B. dorsalis* males to clove bud essential oil (CCEO) were then evaluated. Further investigations were conducted on the attractancy of CCEO against two natural predators *Coccinella septempunctata* L. and *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). In addition, we further tested the cytotoxicity of CCEO to cells of non-target organisms, including human foetal lung fibroblast 1 (HFL1), human breast cancer (MDA-MB-231), mouse embryonic hepatocytes (BNL-CL.2), and insect ovary (SF-9) cells. This study contributes to the development of a safe, efficient, and sustainable attractant for the control of *B. dorsalis*.

2. Materials and Methods

2.1. Reagents and Chemicals

Flower essential oils (EOs) under study were purchased from Luxury Bloom Biotech Co., Ltd. (Shanghai, China). Dulbecco’s Modified Eagle Medium (DMEM), a high-sugar medium, was purchased from the HyClone Company (Logan, UT, USA). TNM-FH medium...
was purchased from Sino Biological Inc. (Beijing, China). Other common chemicals necessary for the experiments were purchased from Sigma Aldrich (Shanghai, China).

2.2. Insects Rearing

2.2.1. Bactrocera Dorsalis

*Bactrocera dorsalis* was maintained under laboratory conditions at the Henan University of Science and Technology (HAUST) and reared at 27 ± 1 °C and 75% ± 1% relative humidity with a photoperiod of 14 h:10 h (L:D). *Bactrocera dorsalis* was reared for more than 30 generations. The colony was periodically refreshed with wild flies to maintain genetic diversity. Banana slices were used to collect eggs. Hatching larvae were reared on an artificial diet based on that used in a previous report [11,28]. The adult flies were reared in mesh cages (30 cm × 30 cm × 30 cm) and fed on an artificial diet consisting of dry sugar:yeast extract (1:1, w/w) and water [3,27].

2.2.2. Ladybirds

Adults of the natural predators *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) (seven-spotted ladybeetle) and *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) were collected from the research experimental field of HAUST. Both larval and adult ladybirds were fed with green peach aphids and reared under laboratory conditions of 25 ± 1 °C, 65% ± 5% relative humidity, and a 12 h light regime [29,30]. Cotton wool soaked in water served as a source of water. Larvae and adult ladybirds (3 days after eclosion) were chosen for tests.

2.3. Cell Culture

Cells of human foetal lung fibroblast 1 (HFL1), human breast cancer cell (MDA-MB-231), and mouse embryonic hepatocytes (BNL-CL.2) were cultivated in DMEM supplemented with 100 µg/mL of streptomycin, 100 U/mL of penicillin, and 10% foetal bovine serum (FBS) in an incubator with a humidified air atmosphere of 5% CO2 at 37 °C. The MDA-MB-231, HFL1, and BNL-CL.2 cells were purchased from Procell Life Science and Technology Co., Ltd. (Wuhan, China). *Spodoptera frugiperda* (Lepidoptera: Noctuidae) vary cells (SF-9) were kindly donated by the Fruit Tree Research Institute, Guangdong Academy of Agricultural Sciences (Guangzhou, China). The SF-9 cells were maintained in TNM-FH medium supplemented with 1% penicillin and 10% FBS and grown in a humidified incubator at 28 °C. All tests were conducted with exponentially growing cells [3,27].

2.4. Responsiveness of B. Dorsalis Flies to Flower-Derived EOs in an Indoor Trapping Assay

Based on recently reported methods [3,27], we compared the taxes of *B. dorsalis* mature flies to the flower-derived EOs, including clove bud, *Michelia alba*, peony, gardenia, peach blossom, jasmine, pear blossom, chrysanthemum, *Rosa chinensis*, *Osmanthus fragrans*, and rose EOs. Initially, EO solutions were prepared using 5% Tween-80, with final concentrations of 50, 100, 200, 400, 800, and 1000 µL/mL. Subsequently, 40 mature flies (16 days old, male:female = 1:1) were randomly selected as subjects and placed in a cubical screen cage (35 cm per side). After approximately 30 min, a fly trap was placed inside each cage. The fly trap was constructed from 150 mL conical flasks on which a silicone top containing a 15 mL centrifuge tube (cut for 3.5 cm) was securely placed. Each trap contained a 1.5 mL tube with a cotton wick with 100 µL of the trial concentration of the EOs. In the control groups, 5% Tween-80 was used in the traps. Traps were removed after 2 h, and trapped flies were counted. Experiments were biologically independently replicated five times. Experiments were conducted from 09:00 to 11:00. During the experimental period, the ambient temperature and relative humidity were controlled at 27 ± 2 °C and 75% ± 5%, respectively. The responsiveness ratio of *B. dorsalis* flies to the flower-derived EOs was calculated with the following formula:

\[
\text{Response rate (\%)} = \frac{\text{No. of flies trapped in treatment} - \text{No. of flies trapped in control}}{\text{No. of test flies}} \times 100\%
\]
2.5. Effect of Age, Daily Rhythm, and Physiological Condition on Male Taxis to CBEO

For this experiment, 40 male flies aged 2, 4, 6, 8, 12, 16, 20, 24, 28, and 32 days were selected randomly to determine the influence of age on CBEO sensitivity in male flies. A trapping assay was conducted as previously described. Sixteen-day-old male flies were assessed in three separate experiments at 09:00 (morning), 13:00 (early afternoon), and 17:00 (near dusk) to assess whether the responses of mature males to CBEO varied throughout the day. Furthermore, to determine whether mating status affects the responsiveness of males to CBEO, a comparison between virgin and mated males (16 days old) was conducted at 09:00, 13:00, and 17:00. Finally, the responses of starveling or thirsty males (16 days old) to CBEO were assayed to determine whether the responses of mature males to CBEO vary according to their physiological condition. Each bioassay was repeated five times.

2.6. Attractancy of CBEO to Natural Predator Ladybirds

In this experiment, the responses of larval (1st instar, 2nd instar, 3rd instar, and 4th instar) and adult (3 days old) ladybirds to CBEO were investigated using a Y-tube olfactometer apparatus (20 cm upstream arm, 25 cm common arm, 3 cm internal diameter, and 60° branching angle). Bioassays were conducted in accordance with a previously published protocol [31–33]. Treatment with CBEO at 400 µL/mL was applied as a test stimulus. An olfactometer arm was fitted with a filter paper strip containing 10 µL of an odour stimulus solution. The opposite arm, which served as the control, was coated with 5% Tween-80 (10 µL) on filter paper. Each olfactometer arm was pumped with 100 mL of purified air every minute. The Y-tubes were enclosed in a white fabric box to reduce differences in light intensity.

After turning on the air pump for 5 min, ladybirds pre-starved for 4 h were used in the olfactory experiments. Each group of 10 samples was selected randomly and placed into the common arm tube simultaneously. Each bioassay test was replicated five times. After acclimatization for 2 min, participants were allowed to move freely in the Y-tube for 8 min. It was deemed an effective choice when the individual entered one arm tube, passed one-third of its length, and remained for 30 s. Non-responders were those that did not meet the abovementioned criteria. After testing 10 ladybirds, odour sources were interchanged, and the positions of the stimuli were rotated to avoid any influence of unforeseen asymmetries in the setup. Following each test, acetone and distilled water were used to clean the Y-tube testing devices. Each ladybird was tested only once. All bioassays were conducted daily from 09:00 to 17:00 under 70% ± 5% relative humidity and 27 ± 1°C. The following formula was used for the calculation of ladybird response ratios to CBEO [3]:

\[
\text{Response ratios (％) = \frac{\text{Number of responding ladybirds for one arm}}{\text{Total number of responding ladybirds}} \times 100\%}
\]

2.7. Cytotoxicity Test

MTT assays were used to test the cellular toxicity of CBEO against MDA-MB-231, HFL1, SF-9, and BNL-CL2 cells using a previously described method [3,34,35]. Briefly, cells (1 × 10⁵ cells/well) were seeded in a 96-well plate in a final volume of 100 µL/well. After 24 h of priming, cells were treated with different concentrations of CBEO solutions in dimethyl sulfoxide (DMSO) (15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL) and incubated for 24 h. Controls received fresh media containing 0.5% DMSO solution. After 24 h, the cell viability was investigated by adding 10 µL of MTT solution (5 mg/mL) to the medium. After incubation for a further 4 h, the supernatant was removed and 100 µL DMSO was added to dissolve formazan crystals. Subsequently, the optical density (OD value) of the mixture was measured at a wavelength of 570 nm. The experiment was conducted independently in triplicate. Cell viability was calculated with the following formula:

\[
\text{Cell viability (％) = } \frac{OD_{\text{treatment}}}{OD_{\text{control}}} \times 100\%
\]
2.8. Data Analysis

Data analysis was conducted using SAS v9.20 (SAS Institute, Inc., Cary, NC, USA). GraphPad Prism 8.0 software was used to draw the figures. Each variable was expressed as the mean and standard error (SE). The statistical significance of differences between experimental and control groups was assessed using one-way ANOVA and Dunnett’s multiple comparison post hoc test. Ladybird distribution in the Y-tube olfactometer bioassays was compared by chi-square analysis. Unresponsive ladybirds were excluded from the analyses. Prior to analysis, the percentage data were transformed with arcsine. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Behavioural Response of B. Dorsalis Mature Flies to Flower-Derived EOs

We compared the attractant activities of different doses of flower-derived EOs to mature flies by performing an indoor trapping assay (Figure 1). The EOs at different concentrations exhibited different levels of attractiveness toward B. dorsalis adults. In comparison with the other flower-derived EOs, the CBEO was more effective at attracting mature flies (Figure 1A). At concentrations ranging from 50 to 400 μL/mL, the CBEO showed significant enhancement of attractiveness. In contrast to the other concentrations, the CBEO at 400 μL/mL exhibited the highest attractant activity (51.00% ± 2.45%) (F = 32.19; df = 5; p < 0.0001). The trapping frequency of flies attracted to the Michelia alba EO was highest at 1000 μL/mL (27.00% ± 2.00%) (F = 19.64; df = 5; p < 0.0001), which was significantly lower than that of the CBEO at 400 μL/mL (t = 5.58; df = 4; p = 0.0051).

![Figure 1](image-url)

**Figure 1.** Behavioural response of Bactrocera dorsalis mature flies to flower-derived essential oils (EOs). Attractant activity to mature flies was assessed using a trapping method for different concentrations of (A) clove bud, Michelia alba, and peony EOs; (B) gardenia, peach blossom, and jasmine EOs; (C) pear blossom, chrysanthemum, and cerasus EOs; and (D) Rosa chinensis, Osmanthus fragrans, and rose EOs. Each bar represents the mean ± SEM of five replicates. Different lowercase letters indicate significant differences (ANOVA, p < 0.05).
3.2. Behavioural Activities of Male and Female Flies in Response to Flower-Derived EOs

Several of the tested EOs had clear levels of attractiveness to B. dorsalis males and females at various concentrations (Figure 2). Females and males were not attracted by the peony EOs source (Figure 2C). Notably, female and male flies showed a significant preference for CBEO (Figure 2A). The highest trapping rate of females by the CBEO at 400 µL/mL was 16.00% ± 1.87%, which is significantly lower than the attractiveness of the same concentration of CBEO to males (86.00% ± 3.67%) (t = 22.14; df = 4; p < 0.0001). Compared with females, males were more likely to be trapped by the CBEO. In addition, the trapping rate of males in response to the CBEO at 1000 µL/mL was the highest (48.00% ± 2.55%) (Figure 2B).

Figure 2. Comparative responsiveness of male and female flies to different concentrations of flower-derived essential oils (EOs). Responsiveness to (A) clove bud, (B) Michelia alba, (C) peony, (D) gardenia, (E) peach blossom, (F) jasmine, (G) pear blossom, (H) chrysanthemum, (I) cerasus, (J) Rosa chinensis, (K) Osmanthus fragrans, and (L) rose EOs was tested. Each bar represents the mean ± SEM of five replicates. Asterisks indicate a significant difference between the sexes (t-tests, p < 0.05). * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant (p > 0.05).

3.3. Attractiveness Characteristics of CBEO to Male Flies

Significant variations in the age-related trappings of CBEO were evident among male flies (Figure 3A). The taxes presented by young males (2- and 4-day-old) to the CBEO were the lowest. In particular, the mean response rates of 12-, 16-, and 20-day-old male flies to the CBEBO were 77.00% ± 3.66%, 85.50% ± 2.15%, and 83.50% ± 2.03%, respectively,
which were significantly higher than that of any other age categories ($F = 128.88; df = 9; p < 0.0001$). Even at 28 and 32 days of age, males were still fairly highly attracted to the CBEO. Interestingly, there was a noticeable difference between males throughout the day in their response to the CBEO. In the morning (09:00) and early afternoon (13:00), sexually mature males responded most strongly to the CBEO, but their response declined dramatically at dusk (17:00) ($F = 356.60; df = 2; p < 0.0001$) (Figure 3B). Moreover, neither virgin nor mated mature males responded significantly to the CBEO ($p > 0.05$) (Figure 3C). In addition, compared with the control groups, male flies suffering from starvation or thirst showed a relatively significant preference for the CBEO ($t = 6.67, df = 4, p = 0.0026; t = 2.89, df = 4, p = 0.0447$) (Figure 3D,E).

![Figure 3](image.png)

Figure 3. Responsiveness of *Bactrocera dorsalis* male flies to clove bud essential oil (CBEO): (A) Age-related response of males to CBEO. (B) Diurnal pattern of mature virgin male responsiveness to CBEO. (C) Diurnal pattern of virgin and mated male responsiveness to CBEO. (D,E) Response of starved or thirsty males to CBEO. Each bar represents the mean ± SEM of five replicates. Different lowercase letters indicate significant differences (ANOVA, $p < 0.05$). Asterisks denote a significant difference between treatments ($t$-tests, $p < 0.05$). * $p < 0.05$, ** $p < 0.01$, ns = not significant ($p > 0.05$).

3.4. Two-Choice Assays of *C. septempunctata* and *H. axyridis* in a Y-Tube Olfactometer

The responses of the tested *C. septempunctata* and *H. axyridis* in the Y-tube olfactometer for the CBEO at 400 µL/mL are shown in Figure 4A, B. In the dual-choice olfactometer assay, more *C. septempunctata* first-instar larvae were attracted by the 5% Tween-80 compared with the 400 µL/mL of CBEO ($\chi^2 = 4.00, df = 1, p = 0.0455$). However, second-instar, third-instar, and fourth-instar larvae as well as 3-day-old adults of *C. septempunctata* showed no significant preference for the CBEO compared with the control ($p > 0.05$) (Figure 4A). Similarly, *H. axyridis* larvae and adults showed no obvious preference between the CBEO and the control ($p > 0.05$) (Figure 4B). Overall, *C. septempunctata* and *H. axyridis* were unable to recognize the odours emitted by CBEO.
Figure 4. Preferences of Coccinella septempunctata and Harmonia axyridis to CBEO were assessed with a Y-tube olfactometer bioassay. (A) Behavioural response of C. septempunctata to 400 µL/mL of CBEO. (B) Behavioural response of H. axyridis to 400 µL/mL of CBEO. CK represents 5% Tween-80 treatment. NR (no response) indicates the number of ladybirds that failed to respond within the allotted timeframe. Each bar represents the mean ± SEM of five replicates. Asterisks represent a significant difference in preference (chi-square test, $p < 0.05$). * $p < 0.05$, ns = not significant ($p > 0.05$).

3.5. Cytotoxicity of CBEO against SF-9, BNL-CL.2, HFL1, and MDA-MB-231 Cells

MTT assays were performed to determine the cytotoxicity of CBEO against SF-9, BNL-CL.2, HFL1, and MDA-MB-231 cells. The trend tests consistently showed a positive correlation between the CBEO concentration and the inhibitory effect (Figure 5). As evidenced by these tests, CBEO at concentrations ranging from 125 to 1000 µg/mL significantly inhibited the viability of MDA-MB-231 cells ($F = 149.07; df = 6; p < 0.0001$) (Figure 5A). Likewise, CBEO at concentrations ranging from 62.5 to 1000 µg/mL significantly inhibited SF-9 cell viability ($F = 174.78; df = 6; p < 0.0001$) (Figure 5B). The $IC_{50}$ values of CBEO on MDA-MB-231 and SF-9 cells were 257.6722 µg/mL and 212.5499 µg/mL, respectively (Table 1). Notably, the CBEO exhibited no significant cellular toxicity on HFL1 and BNL-CL.2 cells (Figure 5C,D). Only exposure to high concentrations (1000 µg/mL) of CBEO for 24 h significantly inhibited the viability of the HFL1 ($F = 135.54; df = 6; p < 0.0001$) and BNL-CL.2 cells ($F = 86.47; df = 6; p < 0.0001$). $IC_{50}$ values of 1053.3570 µg/mL and 1060.4790 µg/mL were observed for the CBEO on HFL1 and BNL-CL.2 cells, respectively (Table 1). Consequently, CBEO was demonstrated to be non-cytotoxic to normal human and mouse cells.
mature adults. Notably, CBEO was significantly more attractive to males than to B. dorsalis females. A dramatic attraction to the CBEO prior to dusk was observed among sexually mature virgin and mated males. In addition, Y-tube bioassays and cellular toxicity tests revealed that CBEO was safe for non-target organisms. In summary, these results provide strategies for safely and biorationally controlling B. dorsalis populations is critical for managing this pest and reducing crop losses. In particular, a search is required for safe and biorational attractant agents from natural sources that could be useful in future B. dorsalis management strategies. In the current study, an indoor trapping trial revealed that CBEO was the most highly attractive EO to B. dorsalis mature adults. Notably, CBEO was significantly more attractive to males than to females. A dramatic attraction to the CBEO prior to dusk was observed among sexually mature virgin and mated males. In addition, Y-tube bioassays and cellular toxicity tests revealed that CBEO was safe for non-target organisms. In summary, these results provide

![Graphs showing cytotoxicity of CBEO to various cell lines](image)

**Table 1.** Assessment of cytotoxicity of CBEO against SF-9, BNL-CL.2, HFL1, and MDA-MB-231 cells for 24 h.

| Cell Line   | Toxicity Regression Equation | Correlation Coefficient | IC$_{50}$ (µg/mL) |
|-------------|-----------------------------|-------------------------|-------------------|
| SF-9        | $y = 1.7605 + 1.3919x$       | 0.9793                  | 212.5499          |
| MDA-MB-231  | $y = 1.6595 + 1.3855x$       | 0.9658                  | 257.6722          |
| HFL1        | $y = 1.3911 + 1.1940x$       | 0.9835                  | 1053.3570         |
| BNL-CL.2    | $y = 1.6807 + 1.097x$        | 0.9596                  | 1060.4790         |

**4. Discussion**

The oriental fruit fly (*Bactrocera dorsalis*) is a globally economically destructive pest that attacks various fruit and vegetable products. At present, the lure-and-kill technology is an important and effective strategy for rapidly controlling *B. dorsalis* [2,3]. The exploitation of environmentally friendly and effective attractants for the early detection and suppression of *B. dorsalis* populations is critical for managing this pest and reducing crop losses. In particular, a search is required for safe and biorational attractant agents from natural sources that could be useful in future *B. dorsalis* management strategies. In the current study, an indoor trapping trial revealed that CBEO was the most highly attractive EO to *B. dorsalis* mature adults. Notably, CBEO was significantly more attractive to males than to females. A dramatic attraction to the CBEO prior to dusk was observed among sexually mature virgin and mated males. In addition, Y-tube bioassays and cellular toxicity tests revealed that CBEO was safe for non-target organisms. In summary, these results provide...
timely, valuable information on the environmental friendliness of CBEO and promote its development as a promising green bait for detecting and controlling *B. dorsalis*.

Plant extracts have been considered for the development of new and less toxic control methods due to their low toxicity and low environmental impact [36]. The biological activity of EOs strictly depends on their chemical compositions, which vary according to the plant parts used, drying method, extraction technique, plant age, plant phenological stage, harvesting season, soil composition, and environmental conditions in which the plant grows [4,13]. The present research revealed that certain EOs were effective as attractants of *B. dorsalis* to some degree in an indoor trapping experiment. The EO from *Michelia alba* (Magnoliaceae) was slightly attractive to *B. dorsalis* males. Among all examined EOs, the CBEO had the highest attractant efficacy for *B. dorsalis* males.

The evergreen tree *Syzygium aromaticum* (L.) Merr. & L.M.Perry, belonging to the Myrtaceae family, produces aromatic flower buds commonly known as cloves [37]. Previous publications confirmed that clove oil extract shows potential attractant activity against males of two species of fruit flies: *C. capitata* and *Bactrocera zonata* (Saunders) (Diptera: Tephritidae). In contrast, female *C. capitata* and *B. zonata* do not show a positive response to the clove oil extract [36]. Furthermore, as reported by Bulawan et al. [38], *B. dorsalis* adults were captured most frequently in clove leaf extract treatments. CBEO is extracted from dried flower buds of the clove tree [39]. The present findings are in agreement with those of Moustafa et al. [36] and Bulawan et al. [38]. Of particular importance is the fact that the attractant efficiency of an EO is associated with its composition. The major constituents identified in CBEO are eugenol, β-caryophyllene, O-allylguaiacol, α-humulene, β-thujaplicin, caryophyllene oxide, and lesser amounts of other components, such as benzyl alcohol, 2-heptanol, 2-nonanone, 2-nonanol, 2-undecanone, and carvone [40–42]. The compound β-caryophyllene was demonstrated to be a more specific and potent male lure for *B. correcta* and *C. capitata* [43,44]. Moreover, it has been demonstrated that the relatively low concentration of β-caryophyllene attracts *B. dorsalis* gravid females [45]. A previous study also revealed that α-humulene is clearly attractive to males of *C. capitata* [45]. Additionally, 2-nonanone and 2-undecanone have been reported to stimulate a strong antennal response and a positive oviposition response on *C. capitata*, acting as an attractant and oviposition stimulant [46]. Although the constituents of clove bud oils can differ, it is highly likely that eugenol is the primary component of clove EO, according to all reports [37,39–41,47–49]. Although the attractiveness of eugenol to *B. dorsalis* has not been reported, several of its analogous compounds have been characterized as male attractants and male sex pheromones of *B. dorsalis* [50]. In addition, eugenol has a broad range of pharmacological effects, including local anaesthetic, analgesic, antimicrobial, anti-inflammatory, antitumor, and hair-growing properties [51–53]. Moreover, eugenol has been generally recognized as safe (GRAS) as a direct human food ingredient [54].

Notably, the positive response of *B. dorsalis* males to CBEO increased with age. Sexually immature males did not respond to CBEO as early as 2 and 4 days after emergence (DAE). Biologically, male adults become sexually mature at approximately 10 DAE [27,28]. It was interesting to observe that the attraction of the male flies to the CBEO peaked during the period from 12 to 24 DAE, which coincided with the sexual maturity of the flies. Immature males of *B. dorsalis* spend most of the day hunting for protein and sugar to supplement their nutritional requirements, whereas mature males respond favourably to sex pheromones or parapheromones [55,56]. These results indicate that CBEO plays a more important role among *B. dorsalis* males for chemical and ecological purposes than for nutritional functions. The attractiveness of a volatile compound is not solely determined by its chemical properties and changes with the physiological state of an insect [3,11,57]. Under starvation and thirst stress, *B. dorsalis* males became significantly more responsive to CBEO. Moreover, the responsiveness of sexually mature males to CBEO fluctuated during the day: they responded most strongly in the morning and afternoon and responded less markedly near dusk. As a dusk-mating species, its courtship behaviour is restricted to the period from 17:00 to 19:00 [11]. In this context, during the courtship phase, *B. dorsalis* males may
transiently cease responding to the CBEO in favour of female sex pheromones. In addition, *B. dorsalis* adults are known to perform multiple mating [3,58]. The behavioural responses to CBEO were not significantly different between virgin and mated males. Undoubtedly, knowledge of the characteristics of male flies’ responsiveness to CBEO is vital in the planning and monitoring of *B. dorsalis* control programmes.

The increasing consumption of natural extracts has raised concerns about their safety. To use natural extracts effectively, an assessment of their safety is necessary [59,60]. In this context, prior to endorsing CBEO as a novel male attractant for *B. dorsalis* control, certain issues regarding its ecotoxicological safety should be considered. The ladybirds *C. septempunctata* and *H. axyridis* are important and beneficial natural predators. Although they are not predators of *B. dorsalis*, they have been widely used as the main biocontrol agents against a variety of aphids in greenhouses and farmland [30,61–63]. These beetles are recognized as widely distributed in agricultural and natural habitats around the world. However, these predators are negatively affected by unintended and indiscriminate exposure to synthetic insecticides [30,64]. Specifically, in the current evaluation, the CBEO possessed no significant attractancy for larvae and adults of *C. septempunctata* and *H. axyridis*. A previous study revealed that clove EO has no acute toxicity to *Coleomegilla maculata* (De Geer) (Coleoptera: Coccinellidae), a non-target ladybeetle. In addition, *C. maculata* exposed to clove EO exhibited unaffected locomotion and aphid predation abilities [65]. Furthermore, clove oil is reportedly non-toxic to fish [39].

Notably, cultivable cell-based systems present rapid in vitro assays for assessment of the potential toxicity and risk of xenobiotic chemicals [3,26,27,66]. In addition to being accurate and effective, these systems use no animals or humans, which eliminates any ethical dilemmas [27,67]. Based on the present cytotoxicity investigation, CBEO was indicated to be highly cytotoxic to human cancer cells (MDA-MB-231) and insect cells (SF-9) but relatively showed low or no toxicity to normal human and mouse cells (HFL1 and BNL-CL.2). Therefore, CBEO is likely to possess anticancer and insecticidal properties. Coincidentally, clove EO shows anticarcinogenic, antimutagenic, and insecticidal potential [39,42,47,68,69]. These observations accord well with the present results. Moreover, clove buds and clove oil have been approved as generally safe by the U.S. Food and Drug Administration [70]. In addition, the U.S. Environmental Protection Agency has classified these as minimum-risk pesticides. Overall, the present cytotoxicity study further reveals that CBEO can be regarded as nontoxic and exhibits a good environmental safety performance.

5. Conclusions

In short, the current results show that CBEO may be developed as a safe and potent lure for *B. dorsalis* male flies, which may help improve and optimize current trapping control techniques. Though the premise is promising, the use of CBEO as bait in attract-and-kill programmes in this field is still poorly investigated and implemented. Moreover, the bioactivity of CBEO on other non-target organisms requires further study.

Author Contributions: Conceptualization, Z.-J.H., J.-W.Y., Z.-H.C. and H.L.; methodology, J.-W.Y., Z.-H.C., C.C., S.-Z.D. and H.L.; software, J.-W.Y., Z.-H.C., C.C., Y.-P.M. and N.L.; validation, Z.-H.C., N.L., G.-L.M. and S.-Z.D.; formal analysis, C.C., Y.-P.M., G.-L.M. and Q.B.; investigation, Z.-J.H., J.-W.Y., Z.-H.C. and N.L.; resources, M.D., G.-L.M. and Q.B.; data curation, G.-L.M. and Q.B; writing—original draft preparation, Z.-J.H., J.-W.Y., Z.-H.C., S.-Z.D. and H.L.; writing—review and editing, Z.-J.H., Q.B., S.-Z.D. and H.L.; visualization, S.-Z.D. and H.L.; supervision, M.D., S.-Z.D. and H.L.; project administration, H.L.; funding acquisition, N.L., G.-L.M., S.-Z.D. and H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Natural Science Foundation of China (No. 32001916), the Young Elite Scientist Sponsorship Program by Luo Yang (No. 2022HLTJ03), and the Open Research Fund of the Key Laboratory of South Subtropical Fruit Biology and Genetic Resource Utilization (MOA) (No. 202101). The funders have no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Data Availability Statement: All data sets presented in this study are included in the article and can be provided by the authors upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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