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INSECTICIDAL ACTIVITIES OF ESSENTIAL OILS FROM PANDAN AND LEMONGRASS AGAINST THE ADULT RED PALM WEEVIL

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Abstract: The goal of this study is to ascertain whether the essential oils (EOs) derived from the plants *Pandanus amaryllifolius* (pandan) and *Cymbopogon citratus* (lemongrass) have any potential to be effective insecticides against the adult *Rynchophorus ferrugineus* (red palm weevil, RPW). The effectiveness of the EOs is evaluated based on their capacity to kill RPW instantly upon contact and also on their ability to block acetylcholinesterase (AChE) enzymes. These EOs' impact on protein synthesis was also identified. After five days of exposure, the mortality rate of RPWs was found to be between 11% and 33%, with female RPWs being more affected than male RPWs. However, increasing the concentration of EOs from 5% to 10% (v/v) had no discernible effect on mortality. Both EOs are ineffective AChE inhibitors because they had no effect and rather increased the activity of the enzyme. The enzyme AChE in female RPWs is significantly inhibited by just 10% of the lemongrass. With the exception of 10% EOs, which markedly increased the TSP of female RPWs, the RPWs' total soluble proteins (TSP) were much lower after treatment. In conclusion, both EOs have the potential to be applied as insecticides against adult RPW. Additionally, it may be said that both EOs function in a similar way towards RPW. These results add to the body of knowledge regarding the search for new insecticides that may be helpful in controlling the RPW because this study has yet to be conducted before.

Keywords: Essential oils, red palm weevil, contact toxicity, acetylcholinesterase, total soluble proteins.

Introduction

Rhynchophorus ferrugineus, commonly known as red palm weevil (RPW), is one of the most destructive pests that threaten palm trees (Mohamed *et al.*, 2020). It was first discovered in Southern and Southeastern Asia in the early 20th century (Nasir *et al.*, 2016). The female RPW lays eggs in wounds as well as in the axils and petioles of fresh leaves. The larvae burrow through the petioles and finish their life cycle at the terminal bud of a date palm. The larval stage is the most destructive stage because it feeds by destroying the internal tissues of the palm tree, which makes it challenging to handle because there are no early signs of an infestation (Hoddle *et al.*, 2013; Vacas *et al.*, 2013; Milosavljević *et al.*, 2019). Once infested, the palm trees die without anyone noticing this silent boring destroyer. The current Integrated Pest Management (IPM) for RPW in Malaysia relies heavily on chemicals (Malaysian Department of Agriculture, 2017). For example, chemical insecticides such as Spirotetramat, Fipronil, Chlorpyrifos, and Methidathion can be applied to RPW-infested palms using the trunk injection method (Shar *et al.*, 2012), or spraying cypermethrin to its canopy for some shorter palm plants (Wahizatul *et al.*, 2017). While using chemical or synthetic insecticides can cause an almost complete removal of the pests from the infested area, they have a negative influence on the natural environment as well as human health (Chowański *et al.*, 2014). Excessive use of these insecticides has made many insect species become physiologically or behaviorally resistant (Boyer *et al.*, 2012). As a result, it is crucial to identify effective replacements for synthetic chemicals that are also more environmentally friendly (Campolo *et al.*, 2018).

Essential oils (EOs) are organic compounds created naturally by plant metabolism, and they give plants their characteristic scent (Pavela, 2015). They are a potential and suitable alternative due to their potent efficiency against a wide range of pests, diverse modes of action, minimal toxicity of residues during application and on non-target organisms, and reasonably cost-effective manufacturing techniques (Pavela & Benelli, 2016). EOs have been proven to exhibit insecticidal activities such as repellency, contact toxicity, ovicidal, larvicidal and antifeedant against important pests. EOs can easily enter an insect's respiratory system, given their highly volatile nature. To name a few, cinnamon's EO exhibits repellent activity against the rice weevil (Shi *et al.*, 2022), while basil oil showed the strongest contact toxicity effect on third instar larvae of the tobacco cutworm as studied by Kim *et al.* (2021). On the other hand, EO from peppermint showed excellent ovicidal and larvicidal activities against the common house mosquito (Hadjira *et al*., 2021). EOs can also be neurotoxic to insects by inhibiting acetylcholinesterase (AChE), an enzyme which degrades acetylcholine (ACh), an essential neurotransmitter in the central nervous system (CNS) of insects. Thus, many insecticides nowadays have been synthesized targeting the AChE of insects, interfering with the breakdown of ACh, leading to the deposition of the ACh in the nerve synapses and causing disrupted neurotransmission (Thapa *et al.*, 2017).

In our study, RPWs were treated with EOs from two plants, namely pandan (*Pandanus amaryllifolius*) and lemongrass (*Cymbopogon citratus*) to determine the potential of these EOs as contact toxicity and AChE inhibitors against RPW. While pandan and lemongrass are widely used by Malaysians as insecticides, the use and study of these plants on RPW are few. In addition, since EOs represent a complex and unique mixture of compounds which are specific for each plant and extraction procedure, it opens up vast opportunities for researchers to study their insecticidal activities and produce the best formulation for bioinsecticide against the targeted pest.

Materials and Methods

Sampling of Adult Red Palm Weevil

A Sampling of the RPW or *Rhynchophorus ferrugineus* adults was done around the vicinity of Universiti Malaysia Terengganu (UMT), Kuala Nerus, Terengganu, Malaysia (5°24'19.03"N, 103°5'15.7"E). Traps were located at places expected to have been infested by RPW. Pineapple was served as food bait and pheromone was hung at the lid of the trap. The traps were observed after a week and the RPWs that were still alive were collected; they were then kept in a container containing sugarcane for their source of food. New food baits and pheromones were refilled into traps and observed again after a week. The collected RPWs were then transported to the Biochemistry Laboratory, Faculty of Science and Marine Environment, UMT. The RPWs were sorted into females and males in the laboratory and were further reared.

Extraction of Essential Oils

Fresh pandan or screw pine (*Pandanus amaryllifolius*) and lemongrass (*Cymbopogon citratus*) leaves were purchased from local markets in Kampung Tok Jembal, Kuala Nerus, Terengganu, Malaysia (5°23'23.02"N, 103°5'59.9"E). The leaves were first cleaned under running tap water to ensure no dust and dirt were left on the leaves. About 4.05 kg and 1.4 kg of pandan and lemongrass leaves, respectively, were then cut into small pieces prior to hydrodistillation. Hydrodistillation was done according to Zakaria *et al.* (2020), where water was heated at 70˚C using n-hexane as a solvent. At the end of hydrodistillation, about 0.081 g of EO was extracted from 4.05 kg of pandan leaves while 4.65 g of EOs were extracted from 1.4 kg of lemongrass leaves. These EOs were then stored in amber glass bottles at 4˚C until further use.

Contact Toxicity of Essential Oils Against the Adult RPW

Contact toxicity was conducted according to the method by Liu *et al*. (1999) with modifications. The RPWs were quarantined for 24 hours without food prior to experiments. Only the healthy and active RPWs were used for treatment. In each treatment, three male and three female RPWs were used, considered as one replicate. There were three replicates overall. About 50 µL of the essential oils (EOs) were topically applied to the head, thorax, and abdomen of the weevils. The weevils were treated with two different concentrations of extracted pandan and lemongrass EOs which were 5% and 10% (v/v). The concentrations were prepared by adding 0.5 (5%) or 1.0 (10%) mL of extracted EOs into 9.3 mL or 8.8 mL distilled water, respectively, with 0.2 mL 2% Triton X-100 as an emulsifier. Weevils treated with 9.8 mL distilled water and 0.2 mL 2% Triton X-100 were used as control. Treated RPWs were then kept in a small container with holes separately. The number of RPWs that were dead was monitored every day for five days. The mortality rate was calculated on day five by taking up the total cumulative of dead RPW divided by the number of samples then times 100. Dead RPWs were collected and stored in the freezer until further use.

Extraction and Quantification of RPW's Total Soluble Proteins (TSP) After Treatment

The heads of dead RPWs collected earlier were separated from the abdomen and used for TSP extraction and quantification. The heads were weighed and ground with liquid nitrogen until they resembled a fine powder. By following the Sami and Shakoori's (2007) method, the suspension was homogenized in 100 mL of 0.5 M Tris-HCl buffer with a pH of 8.5, passed through a sieve, and filtered, then centrifuged at 10,000 Xg for 10 min at 4°C. The supernatant was kept at -20°C until use and utilized as a source for an acetylcholinesterase enzyme assay. The concentration of TSP extracted was quantified using 1 mg/mL bovine serum albumin (BSA) standards according to the Bradford method (Bradford, 1976). The standard concentrations used were prepared in a series of 0.0625 to 1 mg/mL and the absorbance was measured after five minutes using a UV spectrophotometer at 595 nm.

Determination of Acetylcholinesterase (AChE) Activity of Treated RPWs

The AChE activity of treated RPWs was determined using the Acetylcholinesterase Fluorescent Activity Kit (EIAACHEF) from ThermoFisher Scientific, US. The kit utilizes a proprietary non-fluorescent molecule that covalently binds to the thiol product of the reaction between the AChE Substrate (i.e., acetylcholine iodide) and AChE in the standards or samples, yielding a fluorescent product. About 100 µL of extracted TSP samples were first added into the appropriate wells. A Reaction mix (50 µL) containing 10X AChE Substrate Concentrate, 10X Detection Reagent Concentrate and dry dimethylsulfoxide (DMSO) was then added and mixed with the samples. The assay mixture was incubated for 20 minutes at room temperature. Fluorescent emission at 510 nm with excitation at 390 nm were then read in a fluorescent plate reader. The AChE activity of the samples was compared to that of a standard curve prepared from 0-100 mU/mL of AChE Standards provided. One unit of enzyme activity was defined as 1.0 µmol of acetylthiocholine iodide hydrolyzed per minute at pH 7.4 and 37℃.

Statistical Analyses

All data was presented as the mean ± SE value of three replicates unless stated otherwise. Statistical analysis was done using one-way ANOVA with Tukey's post-hoc test. Statistical differences were considered significant at $p < 0.05$. Results of the analysis were generated using SPSS Statistics 20.0 software.

Results and Discussion

The data on the mortality rate of both male and female red palm weevils (RPWs) after five days of treatment with essential oil (EOs) of pandan and lemongrass is tabled in Table 1. It was observed that both the EOs showed greater impact on the death of male than female RPWs. There was no significant difference observed when it came to the concentration of EOs applied. However, there was a decreasing trend in the mortality rate of female RPWs when we increased the concentration of EOs from 5% to 10%. In general, the death of RPWs, in spite of gender, was only observed after day three of treatment for 10% pandan compared to day one of 5% pandan (data not shown). In comparison, both 5% and 10% lemongrass exerted their effect on day one killing at least one of the RPW. The effect was slow at first for the 5% lemongrass but suddenly shot to more than the 10% lemongrass after day three (data not shown).

Table 1: Mortality rate of red palm weevil after treatment with essential oils of pandan and lemongrass after five days

Treatment	Pandan		Lemongrass	
	Male	**Female**	**Male**	**Female**
Control	0.00	0.00	0.00	0.00
5% (v/v) essential oil	22.2 ± 6.4a	22.2 ± 6.4a	33.3 ± 5.6a	27.8 ± 3.2a
10% (v/v) essential oil	22.2 ± 6.4a	11.1 ± 6.4a	33.3 ± 0.0a	22.2 ± 6.4a

[as] For each plant EOs, mean ± SEs in the same column followed by the same letters do not differ significantly (p > 0.05) in ANOVA test.

Contact and fumigant insecticidal actions of plant EOs have been well demonstrated by previous researchers. For example, oil extracts from basil and coneflower were shown to cause more than 95% mortality of *Sitophilus granarius* after 24 hours and 72 hours application, respectively (Teke & Mutlu, 2021). About 2.9 µg/adult of EO from leaves of *Mentha piperita* was needed to kill half of the population of *Tribolium castaneum* (Pang *et al.*, 2020). In a study by Changbunjong *et al.* (2022), they observed that a concentration of EO from *Citrus aurantium* increases, the rate of mortality of stable fly, *Stomoxys calcitrans* also decreased within 24 hours of exposure with a median lethal dose of 105.9 µg/fly. All of these researchers concluded that the contact toxicity effect was due to the chemical composition of the EOs. It is widely known that the phenolic and flavonoid compounds of EOs play a significant part in plant defence mechanisms (Pavela & Benelli, 2016). They can be repulsive, have offensive smells and may be lethal to insects when ingested.

According to Moustafa *et al.* (2021), the main compounds of lemongrass leaves EO were α-citral (36%) and β-citral (35%). Citral (i.e., a mixture of geranial and neral) is considered to be the key contributor to the insecticidal property of lemongrass EO (Eden *et al.,* 2020; Solomon *et al.,* 2012). Pandan, on the other hand, consists mainly of squalene (14.1-33.8%) and phytol (1.4 - 6.2%) (Zakaria *et al.*, 2020). As with other insecticidal EOs, these compounds may be the reason for the effect seen with the mortality rate of adult RPW in this study. However, the effect of both these EOs may not be as effective since the mortality rate of RPW was rather low with less than 50% achieved even after five days (Table 1). The applications of pandan and lemongrass on RPW were scarce to be compared with. With other insects to compare with, pandan has been shown to be effective in controlling the population of *Helicoverpa armogera*, a sweet corn pest (Yunus, 2019), and *Plutella xylostella*, a diamondback moth (Imtithal *et al.*, 2018). Lemongrass, on the other hand, is more commonly used as an insecticides and among others, to control the subterranean termite *Reticulitermes flaviceps* (Chunzhe *et al.*, 2022); the black cutworm, *Agrotis ipsilon* (Moustafa *et al.*, 2012); and the german cockroach, *Blatella germanica* (Rahayu *et al.*, 2018).

The total soluble proteins (TSP) of RPW were also observed to be affected by both the pandan and lemongrass EOs (Figure 1). The impact, however, was greater in the female [Figure 1(b)] than the male RPW [Figure 1(a)]. TSP of both male and female RPWs was found to decrease to as low as 30% of the control. However, in the female RPWs, TSP was found to increase up to 50% of control with 10% lemongrass EO. A decrease in TSP may indicate the inhibitory effect of the EO while an increase in TSP may be due to the trigger of new protein production by the EO. The newly synthesized proteins could be used as a defense mechanism against the EO. EOs are rich in phenolic compounds, such as flavonoids, which can pass through an insect's membrane. Since phenolic compounds are highly lipophilic, it is believed that these compounds can penetrate into the insect's integumentary system through its epi- and exocuticles which are lipophilic and consist of lipids, lipoprotein, and protein (Tak & Isman, 2015). Post-ingestive effects of these compounds typically take place in the insect's midgut through oxidative mechanisms, resulting in the formation of superoxide radicals and other reactive oxygen species (Barbehenn, 2002). Oxidative stress generated can lead to protein oxidation, decreasing its content. Furthermore, flavonoid compounds are cytotoxic and can interact with different enzymes through complexation (Mierziak *et al.*, 2014), leading to the inhibition of these enzymes.

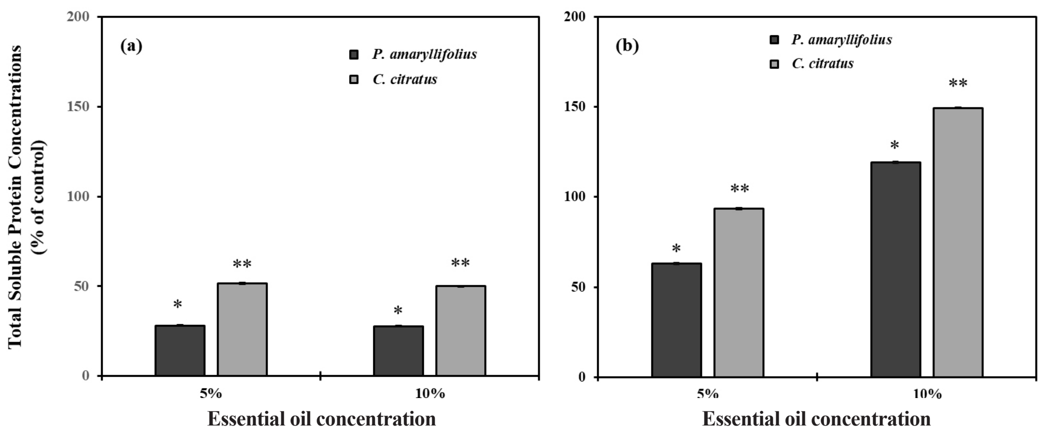

Figure 1: Total soluble protein concentrations of (a) male, and (b) female adult RPW treated with 5% and 10% of *P. amaryllifolius* (pandan) and *C. citratus* (lemongrass). Different asterisks above the bars indicate significant differences in treatments with same amount of essential oil (n = 3, $p > 0.05$).

Ingested phenolics can be tolerated or detoxified depending on the levels of antioxidants and the presence of detoxification enzymes, as well as the pH of the gut (Simmonds, 2003). Detoxification enzymes, play an important role in the survival of insects exposed to endogenous and exogenous xenobiotics (Wu *et al.*, 2013). High levels of glutathione S-transferase (GST) activity are associated with the expression of metabolic resistance to insecticides (Clark, 1990). As an important detoxifying enzyme, carboxylesterase (CarE) has been implicated in insecticide resistance with its relevant metabolic functions, such as catalyzing hydrolysis of ester, sulfate, and amide (Zhang *et al.*, 2011). On the downside, detoxification enzymes in insects can be inhibited by flavonoid compounds which are associated with insecticide resistance (Wang *et al.*, 2016).

Associated with insect resistance against pesticides is the enzyme acetylcholinesterase (AChE) which degrades the neurotransmitter acetylcholine, producing choline and an acetate group (Soreq & Seidman, 2001). In this study, we wanted to determine whether the pandan and lemongrass EOs are capable of inducing mortality in RPW by disrupting AChE activity. Figure 2 shows the three conditions of AChE observed after the treatment. In the first condition, an increase in the AChE activity was observed in male RPWs treated with 10% pandan and lemongrass EOs as well as in female RPWs treated with 5% pandan. The second condition was observed in male RPWs treated with 5% of both EOs as well as in female RPWs treated with 5% lemongrass and 10% pandan, where the activity was on par with the control. In the last condition, we observed a significant decrease in activity as shown by the female RPWs treated with 10% lemongrass.

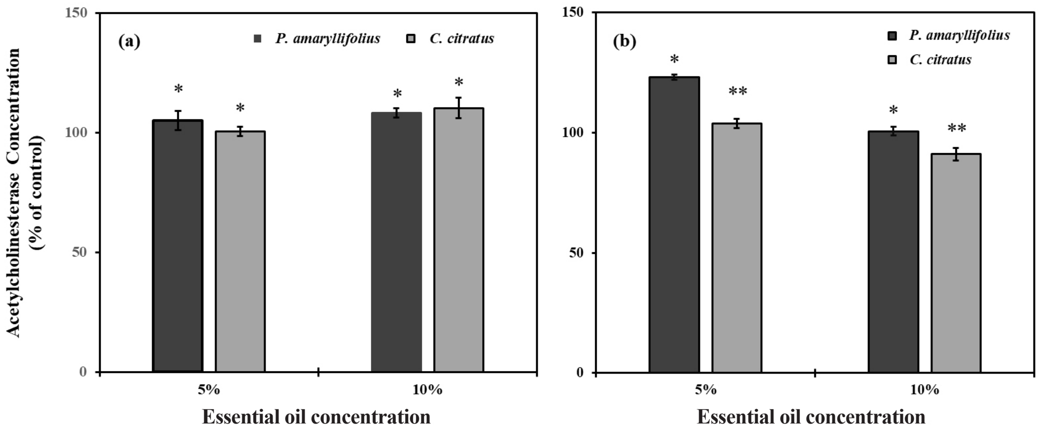

Figure 2: Acetylcholinesterase concentrations of (a) male, and (b) female adult RPW treated with 5% and 10% of *P. amaryllifolius* (pandan) and *C. citratus* (lemongrass). Different asterisks above the bars indicate significant differences in treatments with same amount of essential oil (n=3, p>0.05).

In response to exposure to AChE inhibitors, some insects may increase their AChE activity as a defense mechanism. The increased AChE activity allows the insect to break down acetylcholine more rapidly, which can reduce the toxic effects of the insecticide (Siddiqui *et al.*, 2023). This increase in AChE activity is known as the 'compensatory upregulation' of AChE (Feroz *et al.*, 2020). Another possible explanation for the increase in AChE activity after insecticide treatment could be that the insects developed a resistance mechanism to the insecticide (Li & Han, 2004). Some insect populations can develop resistance to insecticides through genetic changes that result in mutations in the AChE gene, which reduce the binding of the insecticide to the enzyme (Li & Han, 2004; Geresemu *et al.*, 2023).

A decrease in AChE is commonly observed in response to the presence of AChE inhibitors in the insecticides. For example, in a study by Duque *et al.* (2023) using molecular docking analysis, they observed that the bioactive compounds in various EOs exert their inhibitory effect on the AChE activity of *Aedes aegypti* larvae at specific amino residues within the AChE structure leading to the death of the mosquito. A decrease in AChE activity can also occur in response to other factors that affect the insect's nervous system, such as nerve damage which can be seen in pyrethroid insecticides (Vijverberg & Bercken, 1990). In these cases, the decrease in AChE activity may be a symptom of broader neurological dysfunction. If the decrease in AChE activity is due to insecticide exposure, it could indicate that the insect is sensitive to the insecticide and is experiencing its toxic effects. The insecticide may cause overstimulation of the nervous system by preventing the breakdown of acetylcholine, leading to paralysis or death (Buszewski *et al.*, 2019).

No change in AChE activity may indicate that the EOs do not affect AChE or that the insect has not yet developed a compensatory response to the EOs. Some insecticides act by disrupting the insect's nervous system through different mechanisms, such as by blocking ion channels (Ozoe, 2021; Cens *et al.*, 2022). Insects may take time to develop resistance or to regulate their AChE activity in response to insecticide exposure.

Conclusion

The results of this study have demonstrated that pandan and lemongrass essential oils (EOs) both have the potential to be employed as natural insecticides to reduce the red palm weevil (RPW) infestation. These two EOs' modes of action are remarkably similar. They were not entirely effective, but they were still capable of instantly killing the RPW. Acetylcholinesterase (AChE) activity varied significantly when RPW were exposed to these EOs. As shown with 5% pandan and lemongrass for male weevils and 5% lemongrass and 10% pandan for female weevils, the enzyme's activity did not alter from the control. However, although female weevils treated with 5% pandan showed an increase in the activity of the enzyme, female weevils treated with 10% lemongrass showed a decrease in the enzyme's activity. In contrast, the males' enzyme activity was raised by 10% of both EOs. Both concentrations of pandan and lemongrass EOs decreased the total soluble proteins (TSP) in RPW males and the same effect was observed in females treated with 5% of each plant EO. In contrast, 10% of both plant EOs raised the TSPs of RPW females. These findings imply that TSP is being suppressed or that defence proteins are being produced in the weevils to counteract the insecticidal effects of EOs. The results also showed that female RPWs were most negatively impacted by EOs. This might be a result of the physiological differences between male and female RPW. To achieve a complete profile of the EOs as possible insecticides against RPW, more research should be done to examine the specific mechanisms of the EOs' actions as a contact toxicant and an AChE modulator.

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