JBiopest 12(1):76-82(2019)

Bioactivity of *Citrus aurantifolia*, *Citrus limon* and *Piper nigrum* essential oils on *Callosobruchus maculatus* (F.) (Coleoptera:Bruchidae)

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ABSTRACT

In the current study *Callosobruchus maculatus* adults were exposed to essential oils of *Citrus limon*, *Citrus aurantifolia* and *Piper nigrum* and the contact mortality, repellency, fumigation effects and oviposition deterrent activities of each oil were determined over a 72h period. After 72h, 50% mortality of *C. maculatus* adults was observed for *C. limon* at a concentration of < 1.56 µL/mL, for *C. aurantifolia* at a concentration between 12.5–25.0 µL/mL and for *P. nigrum* the concentration causing 50% mortality was 1.56 µL/mL. All oils tested displayed some level of repellency to *C. maculatus* after 12h and 24h. *C. aurantifolia* essential oil at concentrations above 6.25µL/mLwere classified as a Class IV repellent, while *P. nigrum* was classified as a Class IV repellent at 25.0 and 50.0 µL/mL at 24h post application and a Class V repellent only at 50.0 µL/mL at 12h. The results of the 50% fumigant mortality (FC_{50}) indicated that *P. nigrum* essential oil was the most toxic fumigant (FC_{50} = 0.140µL/L air) among the three oils tested. Black pepper oil also took the shortest time (FT_{50} = 7.71h) to cause 50% mortality to a population of *C. maculatus*.The anti-oviposition effects reveals that both *C. limon* and *C. aurantifolia* gave relatively low DQ values at all concentrations. At 25.0 and 50.0 µL/mL, *C. aurantifolia* had higher values (0.62 and 0.64 respectively). However, apart from the lowest concentration, *P. nigrum* essential oil consistently gave high DQ values indicating its potential in preventing egg laying on *Cajanus cajan* seeds. Thus, the use of these essential oils can be considered as alternatives to the use of synthetic insecticides for management of *C. maculatus*

Keywords: Essential oils, bioactivity, *Callosobruchus maculatus*, anti-oviposition

MS History: 26.03.2018 (Received)-30.04.2018 (Revised)-05.06.2019 (Accepted).

Citation: Rayanna Boodram and Ayub Khan. 2019. Bioactivity of *Citrus aurantifolia*, *Citrus limon* and *Piper nigrum* essential oils on *Callosobruchus maculatus* (F.) (Coleoptera:Bruchidae). *Journal of Biopesticides*,**12**(1):76–82.

INTRODUCTION

Callosobruchus maculatus F. (Coleoptera : Bruchidae) or southern cowpea weevil has a tropical and subtropical distribution (Beck and Blumer, 2014). This pest attacks a range of grain legumes in storage, rendering them unfit for human and animal consumption. Damage can be as high as 100% if adequate control is not taken (Owusu-Akyaw, 1991). This has led to extensive use of synthetic insecticides to aid in control; however, numerous problems are associated with excessive pesticide usage ranging from pesticide resistance to high cost (Owusu-Akyaw, 1991). This has increased the demand for alternative, sustainable methods of control with one such control strategy being the use of essential oils. The use of natural oils from plants for management of insect pests in grain storage is an ancient practice as stated by Qi and Burkholder (1981). Additionally, vegetable oils can also be used and appear to be efficient in the control of *C. maculatus* (Qi and Burkholder, 1981).

Several vegetable oils have been evaluated for management of *C. maculatus* including rubber seed oil, palm oil and palm kernel oil. All oil

treatments caused significant mortality ranging from 72-100% to *C. maculatus,* with rubber seed oil being the most effective (Law-Ogbomo and Egharevba, 2006). *Azadirachta indica* A. Juss. is one of the most investigated botanicals for use in insect control, but, in addition to the oil being used as an insecticide, it has also been reported to deter oviposition in *C. maculatus* (Maina and Lale, 2004). Apart from neem, peppermint (*Mentha* x *piperita*), chili (*Capsicum* sp.) and garlic (*Allium sativum* L.) oils which both significantly reduced reproduction of *C. maculatus* in Botswana (Tiroesele *et al.,* 2014). Mahdi and Rahman (2008) investigated the effects of eleven other essential oils including clove (*Syzygium aromaticum*) and black pepper (*Piper nigrum*) which both gave the highest suppressant effect on the pest. The objectives of the current study were to investigate the fumigant and contact mortality and repellent effects of lemon (*Citrus limon*), black pepper (*Piper nigrum*) and lime (*Citrus aurantifolia*) oils on *C. maculatus*

MATERIALS AND METHODS

Insect rearing

Approximately 100g of clean, dried pigeon pea (*Cajanus cajan*) seeds was placed in a 1L glass bottle (9.5cm x 17cm) with a mesh covered lid. Ten to twenty mixed sexed *C. maculatus* adults were placed into the bottle and left in a dark area undisturbed for 4 weeks. Three such arrangements were made for multiplication of *C. maculatus.* The resulting adults were used for conducting bioassays.

Essential oil extraction

Essential oils from *Piper nigrum* L. (Piperaceae), *Citrus limon* (L.) Osbeck (Rutaceae) and *Citrus aurantifolia* (Christm.) Swingle (Rutaceae) were extracted using a Clevenger type steam distillation apparatus. The peels (600g each) of both *C. limon* and *C. aurantifolia* were carefully removed and blended with 500mL of distilled water. The resulting mixture was steam distilled for 5h. In the case of *P. nigrum*, 600g of dried seeds were bought from a grocery, ground with 500mL of distilled water and also steam distilled for 5h. The resulting oil-water mixture for each plant part was separated using a separating funnel and dichloromethane as the solvent.

Bioassays-Contact mortality

Concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56% of each essential oil were made by serial dilution using ethyl alcohol as the solvent. A 1mL of each dilution was used to evenly coat a 9cm Whatman No. 41 filter paper and allowed to air dry for 10 minutes. The treated, dried filter paper was placed in a 9cm petri dish together with 10 pigeon pea seeds and 10 *C. maculatus* adults and covered. Adult mortality and number of eggs oviposited every 24h for 72h was recorded at each concentration. The experiment consisted of 5 replicates for each concentration and a control. The entire contact mortality experiment for a single oil thus consisted of a total of 36 petri dishes. Percent corrected mortality and mean number of eggs oviposited were calculated for each concentration of each essential oil. The 50% lethal concentration (LC_{50}) and 50% lethal time (LT_{50}) were determined using Probit analysis (Finney, 1952).

Repellent bioassay

The repellent effects of each concentration (50, 25, 12.5, 6.25, 3.12 and 1.56%) for each essential oil were determined. Whatman No. 41 filter paper (9cm) was cut in half and one half treated with 1mL of one concentration of an essential oil. The other half was treated with 1mL of ethyl alcohol (control) and both halves allowed to air dry for 10 minutes. Both halves (treated and control) were re-joined using clear Sellotape® and then placed in a 9cm petri dish with the Sellotape® side facing downward. Ten *C. maculatus* adults were placed in the middle of the rejoined filter paper and the number of adults on each side of the filter paper was recorded every 2h for a 24h period. There were 5 replicates for each concentration of each essential oil. The percent repellency was calculated using the formula (Obeng-Ofori, 1995):

$$\% \text{ Repellency} = [(Nc - Nt) / (Nc + Nt)] / 100$$

Where: Nc = Number on the control side
Nt = Number of treatment side

Essential oils were then categorized for their repellency based on the system of Juliana and Su (1983). The Repellent Index (RI) based on the formula of Kogan and Goeden (1970) was also calculated using the formula:

RI = 2G / G + P

Where G = number on treatment side

P = Number on control side

The standard deviations of the mean values of the RI were also calculated and essential oils at different concentrations classified based on whether the oil was an attractant (RI > 1 + SD), the oil was indifferent (= neutral) (RI between 1 – SD and 1 + SD) or the oil was classified as a repellent (RI < 1 – SD). The Discrimination quotient (DQ) was also calculated for C. maculatus exposed tofive concentrations of the three essential oils. The DQ was calculated based on the formula of Messina and Renwick (1983):

DQ= (No. of eggs on control seeds) – (No. of eggs on treated seeds) / Total no. of eggs

The DQ values range from (-1) indicating that all eggs were oviposited on treated seeds to (+1) where all eggs were oviposited on control seeds.

Table 1 Mean number of eggs oviposited by *C. maculatus* on *C. cajan* seeds treated with *C. limon* oil at different concentrations and three time periods

Concentration (µL/mL)	Mean no. eggs ± SE* 24h	Mean no. eggs ± SE* 48h	Mean no. eggs ± SE* 72h
1.56	6.80± 3.15^{aA}	9.20± 4.02^{aA}	11.00±2.41^{aA}
3.12	6.20± 3.88^{aA}	8.80± 4.08^{aA}	9.60 ± 2.11^{aA}
6.25	2.35± 1.75^{aA}	2.44± 0.74^{aA}	2.47± 0.93^{bA}
12.50	1.20± 0.30^{aA}	1.60± 0.53^{aA}	1.79± 0.37^{bA}
25.00	1.00± 0.48^{aA}	1.26± 0.00^{aA}	1.33± 0.32^{bA}
50.00	0.00± 0.00^{aA}	0.00± 0.00^{aA}	0.00± 0.00^{bA}
Control	24.20±1.07^{bA}	43.40±1.21^{bB}	42.4± 2.21^{cB}

*Values followed by the same lowercase letter along a column and the same uppercase letter along a row are not significantly different (P>0.05) from each other based on Tukey-Kramer Multiple Comparisons test

Fumigation bioassay

Fumigation experiments were carried out in 950mL glass jars with concentrations of 5, 2.5, 1.25, 0.63, 0.31 and 0.16% of essential oils corresponding to 5.26, 2.63, 1.32, 0.66, 0.33 and 0.17µL/L air were used. Whatman No. 41 (7cm) filter paper was impregnated with 1mL of each concentration of each essential oil and allowed to air dry for 10 minutes. The air dried filter paper was then attached to the inside cover of the glass jar. Ten pigeon pea seeds along with 10 *C. maculatus* adults were placed inside the glass jar and covered with the treated filter paper-cover combination. There were 5 replicates for each concentration of each essential oil. The number of dead adults was recorded every 24h for 72h. The 50% Fumigant Dose (FD_{50}) and 50% Fumigant Time (FT_{50}) were calculated using Probit analysis. The experiment was repeated for each essential oil.

RESULTS

The mortality of *C. maculatus* adults when exposed for 24h, 48h and 72h to increasing concentrations of the essential oils of *C. limon*, *C. aurantifolia* and *P. nigrum* are presented in Figures 1 – 3. After 72h, 50% mortality of *C. maculatus* adults was achieved for *C. limon* at a concentration of < 1.56 µL/mL of the essential oil. For the same time 50% mortality was achieved for *C. aurantifolia* at a concentration between 12.5 – 25.0 µL/mL while for *P. nigrum* the concentration causing 50% mortality was 1.56µL/mL. Thus, based on the data from these graphs the order of decreasing contact mortality is *C. limon* >*P. nigrum* > *C. aurantifolia.*

C. limon, P. nigrum and *C. aurantifolia* essential oils were tested for their oviposition deterrent ability and for seeds treated with the three essential oils at all concentrations and time periods (24h, 48h and 72h), significantly ($F_{17,\ 882}$= 0.42, P < 0.05) more eggs were oviposited on the untreated seeds compared with treated seeds (Tables 1–3). The mean number of eggs oviposited on seeds treated with different concentrations of *C. limon* oil was not significantly ($F_{11,588}$ = 0.15, P>0.05) different from each other after 24h and 48h. However, after 72h significantly more ($F_{4,\ 245}$=11.60, P<0.05) eggs were oviposited on seeds treated with lower concentrations of *C. limon* oil than at higher concentrations (Table 1)

At lower concentrations, the mean number of eggs oviposited by *C. maculatus* on *C. aurantifolia* oil treated pigeonpea seeds was not significantly different ($F_{2, 147}$=2.24, *P*>0.05) from each other but was significantly different from that at higher concentrations (Table 2). However, at 72h only at 50.0 µL/mL was the number of eggs oviposited significantly lower (, $F_{6, 343}$=2.29, *P*<0.05) than that oviposited at the other five concentrations (Table 2).

Table 2 Mean number of eggs oviposited by *C. maculatus* on *C. cajan* seeds treated with *C. aurantifolia* oil at different concentrations and three time periods

Concentration (µL/mL)	Mean no. eggs ± SE* 24h	Mean no. eggs ± SE* 48h	Mean no. eggs ± SE* 72h
1.56	16.00±1.14^{aA}	18.80±1.16^{acA}	28.00±3.83^{aB}
3.12	13.40 ± 1.63^{aA}	18.00±1.30^{acAB}	23.60±2.44^{aB}
6.25	11.00 ± 2.10^{aA}	15.00±2.30^{acAB}	21.60±2.91^{aB}
12.50	9.40 ± 1.50^{bcA}	14.80±1.39^{acB}	18.60±0.60^{aB}
25.00	5.60 ± 1.03^{bcA}	12.20±1.91^{bcB}	17.80±3.65^{aB}
50.00	2.40 ± 0.51^{bdA}	10.40±1.03^{bdB}	14.00±1.82^{bB}
Control	47.15 ± 2.35^{eA}	84.11± 3.25^{eB}	114.00±5.84^{cC}

Generally, at any concentration, the mean number of eggs was significantly less ($F_{3, 196}$=4.84, *P*>0.05) on 24h treated compared with either 48h or 72h *C. aurantifolia* oil treated seeds. As with *C. aurantifolia,* significantly less eggs ($F_{3, 196}$=22.46, *P*>0.05) were oviposited on seeds treated with higher concentrations of *P. nigrum* essential oil compared with lower concentrations (Table 3). Significantly more ($F_{2, 147}$=12.21, *P*<0.05) eggs were oviposited on seeds treated with any one concentration of *P. nigrum* oil at either 48h or 72h compared with that at 24h (Table 3).

Based on the Discrimination Quotient (DQ) of Messina and Renwick (1983), lower DQ values indicate less effective anti-oviposition oils (i.e. more eggs oviposited on treated seeds than control (=untreated) seeds) and vice-versa. Both *C. limon* and *C. aurantifolia* gave relatively low DQ values at all concentrations which ranged from 0.10 to 0.28 and -0.20 to 0.64 respectively. At 25.0 and 50.0µL/mL, *C. aurantifolia* had higher values (0.62 and 0.64 respectively) than corresponding *C. limon* concentrations. However, apart from the lowest concentration, *P. nigrum* essential oil consistently gave high DQ values (>0.50).

The percentage repellency of each of the three essential oils at six concentrations was determined using the formula of Juliana and Su

Figure 1 Percent corrected mortality of *Callosobruchus maculatus* adults exposed to six concentrations of lemon oil for 72 hours

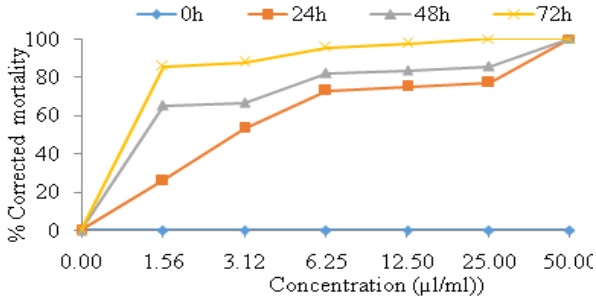


Figure 2 Percent corrected mortality of *Callosobruchus maculatus* adults exposed to six concentrations of lime oil for 72 hours.

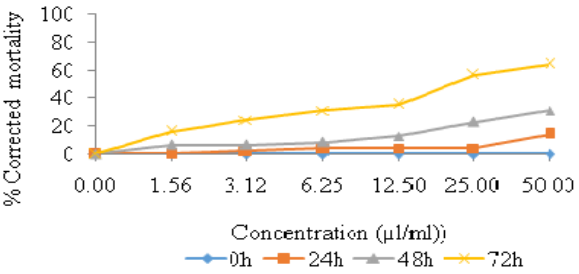
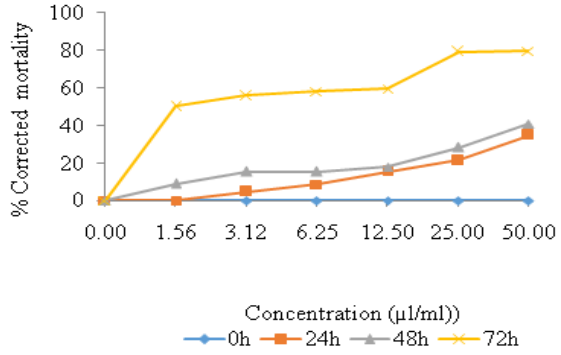


Figure 3 Percent corrected mortality of *Callosobruchus maculatus* adults exposed to six concentrations of black pepper oil for 72 hours



(1983). All oils tested displayed some level of repellency to *C. maculatus* after 12h and 24h. *C. aurantifolia* essential oil at concentrations above 6.25µL/mLwas classified as a Class IV repellent, while *P. nigrum* was classified as a Class IV repellent at 25.0 and 50.0µL/mL at 24h post application and a Class V repellent only at 50.0µL/mL at 12h. Repellent Index also indicated that (apart from *C. limon* at 1.56µL/mL) all oils tested at different concentrations exhibited repellent properties for 24h against *C. maculatus* adults (Table 4).

The contact mortality of *C. limon* (LC_{50} = 0.015µL/mL) was significantly lower ($F_{2, 57}$ = 4238.3, P<0.05) than that of the LC_{50} values of the other two oils indicating that it was the most toxic to *C. maculatus* adults. The essential oil of *C. limon* also took the significantly shortest time

Table 3 Mean number of eggs oviposited by *Callosobruchus maculatus* on *Cajanus cajan* seeds treated with *Piper nigrum* oil at different concentrations and three time periods

Concentration (µL/mL)	Mean eggs ± SE* 24h	Mean eggs ± SE* 48h	Mean eggs ± SE* 72h
1.56	11.00 ± 2.50^{aA}	18.00 ± 3.13^{aA}	28.00 ± 1.73^{aB}
3.12	9.00 ± 1.52^{abA}	16.60 ± 2.16^{aB}	26.40 ± 2.18^{aC}
6.25	8.40 ± 1.50^{abA}	16.00 ± 2.03^{aB}	25.40 ± 0.51^{aC}
12.50	7.80 ± 1.20^{abA}	15.60 ± 3.01^{aAB}	24.60 ± 3.61^{aB}
25.00	4.60 ± 0.98^{bA}	8.20 ± 2.69^{aAB}	16.60 ± 3.72^{bB}
50.00	4.20 ± 0.49^{bA}	7.60 ± 0.93^{bA}	14.40 ± 2.34^{bB}
Control	32.55 ± 3.15^{cA}	52.05 ± 4.53^{cB}	90.35 ± 6.31^{cC}

*Values followed by the same lowercase letter along a column and the same uppercase letter along a row are not significantly different (P>0.05) from each other based on Tukey-Kramer Multiple Comparisons test

Table 4 Repellent effect of six concentrations of three essential oils against *Callosobruchus maculatus* at two time periods.

Essential oil	Concentration (µL/mL)	Repellent Index (RI)[1] (Mean ±SD)	
		12h	24h
Citrus limon	50.0	0.56 ± 0.15 (R)	0.60 ± 0.18 (R)
	25.0	0.64 ± 0.15 (R)	0.64 ± 0.15 (R)
	12.5	0.68 ± 0.27 (R)	0.76 ± 0.23 (R)
	6.25	0.60 ± 0.25 (R)	0.64 ± 0.15 (R)
	3.12	0.48 ± 0.10 (R)	0.76 ± 0.15 (R)
	1.56	0.72 ± 0.35 (I)	0.80 ± 0.25 (I)
Citrus aurantifolia	50.0	0.32 ± 0.16 (R)	0.32 ± 0.10 (R)
	25.0	0.36 ± 0.08 (R)	0.38 ± 0.08 (R)
	12.5	0.32 ± 0.27 (R)	0.32 ± 0.16 (R)
	6.25	0.36 ± 0.20 (R)	0.27 ± 0.08 (R)
	3.12	0.40 ± 0.13 (R)	0.52 ± 0.16 (R)
	1.56	0.72 ± 0.27 (R)	0.36 ± 0.15 (R)
Piper nigrum	50.0	0.12 ± 0.10 (R)	0.24 ± 0.08 (R)
	25.0	0.40 ± 0.13 (R)	0.32 ± 0.16 (R)
	12.5	0.48 ± 0.16 (R)	0.28 ± 0.16 (R)
	6.25	0.52 ± 0.16 (R)	0.52 ± 0.10 (R)
	3.12	0.64 ± 0.08 (R)	0.60 ± 0.13 (R)
	1.56	0.60 ± 0.13 (R)	0.80 ± 0.13 (R)

[1]Repellent Index based on Kogan and Goeden (1970). A – Attractant (RI > 1 + SD), I – Indifferent (RI between 1 – SD and 1 + SD), R – Repellent (RI < 1 – SD).

(LT_{50} = 20.26h, $F_{2, 57}$= 642.07, P<0.05) to cause 50% mortality to a population of *C. maculatus.*

The results of the fumigant mortality (FC_{50}) indicate that *P. nigrum* essential oil was the most toxic fumigant (FC_{50} = 0.140µL/L air) among the three oils tested. Black pepper oil also took the significantly shortest time (FT_{50} = 7.71h, $F_{2, 57}$ = 905.6, P<0.05) to cause 50% mortality to a population of *C. maculatus* compared with that of *C. limon* (FT_{50} = 1.32h) and *C. aurantifolia* (FT_{50}=11.491h).

DISCUSSION

Essential oils can affect insects in several ways including disruption of major metabolic pathways leading to rapid death, acting as contact insecticides (Saxena *et al.,* 1992), fumigants (Shaaya *et al.* 1997), repellents (Plarre *et al.,* 1997) and deterrents or alteration of oviposition.Dugo and Di Giacomo (2002) note that *C. limon* had the highest acaricidal effects and also had the highest larvicidal properties compared to most of the other citrus essential oils tested. *C. limon* oil has been reported to be extremely toxic to insects primarily as a result of high concentrations of limonene which is responsible for its contact insecticidal and fumigant activity (Dugo and Di Giacomo 2002). This was found in the present study as contact mortality decreased among the three essential oils tested in the order *C. limon* (LC_{50} = 0.015µL/mL), *P. nigrum* (LC_{50} = 0.143 µL/mL) and *C. aurantifolia* (LC_{50} = 2.114 µL/mL). A similar observation was made by Mahdi and Rahman (2009) in their experiment conducted to investigate the insecticidal potency of 10 spices.

For all the oils tested, more eggs were oviposited on untreated seeds (control) than on the treated seeds during the 24-72h period. Also there were more eggs oviposited on treated seeds after 72h compared to that at 24 and 48h suggesting that effectiveness of the oils as oviposition deterrents was time dependent possibly because of the dissipation of the essential oil over time. Each of the oils displayed some level of repellency against *C. maculatus*. At the highest concentration tested, *P. nigrum* essential oil exhibited the strongest degree of repellency (Class V) against *C. maculatus* followed by *C. aurantifolia* (Class VI) and *C. limon* (Class III) after 12 hrs.

The efficacy of essential oils, *Citrus sinensis, Citrus limonium, Citrus aurantofolia* and *Citrus paradisi* were assessed as cowpea seed protectants against damage by the cowpea bruchid, *C. maculatus* in a laboratory set up at 2.75 and 5.5 mL oil using hydrodistillation technique where the results presented that cowpea seed damage in all the citrus oil treated seeds was expressively low and ranged between 0.50 and 2.50% when compared to the control (Rotimi and Ekperusi, 2012).*P. nigrum* is proven to possess a volatile oil which exhibits insecticidal properties along with fumigation and ovicidal activities to insects where high mortality was noted especially in *C. maculatus* (Ravindran, 2000). *P. nigrum* essential oil acted as the most toxic fumigant (FC_{50} = 0.140µL/L air) among the three oils tested in the present study.

The three essential oils tested can be used for the control of *C. maculatus* where *C. limon* and *P. nigrum* can be used for controlling the adult beetles as contact insecticides as well as repellents and *C. aurantifolia* can aid in the control of oviposition. Citrus essential oils are highly effective as cowpea seed protectant against damage caused by *C. maculatus* and can be used as safe biopesticides for the management of stored cowpea (Rotimi and Ekperusi 2012).

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