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Toxicological and physiological effects of essential oils against *Tribolium castaneum* (Coleoptera:Tenebrionidae) and *Callosobruchus maculatus* (Coleoptera: Bruchidae)

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ABSTRACT

The aim of this study was to investigate the toxicity and physiological effect of cardamom, cinnamon and nutmeg oils against egg, larva, and adult of *Tribolium castaneum* Herbst and *Callosobruchus maculatus* F. Further biochemical tests were conducted to assess the impact of essential oils on total carbohydrate, protein, fat contents and also assess the enzymes esterase and glutathione s-transferase activity. The mortality results indicated that cinnamon oil has the highest efficacy against egg, larva, and adult of *C. maculatus* with an LC_{50} of 0.01%, 0.132%, and 0.186%, respectively compared with *T. castaneum*, which recorded 1.051%, 0.109%, and 1.239% respectively. Furthermore, all essential oils reduce the total carbohydrate, protein, and fat contents, and cinnamon oil demonstrated to be the most effective among the three essential oils. On the same note, cinnamon oil had a greater impact of inhibiting esterase and glutathione s-transferase activity compared to nutmeg and cardamom oils. Thus, from the results, all the tested essential oils produced a significant range of biological effect on *T. castaneum and C. maculatus.* However, cinnamon oil was the most effective making it suitable botanical extract to develop fumigant to control and manage *T. castaneum* and *C. maculatus* with less environmental hazards.

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INTRODUCTION

The red flour beetle, *Tribolium castaneum* Herbst is a cosmopolitan pest which often destroys stored products especially wheat flour. It is also considered the most common species in the pest complex attacking stored wheat. Although it is considered a secondary pest, requiring prior infestation by an internal feeder, it can readily infest with other grains damaged during harvesting (Devi and Devi, 2015). In addition, larvae and adults feed on grain dust and broken grain, but not the undamaged whole grains and spend the entire life cycle outside the grain kernels (Karunakaran *et al.,* 2004). In severe infestation, the flour turns grayish and has a pungent, disagreeable odour- making it unsafe for human consumption. Furthermore, *T. castaneum* causes a substantial loss in storage due to its high reproductive potential (Prakash *et al.,* 1987). *T. castaneum* may also cause an allergic response (Alenko *et al.,* 2000). It is known that they spread diseases since they can breed throughout the year in the warm area. Pulse beetle (*Callosobruchus maculatus*) is a cosmopolitan pest that often attacks leguminous stored seeds and commonly causes serious damage to stored products with an annual loss of nearly 0.21 million (Thakur and Mandeep, 2013). In addition, the insect causes substantial loss to stored black gram (*Vigna mungo* (L.) Hepper) and infested seeds become unfit for either sowing or human consumption. The beetle causes a widespread infestation in the field condition, but most of the damage is caused during storage. The green beans are seriously affected by the beetle infestation and the insect multiplies very fast in storage, giving rise to a new generation every month causing weight losses of up to 60% (Radha and Susheela, 2014). Essential oils are strong volatile aromatic

compounds with a unique odour, flavour or scent extracted from the plant. Moreover, they are metabolic by-products and so-called volatile plant secondary metabolites. Their aromatic characteristics often play an important role by making them attract or repel insects, protecting them from cold or heat and their chemical is used to develop defendant material of insecticides (Mohan *et al.,* 2011). Due to their distinctive chemical and physical properties, essential oils have been widely applied as an alternative insecticide. In addition, bioactivities of essential oils have shown a variety of activities in controlling pests extending from toxicity of ovicidal, larvicidal, pupicidal and adulticidal activities. Additionally, essential oils have shown sub-lethal effects on oviposition deterrence, antifeedant activity and repellent actions as well as their effect on biological process such as growth rate, lifespan and reproduction (Bakkali *et al.,* 2008; Isman, 2008; Tripathi *et al.,* 2009; Ebadolahi, 2011; Regnault *et al.,* 2012). Cardamom (*Eletaria cardamomum*) Maton (*Zingiberaceae*) is an herbaceous plant; the fruits are often used as a spice for cooking and for medicinal purposes.

In addition, the chemical ingredients contained in cardamom include limonene, cineol, terpineol, borneol acetate terpinyl, and some other types of terpenes (Keezheveettil *et al.,* 2010). *Myristica fragrans* Houtt (*Myristicaceae*), also known as nutmeg commonly found in Banda Islands in Maluku, Indonesia. Nutmeg is known for its commercial value. It has also been used as a cooking spice and has been utilized as a bactericide (Radwan *et al.,* 2014) and insecticide (Tripathi *et al.,* 2015).

The chemical composition of nutmeg includes sabinen, terpinen 4-ol, α-pinene, β-pinene, and β-phellandren (Rastuti *et al.,* 2007; Piras *et al.,* 2012). *Cinnamomum aromaticum* is one of the indigenous plants used as cooking spice and has also been used for medicinal purposes (Hertika, 2011; Ranasinghe *et al.,* 2013). Some of the important compounds in cinnamon oil are limonene, cineol, terpineol, borneol, acetate terpinyl and other numerous types of terpenes (Abdelwahab *et al.,* 2014).

Studies indicate that the compound monoterpenoid causes the death of insects by inhibiting the activity of the enzyme acetyldholinesterase (AChE) (Houghton *et al.,* 2006; Lopez and Maria, 2015). However, other monoterpenoid compounds have shown no effect of inhibiting enzyme activity (Grundy and Still, 1985; Dohi *et al.,* 2009). Later studies reported the presence of the fumigant of essential oils of terpene compounds (ZP 51 and SEM 76) in plants. Labiatae and (+) - limonene exuberate inhibition of AChE in the adults *Ryzopertha dominica* by 65% (Kostyukovsky *et al.,* 2002; Anderson and Coats, 2012). Furthermore, studies have shown that most xenobiotics tend to cause enzymatic transformation after penetration into binding sites of protein and transportation of biological interaction. Glutathione S-transferase (GST) is one of the most significant enzymes for detoxification mechanism owing to its engagement intolerance to pesticides (Gui *et al.,* 2009; Afify *et al.,* 2011). Studies have also indicated that esterases (EST) play a crucial role in the detoxification of xenobiotics to nontoxic materials (Afify *et al.,* 2011).

The aims of this study are: first to investigate the toxicity effects of essential oil against egg, larva, and adult of *T. castaneum* and *C. maculatus*; secondly the effects of essential oil on total carbohydrate, protein fat contents and further esterase and glutathione s-transferase activity.

MATERIALS AND METHODS

Insect maintenance

A population of 500 adults of *T. castaneum* or *C. maculatus* were inserted into glass jar containing wheat flour or green beans for *T. castaneum* and *C. maculatus* respectively at 25^0C and 75% RH, light (16:8 h light: dark). All the insects were bred in the bottle and maintained in the laboratory for two weeks. After two weeks, all adults were removed from the glass jar and further incubated for 4 weeks; this process was aimed at producing a uniform F1 generation (first filial progeny). Adults between the ages 7-14 days were used

for the mortality test while the third instar larvae were used for the biochemical test.

Preparation of essential oils

The three essential oils used in this study were distilled from *C. aromaticum* (cinnamon), *E. cardamomum* (cardamom) and *M. fragrans* (nutmeg). The treatment essential oils were prepared by dilution methods; initially the pure essential oil was measured at desired volume and then the concentration with the dilution equation of $C_1V_1=V_2C_2$ was used for a series of dilution of essential oils preparation using acetone as a solvent.

Mortality test

Mortality test was conducted by placing adults, larvae, and eggs in different Petri dishes (7cm diameter) and then 0.5 mL of essential oil was dripped uniformly on Whatman filter paper using 1.0 mL Mohr pipette after which the filter paper was stacked onto the inner surface of Petri dish. On the other hand, the filter paper was dripped with 0.5 mL acetone for control. Once the treatment was done, both the treated and control filter papers were allowed to dry for 1 minute with the lid slightly open to enable the solvent to evaporate. In this test, 30 individual adults per replication were used whereas for the larvae test 20 individuals in the third instar larvae were tested. Furthermore, in the analysis of egg, 30 two-day old eggs were subjected to fumigation for 72 hours, then incubated for a period of two weeks inside the Petri dish with sealed lid to avoid leakage of volatile oils. Thereafter, the mortality of adults and larvae were recorded 72 hours after treatment (HAT). Furthermore, during this test the eggs, which fail to hatch in 2 weeks were further recorded. Lastly, probit analysis was used to determine LC_{50} and LC_{95} of essential oils on both insect species.

Larvicidal bioassay

Five concentrations of 1.5%, 2.5%, 5%, 10% and 15% of essential oils were prepared for larvicidal bioassay with acetone as solvent. This was followed by uniformly admixing 1000 µL of each concentration with 0.5 g of wheat flour for *T. castaneum* and 0.5 g of green beans C. *maculatus* in a 7-cm diameter Petri dish. The Whatman filter paper was then left to dry at room temperature for 1 minute. Control samples were treated only with pure acetone and dried in the same way. A total of ten third instars larvae were randomly selected placed with treated diets and kept at 27 ± 2ºC and 60 ± 5% RH. The experiment was replicated four times and larvae mortalities were recorded after 72 hours of treatment. Toxicity of larvicidal activity was then calculated based on the 50% mortality of subjected insects (LC_{50}) 72 HAT. The mortality was then analyzed using POLO-PLUS software.

Ovicidal test

Thirty eggs of *T. castaneum* and *C. maculatus* were placed into different Petri dish containing wheat flour and green beans for *T. castaneum* and *C. maculatus* respectively. 0.5 mL essential oil was then added and the Petri dish sealed. The placebos were treated with only 0.5 mL acetone, after 14- days, the mortality of eggs was counted under the stereo microscope. Sterile eggs (eggs that fail to hatch) which died were then counted thereafter; the mortality was evaluated using probit analysis.

Test for total fat, carbohydrate and protein contents

In the analysis, the method by Ebadolahi *et al.* (2013) was adapted. A population of 540 third instar larvae of *T. castaneum* and *C. maculatus* were initially treated with essential oils prepared at a concentration of 1.5%, 2.5%, 5%, 10% and 15% for a period of 24 hours after which the surviving larvae were used to analyse the total carbohydrate, protein, and fat contents. For determination of carbohydrate six treated larvae were placed in a bowl and mixed with the stock solution prepared during the analysis of fat content, then 150 µL anthrone (500 mg anthrone in 500 µL H_2SO_4) was added. Subsequently, the resulting mixture was placed in water bath at 90^0C. The concentration of carbohydrate was then read at an absorbance of 630 nm using spectrophotometer. In the case of fat content larvae were kept in a bowl then mixed with 100 µL sodium sulphate (2% Na_2SO_4) and 750 µL chloroform: methanol (2:1), then stirred

until it become homogeneous. The resulting mixture was then centrifuged at 8000 rpm for 10 minutes at 4^0C. After it 250 of the supernatant was obtained and added to 500 µL of H_2SO_4 then the mixture was placed in water bath at 90^0C. Subsequently, 30 µL of vanillin solutions (600 mg vanillin in100 mL distilled water and H_3PO_4 (400 mL, 85%)) was added to the mixture. After 30 minutes, the absorbance was read at 545 nm using spectrophotometer to determine the concentration of fat content. For determination of total protein six surviving larvae were dissolved in 350 µL distilled water then centrifuged for 5 minutes at 10.000 rpm at a temperature of 4ºC. Then, 10 µL of supernatant was mixed with 90 µL distilled water and 2500 µL dye. The absorbance was then read at 630nm using spectrophotometer to determine the concentration of total protein.

Enzyme analysis

To determine esterase activity the method described by Han *et al*., (1995) was adopted. Six individual third instar larvae were kept in a bowl. 1mL 0.1 mol phosphate buffer solution was then added and homogeneously mixed till the pH stabilized. This was followed by centrifugation for 10.000 rpm for 10 minutes at 4^0C. 75 µL α-naphthyl acetate and 75 µL of saline RR (CH_3CH_2-Na) were again added in each bowl. The reaction was then catalyzed by adding 50 µL of enzyme solution. The absorbance was then read at 450 nm using a spectrophotometer to determine the concentration of esterase concentration.

Glutathione-S transferase

To determine the activity of glutathion S-transferase Habing *et al.,* (1974) method was adopted. Six third instar larvae were initially dissolved in 20 µL distilled water then the resulting homogenize mixture was centrifuged at 12500 rpm for 10 minutes at 4^0C. 15 µL of the resulting solution was the mixed with 135 µL phosphate buffer solutions (Ph=7; 1 mL; 0.1 M) this was followed by addition of 50 µL of 1-chloro-2, 4-dinitrobenzen (CDNB) substrate and 100 µL GST. Finally, at an interval of 1 minute the absorbance was read at 340 nm to determine the concentration of GST.

Data analysis

In this study, the mortality data were analysed using probit analysis (POLO-Plus) while for biochemical data, such as carbohydrates, proteins, fat, enzymes esterase and glutathione s-transferase on the larvae tested were analyzed using analysis of variance (ANOVA) using SPSS program and Tukey's test with confidence level of 95% was incorporated to further elucidate the difference in the treatment.

RESULTS AND DISCUSSIONS

Mortality test

All the essential oils tested revealed significant toxicity effect against adults, larvae and eggs of *T. castaneum* and *C. maculatus* and the mortality rate was concentration dependent and increase in concentration exacerbated mortality. According to Table 1, *T. castaneum* adults, cinnamon oil presented the highest toxicity with an LC_{50} of 1.239% followed by cardamom oil (LC_{50}=3.344%) and nutmeg (LC_{50}=3.584%). In the case of *C. maculatus* adults, again, cinnamon had the highest efficacy with an LC_{50} of 0.186%, this was followed by cardamom (LC_{50}=0.179%) and nutmeg(LC_{50}=0.214%) (Table1). Surprisingly, in Table 1, for *T. castaneum* larva similar toxicity trend to adults was reported, in which cinnamon had the highest toxicity effect with an LC_{50} of 0.109% followed by cardamom (LC_{50}=0.20%) and nutmeg (LC_{50}=0.414%). For *C. maculatus* larva, again cinnamon presented the highest toxicity effect (LC_{50}= 0.132%) followed by cardamom (LC_{50}= 0.162%) and nutmeg (LC_{50}=0.144%) (Table 1). In the case of *T. castaneum* eggs, again, cinnamon presented its lethal effect in which LC_{50} of 1.051% followed by cardamom (LC_{50}=2.922%) and nutmeg (LC_{50}=3.562%). In the case of *C. maculatus* egg, to our surprise, cinnamon showed similar toxicity effect with an LC_{50} of 0.019% whereas for nutmeg and cardamom LC_{50} of 0.198% and 0.268% respectively (Table 1).

Total carbohydrate, protein, and fat contents

For assessment of carbohydrate, protein and fat contents all the essential oils at

concentrations of 1.5%, 2.5%, 5%, 10% and 15% were used to treat 7 -14 day old larvae of *T. castaneum* and C. *maculatus*. In general total carbohydrate, protein and fat contents in all treatments in the comparison with that of the controls significantly decreased. Result indicated that the total carbohydrates in both *T. castaneum* and *C. maculatus* significantly (df =5, F = 5.64, P≤0.001) got reduced when treated with all the three essential oils. However, according to results in Figure 1 (a), cinnamon had a significantly higher (df = 5, F = 5.64, P≤0.001) toxicity against *T. castaneum* that triggered more reduction of total amount of carbohydrate compared to cardamom and nutmeg oils. In Figure 1 (b), cinnamon also triggered the reduction in total quantity of carbohydrate in *C. maculatus* and to some extent triggered the metabolic activity since in some situations the total quantity of carbohydrate neither increased nor decreased (df =5, F= 3.64, P≤0.001). Nevertheless, for control, there was no reduction (df = 5, F = 3.64, P≤0.001) in total carbohydrates. In Figure 1(b), cinnamon at a concentration of 2.5% and 15% resulted in the reduction of total carbohydrate (df = 5, F = 2.29, P≤0.001) in *C. maculatus* larvae. In the case of nutmeg decrease of carbohydrates to *C. maculatus* larvae occurred at a concentration of 2.5%. However, cardamom essential oil treatment resulted in increasing quantity (df=5, F=130.093, P≤0.001) of carbohydrates in larvae of *C. maculatus* with increasing concentration. At a concentration of 15%, all the tested essential oils resulted in significantly (df=5, F=8.20, P≤0.001) reducing the total quantity of carbohydrate in the treated insects with no effect on controls insects.

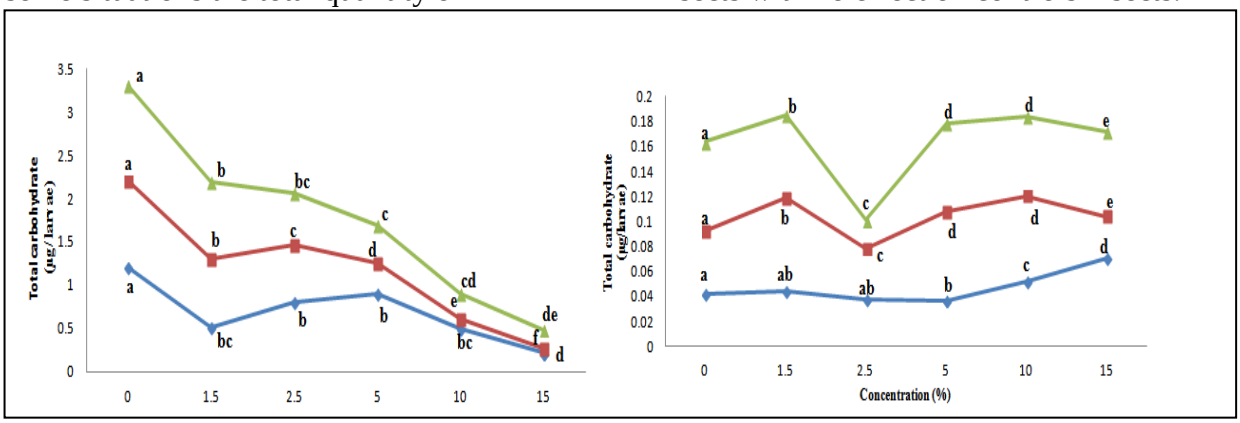

Fig. 1. Amount of carbohydrate in (a); *T. castaneum* and (b); *C. maculatus* larvae treated with different concentrations of cardamom, cinnamon and nutmeg oils after 24 h. Different letters indicate significant differences among concentration level of each essential oil according to Tukey test at p = 0.05.

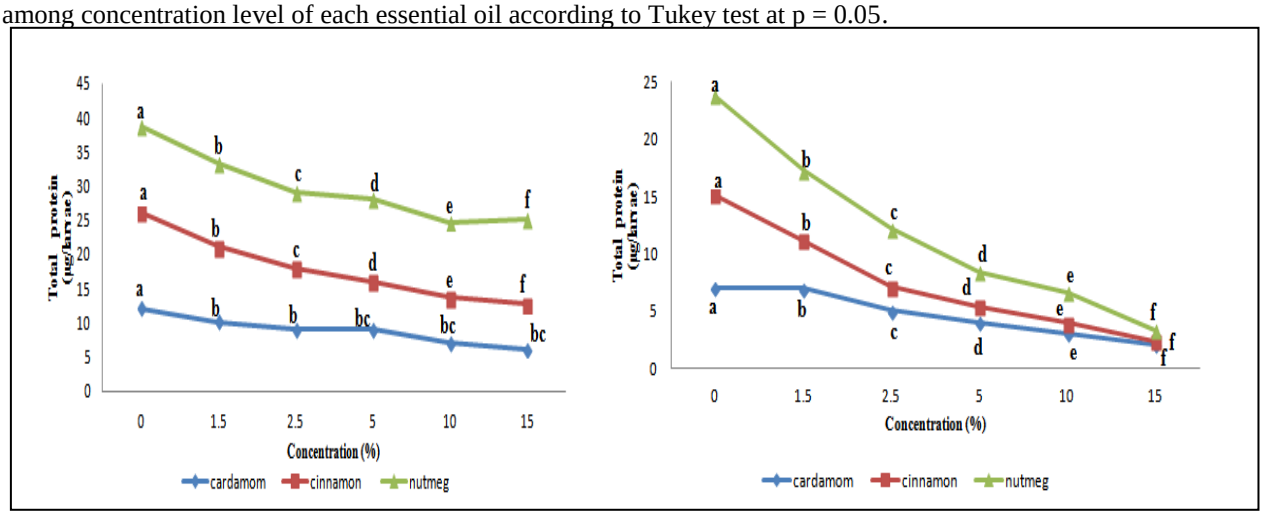

Fig. 2. Total protein in (a); *T. castaneum* and (b); *C. maculatus* larvae treated with different concentrations of cardamom, cinnamon and nutmeg oils after 24 h. Each letter indicate significant differences among concentration level of each essential oil according to Tukey test at p = 0.05.

Moreover, cinnamon oil triggered reduction of total carbohydrate by approximately 1.96 times compared to the control making it the most toxic essential oil compared to nutmeg and cardamom. Thus from the results, it was clearly evidenced that cinnamon had greater effect against larvae of *T. castaneum* compared to *C. maculatus*.

Additionally, all the essential oils reduced the total protein in both treated insects. From the results it was observed that reduction in total protein depended on the concentration of essential oil used for treatment, an increase in concentration resulted in decreasing total protein content. According to Figure 2 (a) and (b), at a concentration of 1.5%, there was a reduction in the total quantity of protein in both *T. castaneum* and *C. maculatus*. Moreover, cinnamon and nutmeg significantly more effective to trigger reduction of the quantity of protein compared to cardamom 2 oil, which had less significant effect on the total protein in *T. castaneum* larvae (refer to Figure 2(a)). According to Figure 2 (b), all the three essential oils resulted in a significant decrease in total quantity of protein in *C. maculatus* egg.

In the analysis of total fat content, all the tested essential oils resulted in a significant decrease in total fat content in both *T. castaneum* and *C. maculatus*.

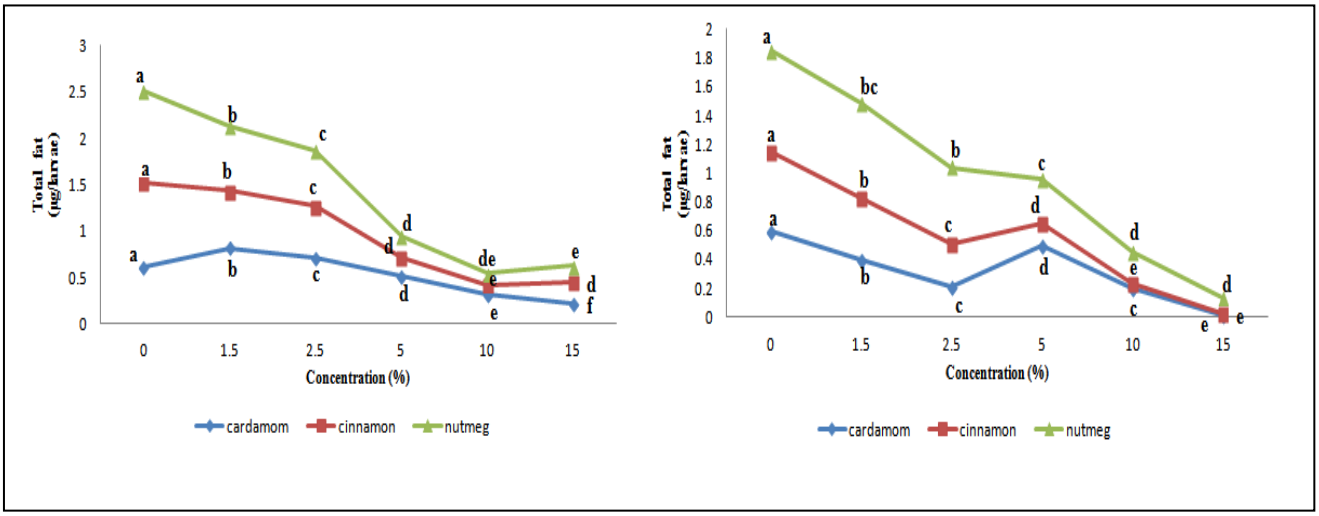

Fig. 3. Total fat content in (a); *T. castaneum* and (b); *C. maculatus* larvae treated with different concentrations of cardamom, cinnamon and nutmeg oils after 24 h. Eachletter indicate significant differences among concentration level of each essential oil according to Tukey test at p = 0.05.

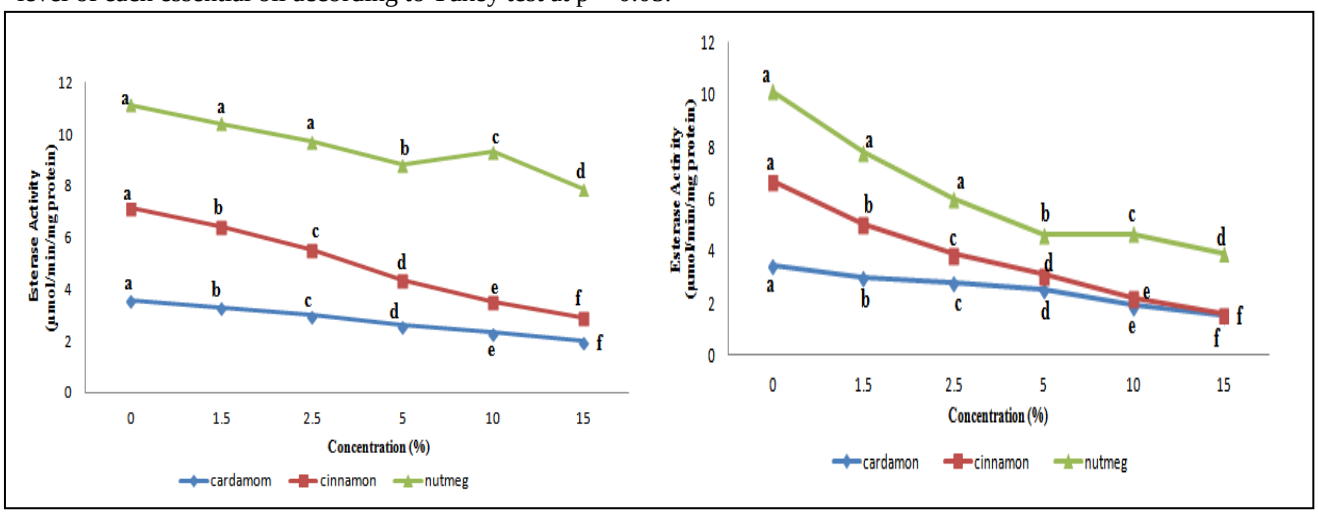

Fig. 4. Activity of esterase in (a) ; *T. castaneum* and (b); *C. maculatus* larvae treated with different concentrations of cardamom, cinnamon and nutmeg oils after 24 h. Each letter indicate significant differences among concentration level of each essential oil according to Tukey test at p = 0.05.

However, according to Figure 3, nutmeg and cinnamon oils showed higher toxic effect that exacerbated a significant reduction in the total quantity of fat content when compared to cardamom oil. In Figure 2 (b), cinnamon oil significantly triggered a reduction in total fat content in the *C. maculatus* compared to cardamom and nutmeg oils.

Esterase and glutathione S-transferase activity

The assessment results on the effect of the essential oils on the enzyme activity indicated reduction in activity of esterase and glutathione transferase in the third instar of both *T. castaneum* and *C. maculatus*. Experimental results in Figure 4 indicated that cinnamon significantly (df=5, F=504.917, P≤0.001) reduced esterases activity in both *T. castaneum* and *C. maculatus* compared to cardamom, although cardamom was significantly (df=5,F=1.404, P=≤0.001) more effective than nutmeg. In addition, according to figure 4 (b) cinnamon and cardamom significantly reduced the activity of esterase in *C. maculatus.* On the other hand, nutmeg had a low effect on esterase activity. At a concentration of 5% both cinnamon and cardamom, had a significant effect on esterase activity. In this analysis all the tested essential oils had resulted in the reduction of esterases activity and cinnamon had a greater effect in reducing enzyme activity in *T. castaneum* and *C. maculatus* when compared with nutmeg and cardamom.

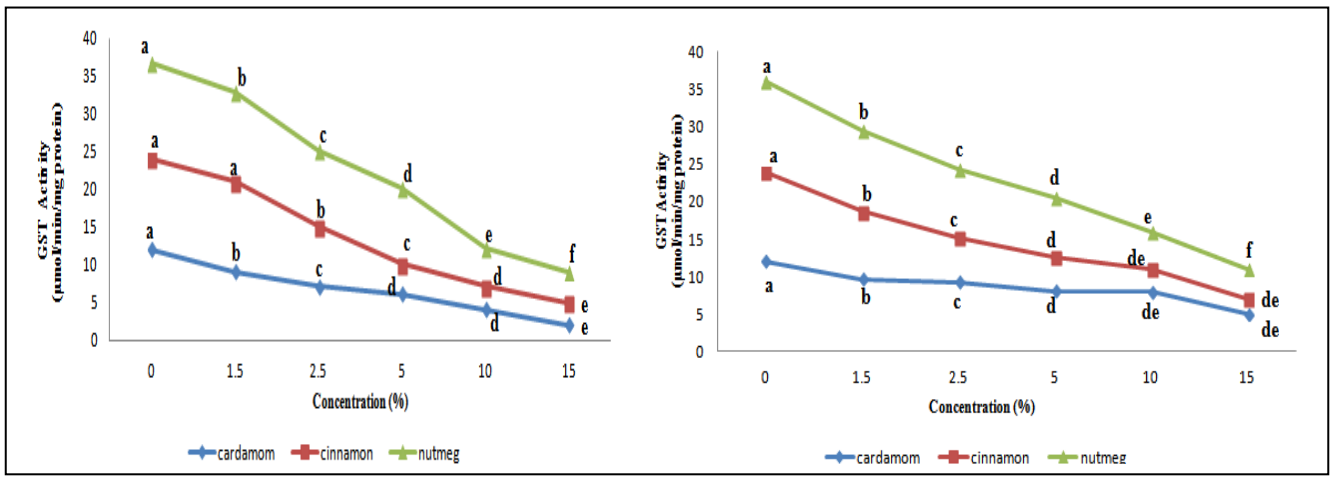

Fig. 5. Activity of glutathione–S–transferase (a); *T. castaneum* and (b); *C. maculatus* larvae treated with different concentrations of cardamom, cinnamon and nutmeg oils after 24 h. Each letter indicate significant differences among concentration level of each essential oil according to Tukey test at p = 0.05.

In the evaluation of glutathione s-transferase activity, all the tested essential oils resulted in significantly reduction in activity glutathione s-transferase in *T. castaneum* and *C. maculatus* larvae. In Figure 5, cinnamon resulted in a significant greater reduction in glutathione s-transferase activity in *T. castaneum* and *C. maculatus* larvae. In general, GST activity was dependent on the concentration of essential oil used to treat both insects. Thus an increase in concentration resulted in a decrease in GST activity. According to results in Table 2, cinnamon oil had a significantly higher effect of inhibiting glutathione s-transferase activity when compared with cardamom and nutmeg oil. Essential oils are naturally complex secondary metabolites derived from aromatic plants, which can be used as a bioinsecticide for controlling some of the pests in the warehouse (Lopez and Pascual, 2010). In this study, the effect of all the tested essential oils against an adults, larvae, and eggs of *T. castaneum* and *C. maculatus* was concentration-dependent. According to the result in Table 1, from the three tested essential oils, cinnamon and cardamom had a significantly higher toxicity against adult, larva, and egg on both insect species. Moreover, nutmeg demonstrated the lowest toxicity. This was in agreement with findings by Wang *et al.*, (2014), where result revealed that the levels of fumigant and contact effect of essential oils largely correspond to dose and exposure time.

Table 1. Toxicity effect of essential oils against *T. castaneum* and *C. maculatus.*

Essential oil	Phase	LC_{50} (%) (95% fiducial limits)	LC_{95} (%) (95% fiducial limit)	Regression equation
		T. castaneum		
	adult	3.344 (3.215-3.454)	5.501 (5.186-5.598)	y=7.610x-3.990
Cardamom	larva	0.2000 (0.171-0.228)	1.008 (0.827-1.302)	y=2.339x+1.637
	egg	2.922 (1.976-3.332)	8.113 (6.138-21.324)	y=3.709x-1.727
	adult	1.239 (1.181-1.285)	1.969 (1.869-2.116)	y=8.160x-0.759
Cinnamon	larva	0.109 (0.087-0.130)	0.568 (0.468-0.741)	y=2.298x+2.209
	egg	1.051 (0.832-1.176)	2.233 (1.969-2.913)	y=5.025x-0.108
	adult	3.584 (3.224-3.926)	16.19 (13.547-20.500)	y=2.512x-1.393
Nutmeg	larva	0.414 (0.351-0.476)	2.253 (1.740-3.260)	y=2.236x+0.856
	egg	3.562 (2.542-4.437)	48.011 (25.216-193.946)	y=1.456x-0.803
		C. maculatus		
	adult	0.179 (0.136-0.221)	1.404 (1..011-2.314)	y=1.841x+1.374
Cardamom	larva	0.162 (0.135-0.188)	0.865 (0.710-1.119)	y=2.258x+1.788
	egg	0.268(0.183-0.355)	21.82 (7.576-183.760)	y=0.861x+0.493
	adult	0.186 (0.158-0.214)	0.962 (0.783-1.261)	y=2.305x+1.684
Cinnamon	larvae	0.132 (0.106-0.155)	0.808 (0.646-1.102)	y=2.087x+1.838
	egg	0.019 (0.001-0.052)	5.023 (1.972-65.089)	y=0.678x+1.169
	adult	0.214 (0.188-0.240)	0.927 (0.782-1.152)	y=2.582x+1.729
Nutmeg	larvae	0.144 (0.111-0.176)	1.383 (1.042-2.045)	y=1.675x+1.409
	egg	0.198 (0.077-0.308)	107.19 (15.235-46783.0)	y=0.602x+0.424

Another study reported that the toxicity of the essential oil is often influenced by the chemical composition. This usually depends on the place of origin, weather, climatic conditions, application method, a period of extraction and plant parts. This explains why nutmeg oil might have low efficacy on adult, larvae, and egg of both insect species (Manal *et al.,* 2013). In addition, the experimental analysis the toxicity effects of all tested essential oils might have been due to their chemical composition. Furthermore, the response of *T. castaneum* and *C. maculatus* to different concentrations of essential oils might have been due to their varied chemical composition. This was similar to the study by Mondal and Khalequzzaman (2009) who observed that contact effect of cinnamon had greater toxicity effect against *T. castaneum* larva (LC_{50} = 0.074 mg cm^{-2}) and *Sitophillus zeamais* adult (LC_{50} = 0.196 mg cm^{-2}). In the analysis of the effect of essential oils on enzyme activity, all the tested oils resulted in reduction in enzyme activity. Nutmeg consists of high aromatic compounds such as myristicin which triggered reduction in enzyme activity. Myristicin in nutmeg acts as a narcotic which interferes with acetylcholinesterase activity resulting in brain damage (Chun *et al.,* 2015). Study by Dhingra *et al.,* (2006) reported that extract of n-hexane in nutmeg at a dose of 100-150 µg mL^{-1} significantly degrades activity of acetylcholinesterase in white mice. On the same note, Kasim *et al.,* (2014) reported that cinnamon consist of compounds such as 1, 2-naphthalenedione ethanone and borneol where cinnamaldehyde is the main toxic compound. Furthermore study by Maina (2013) also indicated that cinnemaldehyde compound (LD_{50}=19.0-24.0 mg mL^{-1}) has the potential to significantly exuberate mortality rate of *Dermatophagoides pteronyssinus* Trouessart (Acari: Pyroglypidae) adult compared with benzyl benzoate and dibutyl phthalate insecticides. In addition, the study has also shown that cinnamon has a fumigant and contact effect against *Lasioderma serricorne*

F., *Sitophilus oryzae*, and *C. chinensis* at a dose of 0.7 mg cm^{-2} with a percentage of 100% within 24 HAT (Kim *et al.,* 2003). In this study, the essential oils prepared at different concentrations resulted in the reduction of protein content in *T. castaneum* and *C. maculatus* larvae. It has been reported that the decrease in protein content is a frequent phenomenon in insects after treatment with toxic compounds (Nathan *et al.,* 2008). Thus there is a possibility that there was depletion of protein affected insects reduction in amino acids to enter the TCA cycle as a keto acid to compensate for the lower energy caused by stress (Nath *et al.,* 1997). Similar results were observed in this study and several reports are there that speak of essential oil leading to reduction in protein content (Smirle *et al.,* 1996; Caballero *et al.,* 2008; War *et al.*, 2011; Roya and Jalal, 2013). The result in this study also indicated that the essential oils resulted in reduction in the total fat contents in *T. castaneum* and *C. maculatus* larvae since lipids are a significant source of energy and are stored in fat bodies. During the feeding period, lipids stored increase but there is decrease in the pupa stage and the quantity of lipids tend to vary with growth stage and feeding condition (Chapman, 1998). Khosravi *et al.,* (2010) reported similar results in the reduction of lipid and carbohydrate rates on *Glyphodes pyloalis* Walker with extract of *Artemisia annua.* Decrease in carbohydrate and lipid rates could be related to a strong deterrence effect of cardamom, cinnamon, and nutmeg.

Esterases constitute is one of the most significant and widely distributed enzymes in the insects, their function is usually to hydrolyze, amide, carboxyl ester, and thioester bonds in numerous compounds and they resist many insecticides (Mukangayama *et al.,* 2003). Esterase is one of the enzymes that respond strongly to the reaction of environmental stimulation (Hemingway and Karunatne, 1998). According to Figure 4, all essential oils caused a reduction in esterase activity of *T. castaneum* larvae after 24 h exposure time. In the two low concentrations (10% and 15 %), essential oil stimulated the expression of EST to increase the detoxification ability. Moreover, at 5%, 10%, and 15%, toxic effect EST activity was suppressed (Fig. 4). Several studies have reported capability of plant products to inhibit esterase activity (Mukangayama *et al.,* 2003; Caballero *et al.,* 2008; Nathan *et al.,* 2008; Malahat *et al.,* 2015). Glutathion s-transferases are often considered as multifunctional enzymes responsible for detoxification but catalyze the conjugation of reduced glutathione and play a critical role in detoxification of insecticides such as organochlorine and organophosphorus compounds, thus making them less or non-toxic (Rufingier *et al.,* 1999). Other xenobiotics such as plant defense allelochemicals against phytophagous insects induce GST activity (Van *et al.,* 2001). In this study, all the essential oils inhibit GST activity and enzyme activity was concentration-dependent, that is GST activity increased with increase in essential oil concentration. The decline of the detoxification ability may be attributed to the insecticidal activities. In the biochemical analysis, cinnamon was observed to have a relatively higher effect to decrease the total carbohydrates, proteins, and fats content compared with cardamom and nutmeg oil. The finding was similar to the study conducted by War *et al.* (2013) who observed a decrease in total quantity of protein, serine protease, esterase and glutathione s-transferase in fat body of *Helicoverpa armigera* after treated with essential oils extracted from Neem (*Azidirachta indica*). The toxicity of plant extract is characterized by its ability to decrease the total quantity of protein in insects. According to Terrie (1984), esterase and GST are a group of enzymes made up of protein (85%) and they play a critical role in the detoxification of toxic compounds that enter and exit from insect body. The decrease in total protein in larvae was postulated as an indicator of toxic exposure to insecticides. The decrease in the total of proteins ultimately decreases total carbohydrates and fats content. Nath *et al.*

(1997) reported that stress due to insecticide exposure might interfere with insect physiology, consequently resulting in a decrease in total protein leading to low amino acids formation in TCA cycle. This further leads to insufficient fatty acid required for synthesis of Adenosine Triphosphate (ATP) energy, thus reduction in ATP energy triggers stress in insects leading to death (Smirle *et al.*, 1996). They further stated that fat acts as a source of energy stored in the body of insects. Moreover, according to the study by Chapman (1998), stored fats in fat body tend to increase during feeding process whereas it decreases when insects are inactive (pupa stages). Nevertheless, a study conducted by Ebadollahi *et al.* (2013) reported that the total of carbohydrates, proteins, and fat content in *T. castaneum* larvae subjected to fumigation with *A. foeniculum* decreased as the concentration of the fumigants increased. In the analysis, it was observed that both larvae tested after fumigated with three essential oils within 24 HAT resulted in a significant decrease in esterase and GST activity compared with control. The experimental result of this study was similar to that of the study by Ebadollahi *et al.* (2013) who reported that essential oil of *A. foeniculum* decreased the activity of esterases and GST on the third instar of *T. castaneum* larvae. A decrease in esterase and GST activity on both the insects might have been triggered by low quantity of protein in fat body. Moreover, War *et al.* (2013) reported that the building blocks of esterase and GST enzyme consist of 60% protein. Consequently, a decrease in the activity of esterase and GST activity in larvae subjected the insect unable to resist the toxic compound. It is still not known whether if the mode of action of essential oils against esterase and GST enzyme activity can interfere with insect defense mechanism against toxic substances. The experiment results in this study indicated that a decrease in esterase and GST activity might have interfered with antioxidant activity of P450 gene, which is responsible for detoxification of toxic compounds in insects. Although the results indicate a decline in enzyme activity in larval, there is still a gap to evaluate the mode of action of the essential oil that might have to induce higher mortality of *T. castaneum* and *C. maculatus*. Finally, this study revealed cinnamon oil and cardamom oil had higher potential to be used as an alternative fumigant for controlling *T. castaneum* and *C. maculatus.* In brief, the result of this study indicates that cinnamon oil is the most suitable essential oil for managing the population of *C. maculatus* and *T. castaneum* in storage facilities such warehouse due to its high toxicity action and its environmental friendliness in nature. However, further studies are required on the safety issues of cinnamon for human health. Future studies are to explore the mode of action of cardamom, cinnamon and nutmeg to develop a formulation to enhance the potency and stability as well as to minimize the cost of production.

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