



Chemical Variability and Fumigant Toxicity of Essential Oils from Peels of *Citrus reticulata* L. Native to Nigeria

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ABSTRACT

Cowpeas infested with *Callosobruchus maculatus* have lower protein quality compared to the uninfected cowpeas. The affected cowpeas are usually treated with synthetic insecticides that are expensive and hazardous to man. Essential oils were established to possess insecticidal properties without detrimental effects on the users. The properties are influenced by environmental conditions that changes with time in a day and as well determine the type of chemical compounds present in the oils. In this study, we investigated the chemical composition and fumigant toxicity of essential oils from rinds of *Citrus reticulata* collected at 7.00am and 2.00pm against *C. maculatus*. Hydrodistillation of the pulverized (500 g each) rinds from morning and afternoon harvests afforded 0.56 ± 0.06 % and 0.38 ± 0.03 % (w/w) of volatile oils. GC-MS was employed to analyze the oils and the results showed abundance of D-Limonene (55.7% and 61.1%), linalool (12.5% and 16.9%), terpinen-4-ol (7.7% and 5.4%), nerol (1.1% and 4.0%) and α -terpineol (3.3% and 4.5%). The insecticidal property of the oils was examined against bean weevils using fumigant toxicity bioassay. The activity of the oils differs significantly with LT_{50} of 1.967 hours and 1.297 hours for the oils from the rinds of fruits harvested at 7.00am and 2.00pm respectively. The oil from the 2.00pm harvest is therefore more potent and thereby recommended as a safer insecticide against stored product insect pest, *C. maculatus*.

Keywords: *Callosobruchus maculatus*, D-limonene, linalool, insecticide, rinds

Introduction

Cowpea weevils (*Callosobruchus maculatus*) is an important insect in post-agricultural practice. Their presence reduces food quality in the affected cowpeas. The infestation of cowpea grains by this insect occurs between harvest and consumption by consumers. Condition of environment, nature of grains during harvest and the use of insecticides to treat grains are among

other factors that dictate the vulnerability of grains to infestation. Globally, chemical methods using synthetic fumigants are the common practice employed to combat storage pests. Despite their effectiveness, these carcinogenic substances constitute a threat to the life of man. In addition, these chemicals are expensive and the insects may develop resistance to the fumigants in the long run (Choi *et al.*, 2001; Ngom *et al.*, 2020). As a result of these

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drawbacks in their uses, efforts are essential in developing new chemical substances from plants to replace the currently used synthetic fumigants.

Ethereal oils are potential sources of chemical compounds and their insecticidal property has recently been explored on stored grain insects using fumigant toxicity (Shaya *et al.*, 2008). The different types of chemical compounds found in the oils vary from one geographical location to another. These compounds also determine the insecticidal activity of the volatile oils (Shaaya *et al.*, 1997; Lopes *et al.* 2008). The essential oil distilled from the leaves of *Citrus limonium* native to Algeria was evaluated for insecticidal activity using fumigant toxicity against adult grain weevils (*Sitophilus granaries* L.). The oil caused mortality of the insect within the treatment hours. The activity was linked to the greater percentages of citral, geranyl propionate and D-limonene in the oil (Soltani *et al.*, 2020). The fresh and dried leaf volatile oils of *C. meyerii* from Nigeria caused mortality of *C. maculatus* after the infested grain was treated for 48 hours. The predominant and synergistic effects of 3-carene, α -pinene, D-limonene, β -ocimene, citronellal and β -elemene were reported to be responsible for the activity (Usman *et al.*, 2016).

Essential oil from *C. aurantifolia* growing in Brazil was characterized and screened for an insecticidal property using ingestion, fumigant and contact toxicity bioassays against *Sitophilus zeamais*. The oil was toxic to the insect irrespective of the method used (Fouad *et al.*, 2017). The oils from the rinds and leaves of the plant from Egypt contained predominant amounts of limonene, decanol, citral, geraniol and geranyl acetate. The oils also showed ovicidal activity against the eggs and larvae of *Aedes aegypti*. The activity was associated to the abundance of the aforementioned chemical compounds in both oils (Khanikor *et al.*, 2019).

D-limonene was identified in highest percentage in the peel essential oil of *C. reticulata* indigenous to Vietnam, Pakistan, Algeria and India (Anwar *et al.*, 2011; Boughendjioua and Boughendjioua, 2017; Goyal and Kaushal, 2018; Boughendjioua *et al.*, 2020; Mai *et al.*, 2020). Interestingly, the oil from the peels of the plant

native to Egypt contained sabinene as the chief constituent (El-Shazly *et al.*, 2016). The peel volatile oil of *C. reticulata* was reported to show fumigant toxicity against stored product pests (Safayi and Mobki, 2012). Meanwhile, there had been no report on how the time of collection affect the chemical composition and insecticidal activity of essential oil from peels of *C. reticulata* of Nigerian origin. On this basis, the aim of this study was to investigate the effect of time of collection on the chemical profile and fumigant toxicity of ethereal oils from rinds of *C. reticulata* for a safer insecticide against cowpea pests.

Materials and Methods

Collection of Samples

Fruits of *Citrus reticulata* were harvested in the month of October, 2023, at the garden of Baptist Theological Seminary, Ogbomosho in Oyo State, Nigeria in the morning (7.00 am) and in the afternoon (2.00 pm). Samples of the fruits were taken to the Herbarium of Department of Plant Biology, University of Ilorin, for identification and deposition of voucher specimens [UILH/003/0123]. The rinds were removed one after the other and cut into pieces.

Essential Oil Extraction

Hydrodistillation process with the use of Clevenger set up was employed to isolate volatile oils from the rinds (500 g each) of the fruits of *C. reticulata* from both harvest in a day (British Pharmacopeia, 1980). The extraction process was repeated three times for each oil. The oils were collected in a sealed sample tube and kept under refrigeration (4 °C) until the analysis was done.

Gas Chromatography (GC) Analysis of the Oils

The characterization of the oils was done using gas chromatography, which was equipped with an Orion micromat 412 double-focusing system consisting of two capillary columns that were coated with Cp-Sil 5 and Cp-Sil 19 fused silica with dimension of 25 m x 0.25 mm and 0.15 μ m film thickness. Flame ionization detector (FID) was used. A volume of 0.2 mL of each oil was injected and the split ratio was 1:30. Hydrogen was the carrier gas and the oven temperature

ranged from 50 to 230 °C at a rate of 3 °C/min. Injection temperature was programmed at 250 °C while the detector temperature was maintained at 200 °C. GC HP-chemstation software was used to calculate the peak area percentage from the FID signal (Kovats, 1958).

Gas Chromatography–Mass Spectrometry (GC/MS) Analysis of the Oils

The chemical compounds that constituted the oils were identified using a Hewlett–Packard HP 5890A GC that was interfaced with a 70-250s double focusing VG analytical mass spectrometer. Ionization voltage of 70 eV, ion source and transfer line temperature programmed at 230 °C were the operating conditions of the MS. An on-line desktop with a computer equipped with disk memory was used to process the acquired MS data. The peak area of each chromatogram was measured to determine the percentage composition of the oil's constituents.

Identification of Constituents in the Oil

To identify the constituents in the oils, (i) a series of n-alkanes (C₇–C₃₀, Supelco Bellefonte, PA, USA) co-injection with standards was employed to calculate the retention indices of all the constituents. The experimental conditions in which the calculation was done were identical; (ii) the fragmentation patterns of the constituents in their mass spectra were compared with the data from Wiley 275 and NIST 08 libraries (Joulain and Koenig, 1998; Adams, 2012). Without using correction factor, the peak area of the GC (FID response) was used to calculate the relative quantity of the constituents in the oils.

Insecticidal Activity

Insect Culture

Cowpea beetles, *Callosobruchus maculatus* L. were obtained from heavily infested cowpea gotten from Oja Waso, Ogbomoso, Oyo State, Nigeria. The insects were rare on clean beans and maintained under ambient environmental conditions (28±2° C). The rearing jars were covered with a muslin cloth and held in place with a rubber band. This allowed aeration and prevented escape of the insects. The jars were placed inside a wire-netted shelf in the laboratory.

Fumigant Toxicity Bioassay

A 3.4-1 round bottom glass flask with a hook fitted glass stoppers was used as fumigation chambers. Portions of each of the essential oils (25 and 50 µL) were introduced into a cotton wool separately and glued to the inner side of cover of the fumigating bottle that was used. Ten active beetles were put inside the bottle. The control was treated with no essential oil. Data were recorded at interval of half an hour for two hours. The LT₅₀ of the oils was calculated using Probit analysis.

Statistical Analysis

The beetle toxicity bioassay was investigated in an experiment with a complete randomized design. Mortality data of treatment groups, expressed as mean ± SD (n = 3), was compared using ANOVA test, followed by Student Newman-Kuels multiple comparison test at 5% level of significance. Probit analysis was used to determine the median lethal time (LT₅₀), which is the time needed to kill fifty percent of the treated insects. Determining the LT₅₀ was essential for comparing the bioactivity of oils from each harvest time. Statistical analysis was conducted using SPSS for Windows version 14.

Results

The quantity of volatile oils gotten from hydrodistillation of rinds of *C. reticulata* collected at 7.00 am and 2.00 pm were 0.56±0.06 % and 0.38±0.03 % respectively. The relative percentage and identities of the chemical compounds of the oils are shown in Table 1.

The total number of chemical compounds present in the oils from 7.00 am and 2.00 pm harvests were twenty-one and eleven respectively. These numbers represented 99.2 % and 98.5 % of the oils. Among the classes of compounds identified, hydrocarbon monoterpenoids were the main class constituting 64.9 % and 62.5 % of the oils from 7.00 am and 2.00 pm harvests separately with D-limonene (55.7 and 61.1 %) as the most significant compound. Sabinene (2.5 %) and β-myrcene (3.9 %) were also identified as major monoterpenes in the oil of the peels of fruits collected at 7.00 am while the oil from the other harvest had only β-pinene (1.4 %) as another hydrocarbon monoterpenoid. Other hydrocarbon isoprenoids that were found in appreciable

quantities in the oil from morning harvest

include β -ocimene (1.6 %), γ -terpinene (1.1 %) and α -terpinolene (1.1 %).

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Table 1: Chemical Composition (%) of Rind Essential Oils of *C. reticulata* harvested at 7.00 am in the Morning and and 2.00 pm in the Afternoon of the same Day

S/N	Compound	% Composition		RI ^a	RI ^b	Mass spectra data
		7am	2pm			
1	Sabinene	2.5	-	976	975	136,105,93,77,41
2	β -Pinene	-	1.4	980	982	136,121,93,69,41
3	β -Myrcene	3.9	-	991	990	136,107, 93, 79, 69
4	D-limonene	55.7	61.1	1031	1027	136,107, 93, 68, 53
5	β -Ocimene	1.6	-	1050	1053	121, 107, 93, 79, 68
6	γ -Terpinene	1.1	-	1062	1057	136,105,93,77,65
7	Linalool	12.5	16.9	1098	1098	136, 121, 93, 71,55
8	Borneol	1.4	1.2	1165	1162	154, 110, 95, 69, 55
9	Terpinen-4-ol	7.7	5.4	1177	1175	136, 111, 93, 71,51
10	α -Terpinolene	1.1	-	1188	1187	136, 121, 93, 53, 27
11	α -Terpineol	3.3	4.5	1189	1189	136, 121, 93, 59,51
12	Nerol	1.1	4.0	1228	1228	156, 82, 69, 41, 27
13	Carveol	0.5	0.6	1217	1216	152, 109, 84, 83, 55
14	Carvone	0.4	0.4	1242	1241	135, 93, 82, 54, 29
15	Geranial	0.8	1.0	1270	1271	162, 109, 69, 41, 27
16	Thymol	0.2	-	1290	1291	135, 121, 91, 77, 51
17	Carvacrol	0.7	-	1299	1298	150, 135, 107, 91, 77
18	δ -Elemene	3.6	1.7	1339	1338	204, 136, 131, 67, 43
19	β -Cubebene	0.4	-	1345	1351	105,119,161,189,204
20	β -Elemene	0.4	-	1391	1390	189, 147, 91, 81, 67
21	δ -Cadinene	0.1	-	1524	1523	204, 161,119, 91, 55
22	γ -Elemene	1.2	0.3	1555	1555	161,121,107, 93, 67
<i>Classes of Compounds</i>						
	<i>Hydrocarbon Monoterpenoids</i>	64.9	62.5			
	<i>Oxygenated Monoterpenoids</i>	27.7	34.0			
	<i>Hydrocarbon Sesquiterpenoids</i>	5.7	2.0			
	<i>Phenyl propanoids</i>	0.9	-			
	Total (%)	99.2	98.5			

Compounds are listed in order of elution from fused silica capillary column coated on CP-Sil 5, RI^a = Literature Retention Indices, RI^b = Calculated Retention Indices, Tr = Compounds present in trace amounts (< 0.1 %);

Bolded name = Chemotype

The relative percentage composition of oxygenated monoterpenoids were 27.7 % and 34.0 % in the rind essential oils from 7.00 am and 2.00 pm harvests apiece. Linalool (12.5 %

and 16.9 %) was detected in highest percentage among other oxygenated isoprenoids in the two oils. Other major isoprenoids in this class of terpenoids were terpinen-4-ol (7.7 and 5.4 %) and

α -terpineol (3.3 and 4.5 %) in the oils as well as nerol (4.0 %) in the oil of the peel of fruits collected in the afternoon. Both oils contained borneol (1.4 % and 1.2 %), carveol (0.5 % and 0.6 %) and geranial (0.8 % and 1.0 %). Carvone (0.4 % each) was found in minor quantity in the oils. Meanwhile, only the oil from morning

harvest contained nerol (1.1 %) and the compound was detected in significant quantity.

The identified sesquiterpenes constituted 5.7 % and 2.0 % of both oils with δ -elemene (3.6 % and 1.7 %) and γ -elemene (1.2 % and 0.3 %) identified in considerable percentages. Like wisely, the rind oil from 7.00 am harvest was constituted by β -cubebene (0.4 %), β -elemene (0.4 %) and δ -cadinene in insignificant quantities. Although; neither of the oils contained oxygenated sesquiterpenoids, only the oil of the morning harvest contained phenyl propanoids (0.9 %). Out of this quantity, thymol constituted 0.7 % while carvacrol was present in 0.2 %.

Table 2 presents the fumigant toxicity of the oils on cowpea beetle insect pest, *Callosobruchus maculatus* L. at different concentrations and exposure periods.

Generally, acute toxicity to beetles was observed to increase with concentration and exposure period. Consequently, complete mortality (100.00 %) occurred when beetles were exposed to oils harvested in the afternoon for two hours at 50 μ L/mL concentration.. In contrasts, the lowest mortality value ($p < 0.05$) was obtained with oils from fruits harvested in the morning and tested at concentrations of 25 μ L/mL and 0.5 hours of exposure. More so, no significant difference ($p > 0.05$) was observed in beetle mortality caused by

exposure to 25 μ L/mL of volatile oil from morning harvests for 0.5 hours and mortality of beetles exposed to the control (0 μ L/mL) treatment. Nevertheless, the oils caused more than 50 % toxicity to the insect when the exposure period reached 2 hours irrespective of the time the samples were harvested and oils' concentration.

The time taken for half of the test population of the cowpea beetle, *Callosobruchus maculatus* L. exposed to oils at a fixed concentration of 25 μ L/mL to be killed (50 % median lethal time or LT_{50}) is presented in Table 3.

The table shows that oil from rinds of fruits harvested in the morning caused 50% insect toxicity in an average of 1.967 hours, with fiducial limits ranging from 2.239 to 1.796 hours. In comparison, the LT_{50} of oil from the afternoon harvest was lower at 1.297 hours (1.0–2.05 hours), indicating that the afternoon rind oil is potentially a more potent and toxic insecticide against *C. maculatus*. However, at a fixed concentration of 50 μ L/mL, both the LT_{50} and fiducial limits decreased for oils from both harvest times (Table 4). A similar trend was observed at the 50 μ L/mL concentration, with the LT_{50} of the oil from the afternoon harvest being lower (1.176 [1.082-1.274] hours) compared to that of the morning harvest (0.638 [0.129-0.957] hours).

Table 2: *Citrus reticulata* rind essential oils harvested at 7 am and 2pm in a day and their toxicity to *Callosobruchus maculatus*

Harvest Time	Conc. (μ L/mL)	Exposure Periods			
		0.5	1.0	1.5	2.0
Morning (7.00 am)	0	0.00a	2.50a	10.00a	15.00a
	25	0.00a	12.50b	22.50a	55.00b
	50	5.00a	45.00a	62.50b	82.50c
Afternoon (2.00 pm)	0	0.00a	2.50a	10.00a	15.00a
	25	15.00b	22.50b	62.50b	75.00b
	50	37.50c	70.00c	95.00c	100.00c

Data having the same alphabet along the column are not significantly different using student newsmen-kuels at $\alpha = 0.05$

Table 3: Median Lethal Time (LT_{50}) of rind Essential Oils of *C. reticulata* that are applied at 50 (μ L/mL) against *Callosobruchus maculatus*

Harvest Time	LT ₅₀ (FL)	Regression Equation	χ^2	df	p-value
7.00 am	1.176 (1.082-1.27)	Y = 0.283-3.602x	2.790	2	0.248
10.00 am	0.683 (0.129-0.957)	y = 0.798+9.263x	7.916	2	0.19

FL: Lower and upper fiducial limit; df: degrees of freedom

Table 4: Median Lethal Time (LT₅₀) of rind Essential Oils of *C. reticulata* that are applied at 25 (μL/mL) against *Callosobruchus maculatus*

Harvest Time	LT ₅₀ (FL)	Regression Equation	χ^2	df	p-value
7.00 am	1.967 (1.796-2.239)	y = 0.1340-2.239x	4.758	2	0.93
10.00 am	1.297 (1.0-2.05)	y = -0.349-4.776x	12.408	2	0.02

FL: Lower and upper fiducial limit; df: degrees of freedom

Discussion

Comparing the quantities of volatile oils obtained in this study with the results from previous studies, more oil yields were obtained from cold expressed peels of the Algerian and hydrodistilled peels of the Indian and Egyptian grown *C. reticulata* (Boughendjioua and Boughendjioua, 2017; Sharma *et al.*, 2019; Javaid *et al.*, 2020). Meanwhile, the peels of the plant from another location in India and Korea afforded lower quantities of oils after hydrodistillation (Kaushal and Goyal, 2018; Lee *et al.*, 2022). The variance of the plant physiology that resulted from differences in environmental conditions of various geographical regions of *C. reticulata* could be accountable for the disparity in the oil yields from the rinds.

Isoprenoid that is present in highest quantity is traditionally used to name an essential oil chemotype (Lahlou, 2004). D-limonene chemotype was reported for essential oil from peels of fruits of *C. reticulata* grown in most parts of the world including Pakistan, Korea, China, Vietnam, Algeria and Morocco (Anwar *et al.*, 2011; Boughendjioua *et al.*, 2020; Mai *et al.*, 2020; Lee *et al.*, 2020; Zhou *et al.*, 2021). Interestingly, our results were similar since the oils were as well of D-limonene chemotypes because D-limonene existed in highest quantities in the two oils.

It has been documented that the biosynthesis of isoprenoids occurs at various parts of aromatic

plants through cationic intermediate mechanistic pathways from their precursors (Wise and Croteau, 1999; Degenhardt *et al.*, 2009). The transformations are usually facilitated by synthases of the most significant isoprenoids in the plant's secretory cells. During the transformations, the cationic intermediates undergo several electrophilic additions, hydride shifts and cyclization until the processes are stopped by loss of proton and hydration (Degenhardt *et al.*, 2009; Usman *et al.*, 2020). It can therefore be inferred that D-limonene and δ -elemene synthases catalyzed the biosynthesis of the isoprenoids in the oils from both harvests.

Comparing the chemical composition of the two oils, it was observed that there exist differences in the type and quantities of compounds in the oils. Qualitatively, the synthase of D-limonene was more active and facilitated the biosynthesis of higher number of monoterpenoids, although in lower total percentage of 92.6 in the oil of morning harvest than the oil of afternoon harvest with a total percentage of 96.5 %. δ -Elemene synthase also catalyzed the formation of more sesquiterpenoids with greater total percentage in the 7.00 am harvested rind oil than the other oil from 2.00 pm harvest. All the monoterpenoids that constituted the oil of the peels collected in the afternoon were constituents of the other oil except β -pinene. Conversely, the oil of the morning harvest contained β -myrcene, β -ocimene, γ -terpinene, and α -terpinolene which were not found in the other oil from morning collection.

Equally, the synthase of δ -elemene mediated the biosyntheses of β -cubebene, β -elemene and δ -cadinene in the oil of the peels collected in the morning only. This revealed that the meteorological conditions of the locality of the plant in the afternoon favour D-limonene synthase's activity for β -pinene biosynthesis as compared to the ecological conditions in the morning. On the contrary, the meteorological conditions in the morning favour the activity of δ -elemene synthase to aid the synthesis of the aforementioned sesquiterpenoids that were present in the oil of the morning harvested fruits while the meteorological conditions in the afternoon did not favour the activity of the enzyme to aid their formation in the oil from afternoon harvest.

The synthases of D-limonene and δ -cadinene aided the conversion of neryl, geranyl, farnesyl and nerolidyl diphosphates to D-limonene, linalool, borneol, terpinen-4-ol, α -terpineol, nerol, carveol, carvone, geranial, δ -elemene and γ -elemene in the two oils. Meanwhile, the compounds were separately found in different quantities in the oils. Borneol, terpinen-4-ol, δ -elemene and γ -elemene were detected in greater percentages in the oil from 2.00 pm collection than the other oil. Comparably, all the monoterpenoids mentioned above (except terpinen-4-ol) were detected in greater amounts in the peel oil from afternoon collection than that of the morning collection. Some phytochemicals were identified in lower percentages in a particular oil because of their premature termination at the early stage of the biosynthesis of the precursors of those compounds. This premature termination might be due to the inactiveness of the synthases of D-limonene and δ -elemene that were supposed to aid their formations as a result of unfavourable meteorological conditions of the plant's locality during period the of harvest (Ijima *et al.*, 2004).

Classical man-made insecticides are commonly employed at higher concentrations to lower the lethal time of insects. However, this may increase the risk of aversion to development in target pests (Liu *et al.*, 2017). In natural insecticides such as essential oils, these risks are nonexistent since they often comprise of complex mixture of biodegradable non-persistent phytochemicals

(Franz and Nova, 2010). As a result, rind essential oils of *C. reticulata* may be adopted as an environmentally safe unconventional fumigant for the control of the cowpea beetle in grain storage facilities. The oils demonstrated toxicity against insects at all the period of exposure and tested concentrations. The insecticidal attribute exhibited by the oils assessed in this study may be ascribed to the complementary effects of its constituent's predominant monoterpenoids (Rajendran and Sriranjini, 2008).

Conclusion

The chemical profiles, quantity of constituents and insecticidal properties of the ethereal oils isolated from the peels of *C. reticulata* collected at 7.00 am and 2.00 pm in the morning and afternoon were compared. Although both oils contained D-limonene in highest percentage as compared to the other constituents, differences were noticed in a number of the phytochemicals in the two oils which also affected their toxicity properties. The peels from the fruits collected at 7.00 am yielded more volatile oil which contained phenyl propanoids that were not found in the oil from the other harvest. Meanwhile, greater number of oxygenated terpenoids constituted the oil from afternoon harvest which showed more potency and caused higher toxicity of the insect (*C. maculatus*) than the other oil. The oil therefore provides an alternative biodegradable fumigant to the conventional non-biodegradable pesticides for the control of cowpea beetle in grain storage facilities.

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Conflict of Interest

The authors declare that there is no conflict of interest associated with this research study.

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