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COMPARATIVE EFFECTS OF *Melaleuca cajuputi* AND *Vitex rotundifolia* ESSENTIAL OILS ON CELLULASE INHIBITION AND IMMUNE RESPONSE MODULATION IN *Rhynchophorus ferrugineus*

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ABSTRACT

The increasing destruction of palm oil crops by the Red Palm Weevil (*Rhynchophorus ferrugineus*, RPW) has prompted the pursuit of environmentally sustainable pest management measures. Essential oils (EOs), recognized for their bioactive characteristics, provide a promising alternative. This study examines the effectiveness of *Melaleuca cajuputi* (Gelam) and *Vitex rotundifolia* (Beach Vitex) EOs in inhibiting RPW cellulase activity and altering its immunological response. The cellulase inhibitory action was evaluated using a carboxymethyl cellulose (CMC) assay, and immune response modulation was examined by haemocyte counts (THC) and differential haemocyte (DHC) classification. The findings show that Gelam EO had significant cellulase inhibition at both 5% and 10% concentrations. Conversely, Beach Vitex EO exhibited a dose-dependent inhibitory effect, showing no effects at 5% and substantial inhibition at 10%. Haemocyte analysis indicated that THC was markedly decreased in both 10% Gelam and Beach Vitex EO treatments relative to controls. Prohemocytes and coagulocytes exhibited a considerable rise in the 10% Gelam EO treatment, whereas plasmatocytes demonstrated a significant decrease, suggesting immunological stress and potential inhibition of essential immune processes. The findings indicate that Gelam EO is a more potent cellulase inhibitor, whereas Beach Vitex EO requires larger dosages to attain similar inhibition levels. Furthermore, EO-induced immunological regulation, especially the inhibition of plasmatocytes, may heighten RPW's vulnerability to environmental stressors and biological control agents. Incorporating these EOs into an integrated pest management (IPM) framework may provide a sustainable substitute for synthetic pesticides. Future research should investigate advancements in EO formulations and their field applications to optimize stability and efficacy for prolonged pest control.

Keywords: Botanical insecticides; Red Palm Weevil; Gelam; Beach Vitex; cellulase; insects immunity

ABSTRAK

Peningkatan kemusnahan tanaman kelapa sawit akibat serangan Kumbang Merah Palma (*Rhynchophorus ferrugineus*, RPW) semakin membimbangkan dan memerlukan pendekatan pengurusan perosak yang lebih lestari. Minyak pati (MP), yang terkenal dengan ciri-ciri bioaktifnya, menawarkan potensi sebagai alternatif kepada racun perosak sintetik. Kajian ini menilai keberkesanan minyak pati *Melaleuca cajuputi* (Gelam) dan *Vitex rotundifolia* (Lemuni Pantai) dalam merencat aktiviti selulase RPW serta permodulatan tindak balas imun serangga tersebut. Tindakan perencatan selulase telah dinilai menggunakan ujian karboksimetil selulosa (CMC), dan modulasi tindak balas imun diperiksa oleh kiraan haemocyte (THC) dan klasifikasi haemocyte pembezaan (DHC). Keputusan menunjukkan bahawa MP Gelam menghasilkan perencatan selulase yang signifikan pada kepekatan 5% dan 10%, manakala MP Lemuni Pantai menunjukkan kesan perencatan yang bergantung kepada dos, dengan perencatan ketara hanya pada kepekatan 10%. Rawatan dengan MP Gelam dan Lemuni Pantai pada 10% juga mengurangkan THC secara signifikan. Tambahan pula, MP Gelam meningkatkan bilangan prohemosit dan koagulosit, serta mengurangkan plasmatosit, menunjukkan tekanan imunologi dan kemungkinan gangguan terhadap fungsi imun utama. Secara keseluruhan, MP Gelam menunjukkan potensi yang lebih tinggi sebagai perencat selulase berbanding MP Lemuni Pantai. Selain itu, kesan imunomodulasi yang diperhatikan berpotensi meningkatkan kerentanan RPW terhadap tekanan persekitaran dan agen kawalan biologi. Penggunaan MP ini dalam strategi Pengurusan Perosak Bersepadu (IPM) berpotensi menjadi pendekatan kawalan yang mampan. Kajian lanjutan harus mengkaji kemajuan dalam formulasi MP dan aplikasinya di lapangan bagi mengoptimumkan kestabilan dan keberkesanan untuk kawalan perosak yang berpanjangan.

Kata kunci: Serangga perosak botanikal; Kumbang Palma Merah; Gelam; Lemuni Pantai; selulase; sistem imun serangga

INTRODUCTION

The Red Palm Weevil (RPW, *Rhynchophorus ferrugineus*), a formidable adversary to palm growers worldwide, has cemented its status as a major agricultural pest. With origins traced back to Asia, its infestation has spread, leaving a trail of destruction across palm plantations in Africa, the Middle East, and parts of Europe (Al-Dosary et al. 2016). This pest's larvae bore into the palm, causing fatal damage that leads to significant economic losses and ecological disruption. The agricultural sector faces billions in financial setbacks due to control measures, loss of yield, and the replacement of dead palms. Moreover, the ecological impact extends beyond mere economic loss, affecting biodiversity and the stability of ecosystems reliant on palm species (Hussain et al. 2013). Efforts to mitigate the RPW's impact have historically spanned chemical, biological, and manual interventions. Despite the efficacy of chemical pesticides for immediate pest reduction, their environmental toll and potential harm to human health raise significant concerns (Oaya et al. 2019; Zakaria et al. 2024). Biological controls, while environmentally friendly, often present challenges in effectiveness and implementation (Verma et al. 2023). The manual removal of infested palms, though direct, is labour-intensive and not always feasible on a large scale (Faleiro et al. 2019). The limitations of these approaches, coupled with the weevil's evolving resistance, highlight the critical need for innovative and sustainable pest management solutions.

In the quest for sustainable agricultural practices, EO from plants have emerged as a viable alternative to traditional pest control methods (Isman & Machial 2006). These natural compounds, known for their bioactive properties, offer an eco-friendly solution with minimal environmental impact. EOs have been recognised for their antimicrobial, insecticidal, and repellent capabilities, making them an attractive option for pest management. Their complex compositions, including terpenes and phenolics, provide a multifaceted approach to pest control, potentially reducing the likelihood of resistance development among pest populations (Koul et al. 2008).

Gelam (*Melaleuca cajuputi*) and Beach Vitex (*Vitex rotundifolia*) EOs stand out for their promising bioactive properties (Azizul et al. 2021; Isah et al. 2023). Gelam oil, derived from a species well-regarded for its medicinal properties, contains compounds that have shown potential in antimicrobial and insecticidal applications. Similarly, Beach Vitex oil, with its unique blend of bioactive components, has been noted for its effectiveness against various pathogens and pests. The exploration of these oils in the context of pest management, particularly against the RPW, offers a novel approach to addressing this agricultural challenge. The RPW's ability to degrade cellulose through its cellulase enzymes is central to its destructive impact on palm trees (Liu et al. 2021). These enzymes facilitate the weevil's digestion of plant material, enabling extensive damage. Hence, the oil cellulase inhibition ability through degradation of glycosidic bond is an option to mitigate the damage from phytophagous insects (Mandels & Reese 1965). By investigating the potential of Gelam and Beach Vitex EOs to inhibit cellulase activity, this study aims to explore new avenues for controlling the weevil's ability to feed and damage palm crops. On the other hand, the immune system of the RPW plays a critical role in its survival and adaptability. Understanding how Gelam and Beach Vitex EOs influence the weevil's immune responses could reveal innovative strategies for enhancing the pest's susceptibility to natural predators and control measures, offering a new dimension to pest management practices.

While EOs are widely recognized for their pest control potential, limited research has explored their effects on RPW’s cellulase activity and immune system, as there were limited up to date studies which focus on effects of EO toward RPW hemocytes (Al Dawsari & Alam 2022; Mady et al. 2023). This study seeks to bridge this gap by conducting a thorough investigation into how these EOs affect crucial biological functions in the weevil, potentially contributing to the development of effective, environmentally friendly pest control strategies. The present study investigates the effects of Gelam and Beach Vitex EOs on cellulase activity and the immune response of the RPW. By shedding light on the biochemical interactions between these oils and the weevil, the study aims to contribute significant insights to the field of pest management. The expected findings could lead to the development of new biopesticide formulations, offering a sustainable alternative to chemical pesticides. This research endeavours to support environmentally responsible agricultural practices, reduce the reliance on harmful pesticides, and provide effective solutions to combat the pervasive threat of the RPW.

MATERIALS AND METHODS

Sampling Of Red Palm Weevils

The Red Palm Weevils (RPWs), *Rhynchophorus ferrugineus*, were collected from infected palm trees around Batu Rakit, Terengganu, Malaysia (5°25'06.0"N 103°04'18.9"E) by using pheromone traps. The weevils were subsequently maintained in a controlled environment that closely mimicked their natural habitat, consisting of ventilation-equipped containers, a

regulated temperature range (at room temperature), and a continuous food source, such as sugarcane (Yan et al. 2021). The well-being of the weevils was regularly monitored, and any unhealthy or dead individuals were promptly removed to prevent the potential spread of disease. Prior to experimentation, the weevils were acclimatized about one week to the specific conditions they would encounter during the study (Harith-Fadzilah et al. 2020). Gentle handling techniques were employed during experimental procedures to minimize stress and physical harm. The weevils were reared in ventilated plastic containers (10 cm in diameter × 5 cm in height) under controlled laboratory conditions (25±2 °C, 70±5% relative humidity, and a 12:12 h light–dark photoperiod). The sugarcane diet was replaced every four days to maintain food quality and hygiene.

Extraction of Essential Oils from Gelam and Beach Vitex

Plant EOs were extracted from Gelam and Beach Vitex leaves collected near Universiti Malaysia Terengganu (UMT). Gelam leaves were sourced from Bari Kecil (5°33'44.0"N, 102°51'31.5"E), while Vitex leaves were collected from the vicinity of Pantai Tok Jembal (5°24'18.3"N, 103°05'53.2"E). A total of 10 kg of fresh leaves were used in the extraction process. The extraction followed a water distillation method with slight modifications based on previously established protocols. Freshly harvested leaves were thoroughly cleaned and finely chopped before being subjected to distillation using a heating mantle with distilled water. The mixture was gently heated until it reached a simmer, and the distillation was continued for 1 to 3 hours. After distillation, the aqueous-EO mixture was transferred to a separating funnel, where hexane was added to facilitate oil separation. The funnel was sealed and gently agitated, allowing the formation of distinct layers. The upper hexane layer, containing the EO, was collected and subjected to solvent evaporation in a well-ventilated fume hood. The resulting pure EO was transferred into glass vials and stored at 4 °C until further use.

To prepare 5 mL each of 5% and 10% diluted EO solutions, 2% Triton X-100 was used as an emulsifier. For the 5% solution, 0.25 mL of EO was mixed with 4.75 mL of distilled water containing Triton X-100. Similarly, the 10% solution was prepared by mixing 0.5 mL of EO with 4.5 mL of distilled water supplemented with Triton X-100. All measurements were performed using a graduated cylinder. The components were combined in a clean container and thoroughly mixed using a glass stirring rod to obtain a homogeneous solution. The prepared solutions were then transferred into 5 mL bottles using a funnel. Each bottle was appropriately labelled. The bottles were stored in a cool, dark place, away from direct sunlight, and were shaken well prior to each use. This method ensured a standardized and reproducible approach for the preparation of diluted EO solutions. Adherence to safety precautions, including patch testing, was recommended prior to use.

RPW Crude Protein Extraction

The collected wild RPW adults were acclimatized, while good condition RPWs (healthy, proper locomotive, no broken appendage and no fungus/ ectoparasite observed through naked eyes) were selected after one week. The RPW was placed upside down to prevent escape in the mortar. Liquid nitrogen was poured into the mortar to freeze the RPW, then crushed gently using a pestle until the RPW was completely powdered. 10.0 mg of powder sample was transferred into a 1.5 mL centrifuge tube which contained 1 mL of 0.5M Tris-HCl solution. The sample was homogenized for 10,000 xg for 10 minutes at 4°C. The solid pellet was discarded, and the collected supernatant was used as crude protein extract for cellulase inhibition assay.

Cellulase Inhibition Assay

The assessment of cellulase activity inhibition in RPW by the EOs was conducted following the method described by Sami and Shakoori (2007). Carboxymethyl cellulose (CMC) agar plates were prepared by autoclaving a mixture of distilled water, ammonium dihydrogen phosphate, potassium chloride, magnesium sulphate heptahydrate, yeast extract, CMC, and agar at 121 °C for 20 minutes. The sterilized CMC agar was then poured into sterile petri dishes and allowed to cool. Once solidified, three wells were created in each plate using a 1 cm diameter cork borer. For the assay, a 1:1 mixture of crude protein extract from RPW and EO (50 µL each) was prepared for the two EO concentrations: 5% and 10%. These mixtures were loaded into the wells of the CMC agar plates. Control wells received crude protein extract mixed with distilled water containing 2% Triton X-100 (emulsifier) in place of EO. The plates were incubated overnight at 50 °C. After incubation, the plates were stained with 0.1% Congo red solution for 15 mins with intermittent shaking, followed by multiple washes with 1 M NaCl solution to remove excess dye. Cellulase activity was assessed based on the formation of halo or clearing zones around the wells. The diameter of the clearing zones (measured in centimetres) was used as an indicator of cellulase inhibition. The percentage of inhibition was calculated using the following formula:

$$Inhibition\ rate\ (\%) = [1 - (T/C)] \times 100$$

where T = diameter of the halo zone in the treated sample, and C = diameter of the halo zone in the control. The procedures above were triplicated. This assay served as a critical step in evaluating the efficacy of Gelam and Beach Vitex EOs in inhibiting RPW cellulase activity, offering valuable insights into their potential application as natural biopesticides.

Topical Application of Essential Oils and Sample Preparation for Haemolymph Analysis

For topical application, 0.5 µL of either 5% or 10% Gelam or Beach Vitex EOs was applied to the posterior region of each RPW. Each treatment group consisted of at least 10 adult individuals per replicate (triplicated). The control group received 0.5 µL of distilled water containing 2% Triton X-100, serving as the emulsifier. Following treatment, both control and treated RPWs were transferred into appropriate containers and maintained in dark conditions at room temperature. After one-day interval exposure period, only surviving weevils were selected for subsequent haemolymph collection and analysis.

Haemolymph Collection and Preparation

Haemolymph collection was performed following the method described by Manachini et al. (2011), with slight modifications. Adult RPWs were first surface-sterilized using 70% ethanol and subsequently rinsed with sterile distilled water. Haemolymph was then carefully extracted from the abdominal region using a sterile 23G needle attached to a 1 mL syringe. Approximately 50 µL of haemolymph was collected from each adult RPW. The collected haemolymph was immediately diluted at a 1:3 ratio using 1× phosphate-buffered saline (PBS) solution.

Total and Differential Haemocyte Count Analysis

Total haemocyte count (THC) and differential haemocyte count (DHC) were determined using standard light microscopy techniques. For THC, diluted haemolymph samples were loaded onto a 0.0025 mm² Neubauer hemacytometer, covered with a coverslip, and observed under a light microscope (DLMB LEICA) at 100× magnification. Haemocyte numbers were recorded

from four corner grids and the central grid. The counts were expressed as $\times 10^6$ cells/mL and calculated using the formula adapted from Li-Miao et al. (2024):

$$THC = \frac{Average\ of\ highest\ and\ lowest\ counts \times number\ of\ squares\ counted}{total\ number\ of\ grids} \times dilution\ factor \times 10{,}000$$

This formula enables the conversion of haemocyte counts into a standardized concentration per millilitre.

For DHC, haemocyte classification was performed using a blood smear technique. A 20 µL aliquot of diluted haemolymph was pipetted onto a clean glass slide, smeared evenly, and air-dried at room temperature. The smear was stained with one to two drops of Giemsa aqueous solution, incubated for 10 minutes, then gently rinsed with distilled water to remove excess stain. After drying, a coverslip was mounted, and the slide was examined under a light microscope with oil immersion at 1000× magnification. The procedures were then triplicated. Haemocytes were identified and classified into five major types: prohemocytes, plasmatocytes, coagulocytes, oenocytes, and spherule cells, based on established morphological criteria (Al Dawsari & Alam 2022; Gadelhak 2005; Manachini et al. 2011; Zhang & Zhang 2021). The proportion of each haemocyte type was calculated using the formula:

$$\%\ of\ haemocyte\ type = \frac{Number\ of\ each\ haemocyte\ type \times 100}{Total\ number\ of\ haemocytes\ examined}$$

Data Analysis

Data analysis was aided by Microsoft Excel 16 and IBM SPSS Statistics v20, Shapiro-Wilk test was used to assess data normality, One-way ANOVA was used to evaluate significant differences among treatments, followed by Tukey's post hoc test, in which p-value was set at 0.05.

RESULTS AND DISCUSSION

Cellulase Inhibitory Effect of Gelam and Beach Vitex Essential Oils

The findings in Figure 1 indicate that Gelam and Beach Vitex EOs inhibit cellulase activity in red palm weevils (RPWs), but with distinct inhibitory patterns. Gelam EO exhibited strong cellulase inhibition at both 5% and 10% concentrations, with no statistically significant difference between the two concentrations (df=17, F=0.286, *P=0.6*). This suggests Gelam EO signifying its active compounds effectively bind to and inhibit cellulase without requiring higher concentrations. In contrast, Beach Vitex EO showed a concentration-dependent inhibition, with minimal effects at 5% but a significant increase at 10% (df=17, F=22.17, *P<0.05*), suggesting that higher doses are required to achieve comparable inhibitory effects.

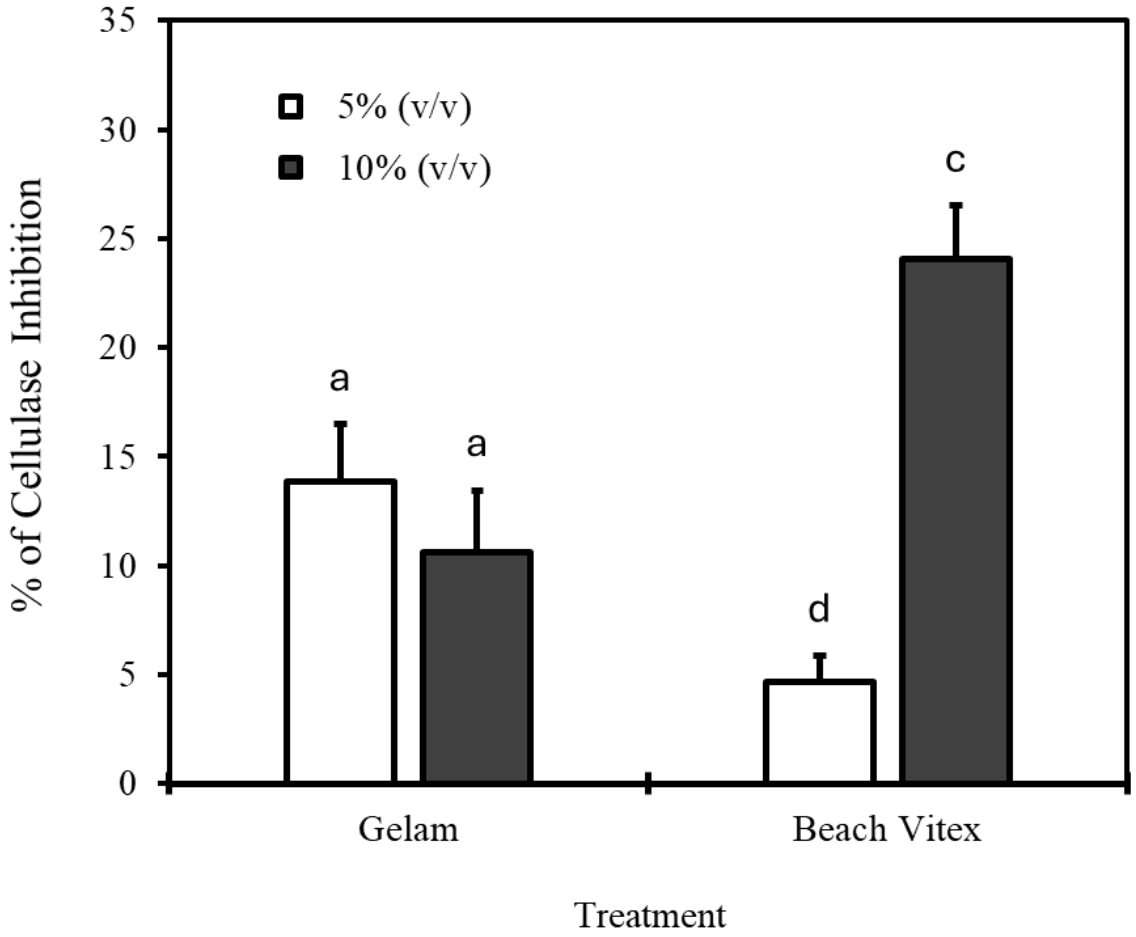

Figure 1. Effect of Gelam and Beach Vitex EOs on cellulase inhibition in red palm weevils at 5% and 10% concentrations. Gelam EO exhibited strong inhibition at both concentrations, with no significant difference between 5% and 10%. In contrast, Beach Vitex EO showed a concentration-dependent response, with significantly higher inhibition at 10% compared to 5%. Different letters above bars indicate statistically significant differences between treatments (one-way ANOVA, $P<0.05$, n = 3)

The findings highlight the stronger inhibitory potential of Gelam EO on cellulase activity, suggesting that its chemical composition contains more potent or synergistic bioactive compounds compared to Beach Vitex EO. This differential efficacy supports Gelam EO as a more effective biopesticide candidate capable of disrupting the digestive processes of RPW. Statistical analysis further reinforces this conclusion, showing that Gelam EO at 5% was as effective as Beach Vitex EO at 10% in inhibiting cellulase activity.

The observed cellulase inhibition by Gelam and Beach Vitex EOs underscores their potential as natural insecticidal agents. The significant reduction in enzyme activity, particularly in response to Gelam EO, suggests that these oils target a crucial physiological process in RPW. This aligns with previous studies demonstrating the insecticidal and antimicrobial properties of *Melaleuca* species oils (Ismail et al. 2022; Liao et al. 2017; Zimmermann et al. 2022). However, the novel application of Gelam and Beach Vitex EOs in targeting cellulase activity offers an approach by interfering with the weevil's ability to digest plant material, these oils could significantly reduce its survival and feeding efficiency on palm

crops. The greater efficacy of Gelam EO over Beach Vitex EO in inhibiting cellulase activity suggests that its bioactive components such as phenolics and flavonoids play a critical role in enzyme inhibition (Yan et al. 2024). Flavonoid molecules have been reported as cellulose inhibitors (Sami & Shakoori 2007). The distinct chemical composition of Beach Vitex EO may explain its lower efficacy, reinforcing the importance of understanding plant-insect chemical ecology for developing targeted botanical pest management strategies.

Variations in phytochemical content and enzyme binding affinities help explain the differing inhibitory patterns between the two EOs. Previous studies on *Melaleuca* species have shown that certain terpenoids, such as eucalyptol (1,8-cineole), α-terpineol, and viridiflorol, strongly inhibit enzymes (Isah et al. 2024; Patramurti et al. 2020). Similar inhibitory effects have been observed in other phytophagous insects exposed to Melaleuca EOs, where digestive enzyme activity was reduced due to direct enzyme binding (Chintalchere et al. 2021; Liao et al. 2017; Liao et al. 2018). In contrast, Beach Vitex EO contains phenolics such as vitexin, luteolin, and flavonoids, which possess antioxidant and enzyme-modulating properties (Chaudhry et al. 2020; Sousa et al. 2021). The delayed but substantial inhibition at 10% suggests a competitive inhibition mechanism, where active molecules compete with the natural substrate (cellulose) for enzyme binding sites. Additionally, the structural complexity of flavonoids may slow enzyme interactions, requiring a higher concentration threshold to disrupt cellulase activity effectively (Stamogiannou et al. 2021).

RPWs rely on cellulase to break down cellulose into glucose for energy metabolism, enabling them to digest fibrous plant material. Inhibiting this process is likely to disrupt nutrient absorption, impair developmental progression, and reduce feeding efficiency (Antony et al. 2017; Osman et al. 2018). The pronounced cellulase inhibition observed with Gelam and Beach Vitex EOs highlights their natural insecticidal potential. The significant enzyme activity reduction, particularly in response to Gelam EO, suggests that these oils disrupt a fundamental physiological process in RPWs, potentially reducing feeding efficiency and survival rates. This aligns with prior research emphasizing the insecticidal and antimicrobial properties of Melaleuca species oils (Ismail et al. 2022; Liao et al. 2017; Zimmermann et al. 2022). However, the novel application of these EOs in targeting cellulase activity offers an alternative pest control strategy, which impair digestion to limit the weevil's ability to infest and damage palm crops, at the same time to reduce host plant damaged by RPW. Future studies should focus on optimizing EO formulations, such as nanoemulsions and encapsulated delivery systems, to improve stability and efficacy. Additionally, field tests and ecotoxicological research will be essential for evaluating practical relevance and potential impacts on beneficial species. Advancements in this area could position these plant-based EOs as sustainable alternatives to synthetic pesticides, reducing environmental hazards while aiding in RPW control.

Immune Response of Red Palm Weevils

The immune system of insects relies on haemocytes, which play a crucial role in cellular immune responses such as phagocytosis, encapsulation, coagulation, and antimicrobial defence (Eleftherianos et al. 2021). Haemocytes are broadly categorized into granulocytes, plasmatocytes, prohemocytes, coagulocytes, oenocytes, and spherule cells, each serving distinct functions in immunity and homeostasis (Castillo et al. 2006; Ribeiro & Brehelin 2006). Disrupting the immune function of the red palm weevil (RPW) using botanical insecticides could provide an eco-friendly approach to pest management. However, the effects of Gelam and Beach Vitex EOs on RPW haemocytes remain unexplored.

The results on total haemocyte count (THC) indicate a concentration-dependent response to EO treatments (Table 1). At 5% EO, haemocyte counts increased moderately by 16.5% for Gelam EO and 57.7% for Beach Vitex EO, suggesting that Vitex EO had a stronger stimulatory effect than Gelam EO at this concentration. However, these increases were not statistically significant compared to the control group (df=39, F=2.306, *P=0.114*). In contrast, at a higher concentration (10%), both EOs significantly reduced total haemocyte counts (df=27, F=41.942, *P<0.05*). Gelam EO treatment led to a decrease by 57.7%, while Beach Vitex EO caused a further reduction to 75.8%. As at low concentration, Beach Vitex EO showed the most drastic reduction than Gelam EO. These results suggest a biphasic response, where lower EO concentrations may trigger immune activation, while higher concentrations induce cytotoxicity, leading to a decline in haemocyte populations. As field application, these EOs possible to weaken RPWs, while insect that consisting lower THC values may increase in susceptibility against pathogens (Siddiqui & Al-Khalifa 2014).

Table 1. Total haemocyte count (THC) of red palm weevil (RPW) adults following topical application of Gelam and Beach Vitex EOs at 5% and 10% concentrations. Results are expressed as mean±standard error (×10^6 cells per mL). Both EOs at 10% concentration significantly reduced haemocyte count compared to control and 5% treatments. Different superscript letters indicate statistically significant difference between treatments (one-way ANOVA, *P<0.05*, n=5)

Treatment	**Concentration (%)**	**Cell Count (x10^6 per mL)**
Control		18.45 ± 1.26^b
Gelam	5	21.5 ± 1.83^b
	10	7.81 ± 0.62^a
Vitex	5	29.1 ± 2.01^b
	10	4.46 ± 3.95^a

The moderate increase in THC at 5% EO treatment but decrease at 10% concentration suggests EO exposure may be due to hormesis phenomenon. The increasing in hemocytes could be contributed through initial immune response activation. Bergmann et al. (2021) reported that low-level immune challenges can induce haemocyte activation as a protective response. Similarly, in *Spodoptera litura* larvae, exposure to cassava (*Manihot esculenta*) extract increased haemocyte counts, suggesting a stress-induced immune activation (Manjula et al. 2020). However, this increase in haemocytes may also indicate potential risks for pest resistance. If EO exposure primes RPW immunity, there is a possibility of adaptive responses, leading to increased resilience against pathogens (Subhagan et al. 2024). At higher EO concentrations, total haemocyte counts dropped significantly, suggesting a cytotoxic effect. Several studies have reported similar EO-induced immune modulation in insects. In one example, THC in *Zeugodacus cucurbitae* (melon fruit fly) larvae showed a significant decline when treated with γ-terpinene, a monoterpene widely present in EOs of many medicinal and aromatic plants (Singh et al. 2023). In a study by Mady et al. (2023), a reduction in THC was also observed with *Origanum majorana*-exposed RPW 4th larval instar. The immune modulation of EOs may be due to many factors. EOs can disrupt cell membranes, leading to haemocyte cytolysis and apoptosis (Al Dawsari & Alam 2022; Yap et al. 2021). Deltamethrin, an EO from *Cymbopogon citratus* and *Cinnamonum camphora* has been shown to reduce haemocyte viability in *Trogoderma granarium* (Feroz 2020). Furthermore, EO-induced

apoptosis has been linked to oxidative stress DNA fragmentation, and mitochondrial dysfunction (Sharifi-Rad et al. 2017), which can eventually reduce haemocytes count.

The analysis of differential haemocyte count (DHC) revealed significant alterations in immune cell distribution (Figure 2) as well as Figure 3 showed the observation of haemocyte types at 100x magnification under light microscope. While granulocytes (df=14, F=1.338, *P=0.322*), oenocytes (df=14, F=1.893, *P=0.188*), and spherule cells (df=14, F=0.75; *P=0.988*) showed no significant changes across treatments, prohemocytes (df=14 F=4.417, *P=0.026*) and coagulocytes (df=14, F=7.024, *P=0.006*) exhibited a significant increase in response to 10% Gelam EO treatment, while it suggests an immune modulation effect. Conversely, plasmatocytes were significantly reduced (df=14, F=6.115, *P=0.009*) in the same treatment, indicating immune modulation or disruption of haemocyte differentiation. These findings suggest EO exposure influences haemocyte differentiation, proliferation, and survival, leading to immune modulation in RPW. The ability of Gelam and Beach Vitex EOs to alter RPW haemocyte profiles is likely attributed to their bioactive compounds. EOs contain terpenoids, phenolics, and flavonoids, which can interfere with cellular function and immune homeostasis. One possible mechanism involves membrane permeability disruption. Certain EO constituents, such as eugenol, thymol, and cineole, have been reported to damage cell membranes, leading to haemocyte lysis and depletion (Regnault-Roger et al. 2012; Yap et al. 2021). Additionally, EOs can induce oxidative stress, generating reactive oxygen species (ROS) that trigger haemocyte apoptosis (Fergani et al. 2020; Ghoneim et al. 2021).

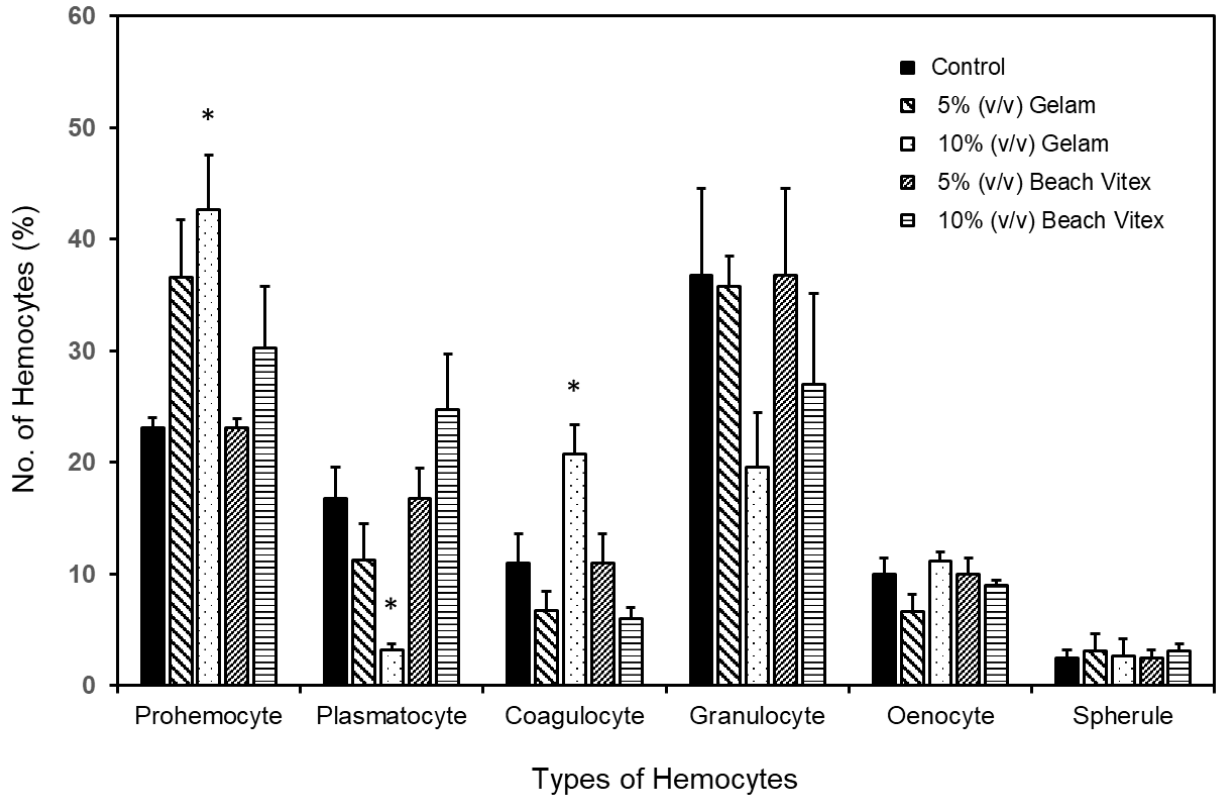

Figure 2. Differential haemocyte count (DHC) in red palm weevil (RPW) adults following topical application of Gelam and Beach Vitex EOs at 5% and 10% concentrations. Data are presented as the percentage of each haemocyte type (mean±standard error). The treatment influenced the distribution of haemocyte types, with notable increases in prohemocyte and coagulocyte proportions at

higher EO concentrations, while plasmatocyte percentages were significantly reduced. Asterisks indicate significant differences (One-way ANOVA, $P<0.05$, n=3) compared to the control group

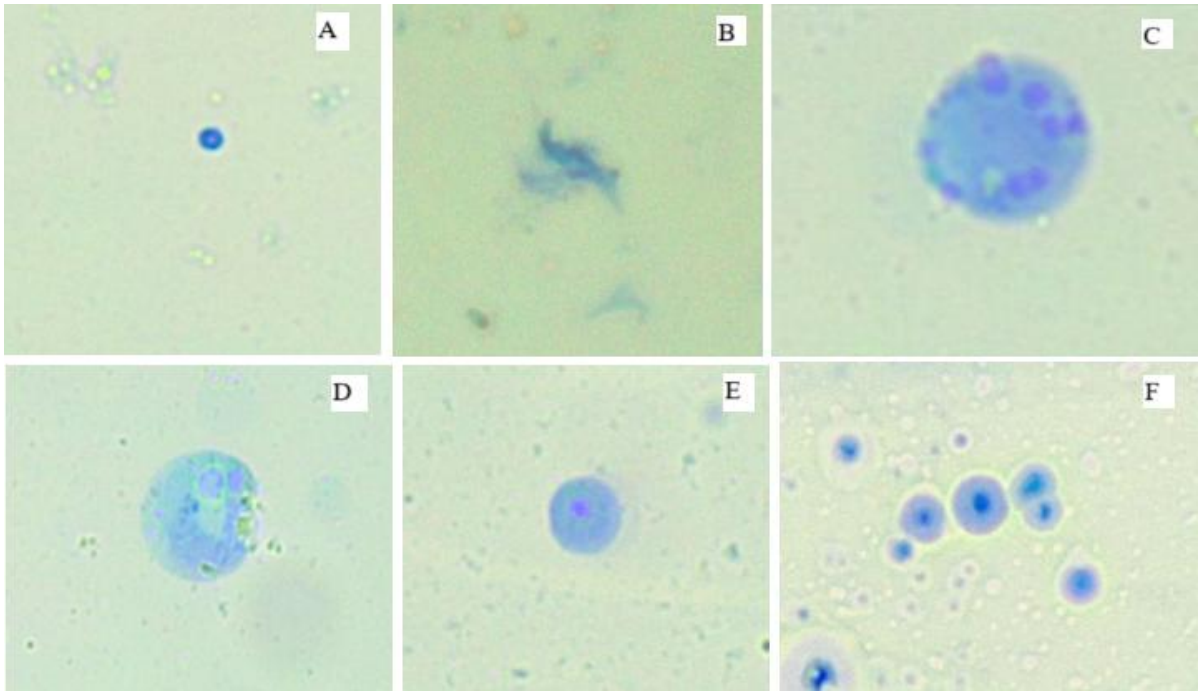

Figure 3. Haemocyte types under 100x magnification under light microscope with the aid of oil immersion using Giemsa staining. A as prohemocyte, B as plasmatocyte, C as coaguloyte, D as granulocyte, E as oenocyte and F as spherule cell

Prohemocytes, which serve as undifferentiated precursor cells for other haemocyte types, were significantly increased following 10% Gelam EO treatment. This suggests that EO exposure induces immune stress, prompting higher hematopoietic activity to compensate for haemocyte depletion. Similar findings have been reported in *Ephestia kuehniella*, where exposure to insect growth regulators led to increased prohemocyte production as a compensatory mechanism (Ghasemi et al. 2014). Likewise, in RPW 4th larval instar, treatment with *O. majorana* EO for 48 hours resulted in increased prohemocytes while reducing granulocytes and plasmatocytes, indicating immune modulation, stress-induced haematopoiesis, or disrupted haemocyte differentiation (Mady et al. 2023). The observed increase in coagulocytes suggests an immune activation response, as these cells play a key role in wound healing and coagulation (Dushay 2009). Haemocytes contribute to clot formation post-injury, reducing haemolymph loss, creating a barrier against pathogens, and promoting wound healing (Dziedziech et al. 2020). An increase in coagulocytes typically signals an active immune response, demonstrating the adaptability of the insect immune system in maintaining equilibrium and survival (Eleftherianos et al. 2021). This suggests that RPW may attempt to repair cellular damage induced by Gelam EO exposure by activating immune defences, as evidenced by increased coagulocyte counts. In contrast, plasmatocytes, which are functionally active haemocytes involved in phagocytosis and pathogen encapsulation, showed a significant reduction in the 10% Gelam EO treatment group. This indicates that EO exposure disrupts plasmatocyte differentiation and survival, potentially weakening immune defences. Certain EOs have been shown to impair insect immune responses by reducing circulating plasmatocyte

counts. For example, cinnamon EO significantly lowered plasmatocyte counts in the rice weevil (*Sitophilus oryzae*) and lesser grain borer (*Rhizopertha dominica*), demonstrating cytotoxic effects on these cells (Tawfeek et al. 2017). Similarly, Ali and Ibrahim (2018) reported that plasmatocyte populations in *S. littoralis* were adversely affected by castor and camphor EOs, with castor oil exhibiting stronger immunomodulatory effects. The reduction in plasmatocytes suggests potential immunodeficiency, increased oxidative stress, and impaired haematopoiesis. Additionally, the observed increase in prohemocytes but a decrease in plasmatocytes (Figure 2) suggests a disruption in immune signalling pathways, preventing prohemocytes from differentiating into plasmatocytes (Lavine & Strand 2002; Morin-Poulard et al. 2021). Since plasmatocytes play a critical role in insect immunity, their depletion could render RPWs more vulnerable to infections and biological control agents. Furthermore, the alterations in RPW haemocyte profiles following EO exposure resemble immune responses observed in other insect species treated with EOs. These findings suggest that Gelam EO, and to a lesser extent Beach Vitex EO, disrupt haemocyte function, potentially weakening the immune system of various insect pests and enhancing their susceptibility to biopesticides.

The observed changes in haemocyte profiles underscore the potential of EOs as immunomodulatory biopesticides. By disrupting plasmatocyte differentiation and increasing immune stress, EOs could weaken RPW immune defences, making them more susceptible to microbial biocontrol agents. This suggests that combining EO treatments with entomopathogenic fungi, such as *Beauveria bassiana*, could enhance efficacy, as immunomodulatory insects may have reduced resistance to fungal infection (Goettel et al. 2005). Moreover, these findings indicate that EOs could be integrated into pest management programs to mitigate insect resistance development, as immune-suppressed pests are less likely to survive repeated infestations. However, EO-based biopesticides must be carefully optimized to minimize unintended effects on non-target species. The observed increase in prohemocytes and coagulocytes at lower EO concentrations suggests that sublethal EO exposure might, in some cases, stimulate immune responses, potentially enhancing pest resilience rather than suppressing it. Therefore, future studies should focus on identifying the optimal EO concentrations that effectively suppress immune function without triggering adaptive resistance.

CONCLUSION

This study highlights the biopesticides efficacy of EOs derived from *Melaleuca cajuputi* (Gelam) and *Vitex rotundifolia* (Beach Vitex) in inhibiting cellulase activity and modulating immunological function in *Rhynchophorus ferrugineus* (RPW). The results indicate that Gelam EO significantly reduced cellulase activity at both 5% and 10% concentrations, with no statistically significant difference seen. In contrast, Beach Vitex EO demonstrated a dose-dependent inhibition, showing no effects at 5% but a substantial increase at 10%. The results indicate that Gelam EO is a more effective cellulase inhibitor at lower doses, while Beach Vitex EO necessitates larger concentrations for similar inhibition. The immune-modulating effects of these EOs were apparent, as total haemocyte counts (THC) reduced at the 10% EO treatment, indicating cytotoxic effects on RPW immune cells. Differential haemocyte examination indicated a significant increase in prohemocytes and coagulocytes in response to 10% Gelam EO, whereas plasmatocytes displayed a significant decrease. These findings suggest that EO exposure triggers immunological stress, potentially compromising RPW defensive mechanisms.

The strong inhibition of Gelam EO can be mechanistically attributed to its terpenoid and flavonoid constituents, which interact with the active sites of cellulase. In contrast, the inhibition exerted by Beach Vitex EO follows to a competitive inhibition model, necessitating elevated concentrations to overcome the natural substrate, cellulose. Moreover, EO-induced immune modulation may increase RPW vulnerability to other biological control agents, presenting a possible synergistic strategy for integrated pest management (IPM). These findings highlight the potential application of Gelam as sustainable biopesticide alternative. Further studies are recommended, such as EO formulations, conduct field validation studies, and examine their molecular interactions with digestive enzymes and immunological pathways. Furthermore, ecotoxicological evaluations can be performed to assess their impact on non-target insect pests and the overall safety of the environment.

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AUTHORS DECLARATIONS

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Conflict of Interest
The authors declare no conflict of interest.

Ethics Declarations
The authors declare that the study did not involve human or vertebrate animals and ethical approval was not applicable.

Data Availability Statements
The results dataset of this study is available from the corresponding author upon reasonable request.

Author's Contributions
Tay Karh Yan (TKY), first author, laboratory work, data analysis, manuscript preparation; Hazlina Ahamad Zakeri (HAZ), corresponding author, manuscript editing, research scheme holder; Muhammad Aieman Asyraf Nuruljaiman (MAAN), laboratory work; Maxswell Junior Presly Gamba (MJPG), laboratory work; Nor Omaima Harun (NOH), supervision of laboratory work.

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