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Effects of Seasonal Variation on Chemical Constituents and Insecticidal Activity of Leaf Essential Oil of *Citrus limonium*

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ABSTRACT: Leaves (500g) of *Citrus limonium* harvested in the morning (7.00 am) during dry and rainy seasons were separately hydrodistilled and yielded 0.46 and 0.30 % (w/w) of essential oils. GC and GC-MS analysis revealed that the oils obtained from dry and rainy season harvests were predominated by monoterpenoids (84.2% and 77.2%). The most abundant constituent of the oils from dry and rainy season harvests was; limonene (17.9% and 15.3%). Other principal constituents of the oils were; β -pinene (8.0% and 1.5%), terpinolene (7.0% and 7.1%), citronellal (12.9% and 8.3%), carveol (8.0% and 9.0%), neral (7.9% and 8.5%), citronellol (4.4% and 5.9%) and β -caryophyllene (3.3% and 5.2%). Contact toxicity bioassay was used to investigate the insecticidal activity of the oils against *Callosobruchus maculatus*. Irrespective of the season of harvest the oils were active against the insect. Interestingly, oils from the two harvests had comparable activity against the adult *C. maculatus*. Hence, seasonal variation has no significant effect on insecticidal activity of the oil on the postharvest insect pest.

KEYWORDS: Post-harvest, *Citrus limonium*, *Callosobruchus maculatus*, Limonene synthase

1.0 Introduction

Citrus limonium is a perennial plant widely grown in Nigeria. It is commonly known as “Jaganyin” by the Yorubas, “lemun tsami” by the Hausas and “Oroma ilu” by the Igbos (Iwu, 1993). The plant is widely used in folk medicine for the treatment of malaria, flu, diarrhea, coughs, acute rheumatism, diaphoretic and diuretic droughts, jaundice and hysterical, palpitation of heart. It also known to counteract the actions of narcotic poisons especially opium. Biological activities of the plant extracts such as antibacterial (Marina *et al.*, 2007), antifungal (Lee *et al.*, 2010), anticancer (Kawaii *et al.*, 2000), antidiabetic and antiviral (Burt, 2004 and Ortuno *et al.*, 2006) justify their use in folk medicine. Phytochemical investigations of the plant led to the isolation of eriocitrin (eriodictyol-7-rutinoside) (Miyake *et al.*, 1997).

Earlier works on essential oils from the leaf

of *Citrus limonium* in different parts of the world revealed that the oils were of limonene chemotype. Interestingly, the quantity of limonene varied significantly. Hence, geographical location affects the quantity of the most abundant compound in the oils. (Oshagi *et al.*, 2003; Muhammad *et al.*, 2006; Marina *et al.*, 2007; Samer *et al.*, 2012 and Luisa *et al.*, 2012).

Meanwhile, it has been reported that the phytochemical profile of some essential oils are affected by season of harvest of plant materials (Hussain *et al.*, 2010; Oziem *et al.*, 2010; Antonio *et al.*, 2011). This may be due to variation in environmental factors from one season to another. The factors determine the physiological conditions that dictate the activity of enzymes which mediate the formation of mono- and sesquiterpenoids from their respective precursors in plants. Hence, could cause variations in phytochemical profile and biological activity of essential oils of the plants. It is on the basis of these that this study aimed at determining the effect of seasonal variation on the constituents and insecticidal activity of

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essential oil from leaf of Nigerian grown *C.limonium* on adult *Callosobruchus maculatus*.

2.0 Materials and Methods

2.1 Materials

2.1.1 Plant Materials

The fresh leaves of *Citrus limonium*, were obtained in the morning (7:00am) during rainy and dry seasons from the Park and Garden of University of Ilorin, Ilorin, Kwara state, Nigeria. The plant was identified at the Herbarium of Plant Biology Department, University of Ilorin, Ilorin, Nigeria, where voucher specimens were deposited.

2.1.2 Cowpea

Clean uninfested 'Oloyin' cowpea cultivar was obtained from Oja Oba market in Ilorin and stored in the refrigerator for 7days prior to use. The cowpea was removed and placed on the bench in the laboratory till the seeds acclimatize before experiment.

2.1.3 *Callosobruchus maculatus*

Adult cowpea beetles (*Callosobruchus maculatus*) were obtained from heavily infested cowpea in storage. They were reared on clean grains and maintained under ambient environmental condition (28±2°C). The rearing jars were covered with muslin fabric to allow aeration and prevent escape of the insects. The jars were placed inside a wire-netted shelf in the laboratory.

2.2 Methods

2.2.1 Isolation of oil

Pulverized leaves (500g) of *Citrus limonium* from dry and rainy seasons were hydrodistilled for 3 hours in a Clevenger-type apparatus, according to the British Pharmacopoeia (1980) specification. The oils were collected into screwed cap sample bottles and were preserved in a refrigerator at 4°C until analysis.

2.2.2 Gas Chromatography

GC analysis was performed on an Orion micromat 412 double focusing gas chromatography system fitted with two capillary columns coated with Cp-Sil 5 and Cp-Sil 19 (fused silica, 25 µm x 0.25 mm, 0.15 µm film thickness) and flame ionization detector (FID). The volume injected was 0.2 mL and the split ratio was 1:30. Oven temperature was programmed from 50 – 230 °C at 3 °C/min using hydrogen as carrier gas. Injection and detector temperature were maintained at 200 °C and 250 °C respectively. Qualitative data were obtained by electronic integration of FID area percent without the use of correction factors.

2.2.3 Gas Chromatographic / Mass Spectrometry Analysis

A Hewlett Packard (HP 5890A) GC interfaced with a VG Analytical 70-250S double focusing mass spectrometer was used. Helium was the carrier gas at 1.2 ml/min. The MS operating conditions were: ionization voltage 70 ev and ion source temperature 230°C. The GC was fitted with a 25 m × 0.25 mm fused silica capillary column coated with CP-Sil 5 and the film thickness was 0.15 µm. The GC operating conditions were identical with those of GC analysis. The MS data were acquired and processed by online desktop computer equipped with disk memory. The percentage compositions of the oil were computed in each case from GC peak areas. The identification of the components was based on the retention indices (determined relative to the retention times of series of n-alkanes) and mass spectra with those of authentic samples and with data from Literature (Joulain *et al.*, 1998; Jennings *et al.*, 1980 and Adams 2012).

2.2.4 *Callosobruchus maculatus* Toxicity Study

The contact toxicity of essential oils from *Citrus limonium* leaves on adult *C. maculatus* was tested for 42 hours. 0.1ml of oils from both rainy and dry season harvested leaves was separately mixed with 10g of clean, uninfested cowpea grains inside a 4cm diameter plastic

container. Ten newly emerged *C. maculatus* adults were introduced into the essential oil coated cowpeas. Each treatment was replicated 3 times. A control experiment without the oils was also set up. Adult mortality was observed at every 6 hours interval.

2.2.5 Data Analysis

The experiments were laid out in a Completely Randomised Design. Treatment means were subjected to one-way ANOVA while mean separation was done using the Duncan Multiple Range Test at 5% level of significance.

3.0 Results and Discussion

Pulverized leaves of *Citrus limonium* harvested during dry and rainy seasons afforded oil in the yields of 0.46 and 0.30% (w/w) on hydro distillation. Thus, dry season harvest yielded more oil than rainy season harvest. The yields compared favourably well with the yield from the leaves of the Brazilian grown *C. limonium* (de Freitas *et al.*, 2011).

Table 1 shows the identities, Kovat indices, relative percentages and mass spectral data of the constituents of leaf essential oils of *Citrus limonium* harvested during dry and rainy seasons. A total of 36 and 37 compounds that represent 98.9 and 96.2% of the oils were identified from their mass spectra.

The oil from dry and rainy season harvests were predominated by monoterpenoids representing 84.2 and 77.2% of the oils respectively. Sesquiterpenoids detected in the oils of dry and rainy season harvests constituted 11.5 and 16.1% of the oils respectively.

The predominant compound in the oils from dry and rainy season harvests was; D-limonene (17.9 and 15.3%). Other principal constituents of the oils were; citronellal (12.9 and 8.3%), carveol (8.0 and 9.0%), α -terpinolene (7.0 and 7.1%), β -pinene (8.0 and 1.5%), citronellol (4.4 and 5.9%), neral (7.9 and 8.5%) and β -caryophyllene (3.3 and 5.2%). Compounds that existed in appreciable quantities were; α -pinene (0.7 and 0.8%), 2-carene (0.5 and 0.7%), trans- β -ocimene (0.8 and 1.0%), γ -terpinene (0.8 and

1.0%), isopulegol (0.6 and 0.5%), α -terpineol (0.7 and 1.1%), β -elemene (0.6 and 0.6%), δ -elemene (1.1 and 1.1%), α -bergamotene (1.1 and 1.9%), humulene (0.7 and 0.8%), germacrene D (0.4 and 0.7%), germacrene A (0.4 and 0.8%), β -bisabolene (1.5 and 2.4%), caryophyllene oxide (0.6 and 0.4%) and phytol acetate (1.6 and 0.6%).

Comparison of the oils reveals that there are both qualitative and quantitative variations in the constituents of the oil. Qualitatively, D-syvestrene, cis- β -ocimene, 2,6-dimethyl-2,6-octadienyl, limonene oxide, cis-verbenol, decanal, geraniol, α -farnesene, spathulenol and 8,9-dehydroneoisolongifolene, that existed in the oil of dry season harvest were not found in the oil of rainy season harvest. Similarly, sucatonene, p-menthene, sabinene hydrate, O-mentha-1(7),8-dien-3-ol, δ -elemene, citronellyl butyrate, α -sinensal, bicycogermacrene and crithmene that were found in the oil of rainy season harvest were not detected in the oil of dry season harvest.

Quantitatively, D-limonene, β -pinene, citronellal and phytol acetate were more abundant in the oil from dry season harvest than the oil from rainy season harvest. Meanwhile, oil of rainy season harvest was richer in; 3-carene, γ -terpinene, terpinolene, terpinen-4-ol, α -terpineol, carveol, citronellol, neral, β -caryophyllene and β -bisabolene than the oil of dry season harvest.

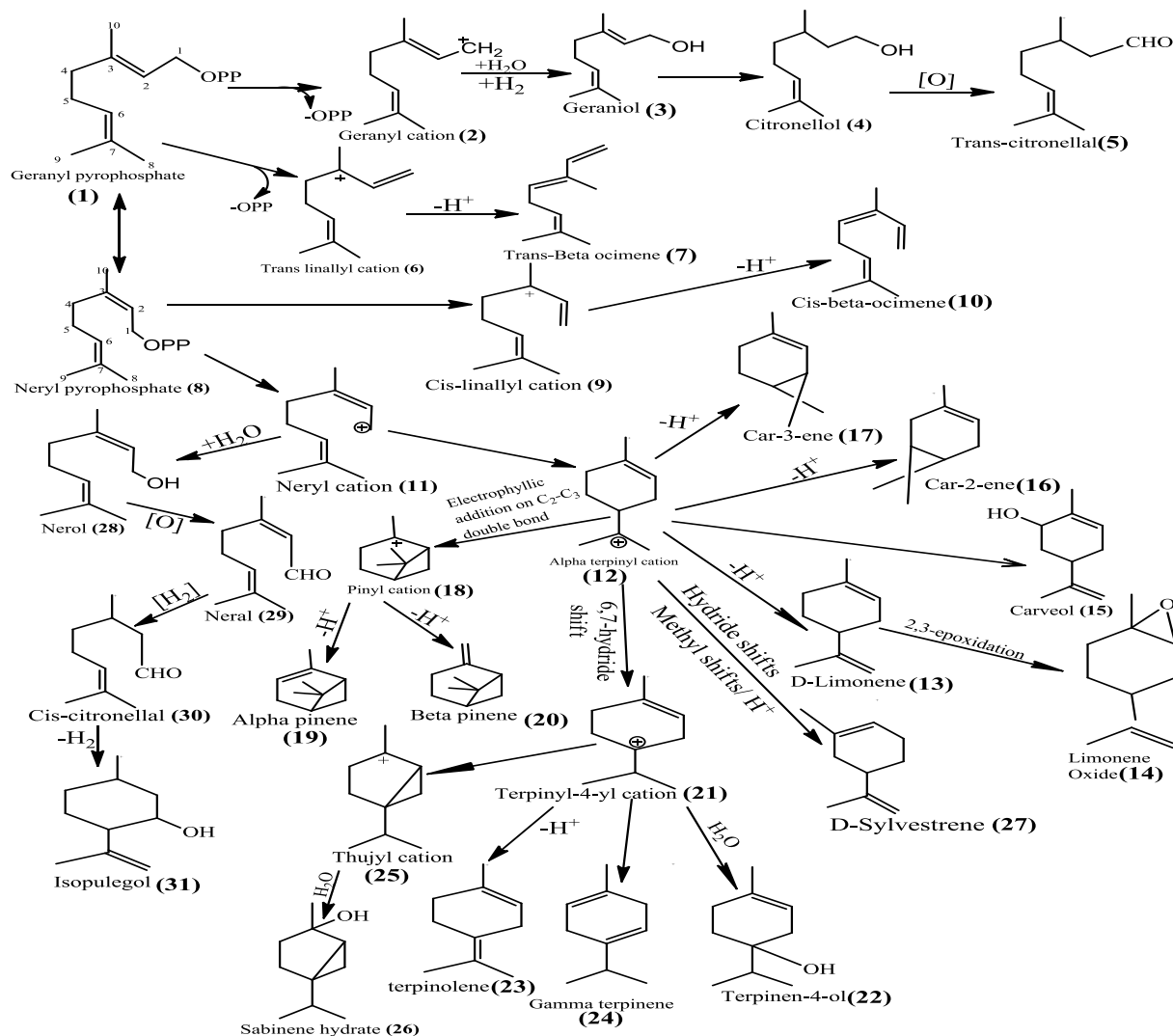
It has been established that, enzymes of the most abundant mono- and sesquiterpenoids mediate the transformations of their precursors (geranyl and farnesyl pyrophosphate) to cationic intermediates (geranyl, linalyl, farnesyl, pinyl, terpinyl, farnesyl, nerolidyl and humullyl cations) (Wise *et al.*, 1998; Degenhardt *et al.*, 2009). The ions subsequently undergo series of rearrangements and cyclization, until it is terminated by deprotonation and hydration to form various terpenoids via cationic mechanisms (Wise and Croteau, 1999).

Table 1: Chemical composition (%) of essential oils from fresh leaves of *citrus limonium* harvested during dry and rainy seasons.

S/N	Compounds	KI	% Composition		Mass Spectra Data
			DSH	RSH	
1	α -pinene	939	0.7	0.8	136,121, 93 ,91,77
2	2,6-dimethyl-2,6-octadiene	978	1.4	-	138,121,95, 69 ,53
3	β -pinene	980	8.0	1.5	136,121, 93 ,69,41
4	Sulcatone	985	-	0.5	126,108, 69 ,55,43
5	p-menthene	1000	-	6.7	138,109, 95 ,81,67
6	2-carene	1001	0.5	0.7	136,121,105, 93 ,41
7	3-carene	1011	0.8	3.4	136,121,105, 93 ,91
8	D-sylvestrene	1027	3.3	-	136,93,79, 69 ,41
9	D-limonene	1031	17.9	15.3	136,121, 93 ,79,68
10	Cis- β -ocimene	1040	3.3	-	136,121, 93 ,79,41
11	Trans- β -ocimene	1050	0.8	1.0	136,121,105, 93 ,79
12	γ -terpinene	1062	0.8	1.5	136,121,105, 93 ,77
13	Sabinene hydrate	1068	-	2.4	154,121, 93 ,77,39
14	Terpinolene	1088	7.0	7.1	136,121,105, 93 ,79
15	Limonene oxide	1133	0.6	-	152,137, 93 ,67,43
16	Cis-verbenol	1140	0.7	-	154,109,94, 59 ,41
17	Isopulegol	1145	0.6	0.5	154,121,81,55, 41
18	Citronellal	1153	12.9	8.3	154,136,121,95, 69
19	Terpinen-4-ol	1177	1.8	2.2	154,136,121,93, 71
20	α -terpineol	1189	0.7	1.1	136,121, 93 , 81,59
21	O-mentha-1(7),8-dien-3-ol	1201	-	0.8	
22	Decanal	1204	0.5	-	156,112,82,70, 57
23	Carveol	1217	8.0	9.0	152, 109 ,84,69,55
24	Citronellol	1228	4.4	5.9	156,138,95,81, 69
25	Neral	1240	7.9	8.5	152,109,94,84, 69
26	Geraniol	1242	1.5	-	154,123,93, 69 ,41
27	δ -elemene	1339	-	0.5	204,161, 121 ,93,77
28	β -elemene	1375	0.6	0.6	204,161,121,93, 81
29	γ -elemene	1378	1.1	1.1	204, 121 ,93,79,41
30	α -bergamotene	1436	1.1	1.9	204,120, 93 ,69,53
31	Humulene	1440	0.7	0.8	204,147,121,107, 93
32	β -caryophyllene	1454	3.3	5.2	204,161,133, 93 ,79
33	Germacrene D	1490	0.4	0.7	204,161,93, 67 ,41
34	Germacrene A	1503	0.4	0.8	204,161,93, 67 ,41
35	β -bisabolene	1508	1.5	2.4	204,161,93, 69 ,41
36	α -farnesene	1508	0.7	-	204,134,93, 69 ,41
37	Citronellyl butyrate	1529	-	0.8	226,109,81, 41 ,27
38	Geranyl butyrate	1562	1.7	1.5	224,154,93, 69 ,41
39	Caryophyllene oxide	1581	0.6	0.4	220,187,161,121, 79
40	Spathulenol	1576	0.7	-	220,205,159,119, 91
41	α -sinensal	1752	-	0.5	218,134, 93, 79 ,55
42	Phytol acetate	1949	1.6	0.6	340,278,123,95, 68
43	8,9-dehydronoisolongifolene		0.5	-	
44	Bicyclogermacrene		-	1.2	204,161, 93 ,79,41
Total (%)			98.9	96.2	
Number of compounds			36	37	

Compounds are listed in order of elution from Silical Capillary Column coated on CP-Sil 5; retention indices on fused Silical Capillary Column coated with CP-Sil 5; *DSH*: Dry Season Harvest; *RSH*: Rainy Season Harvest

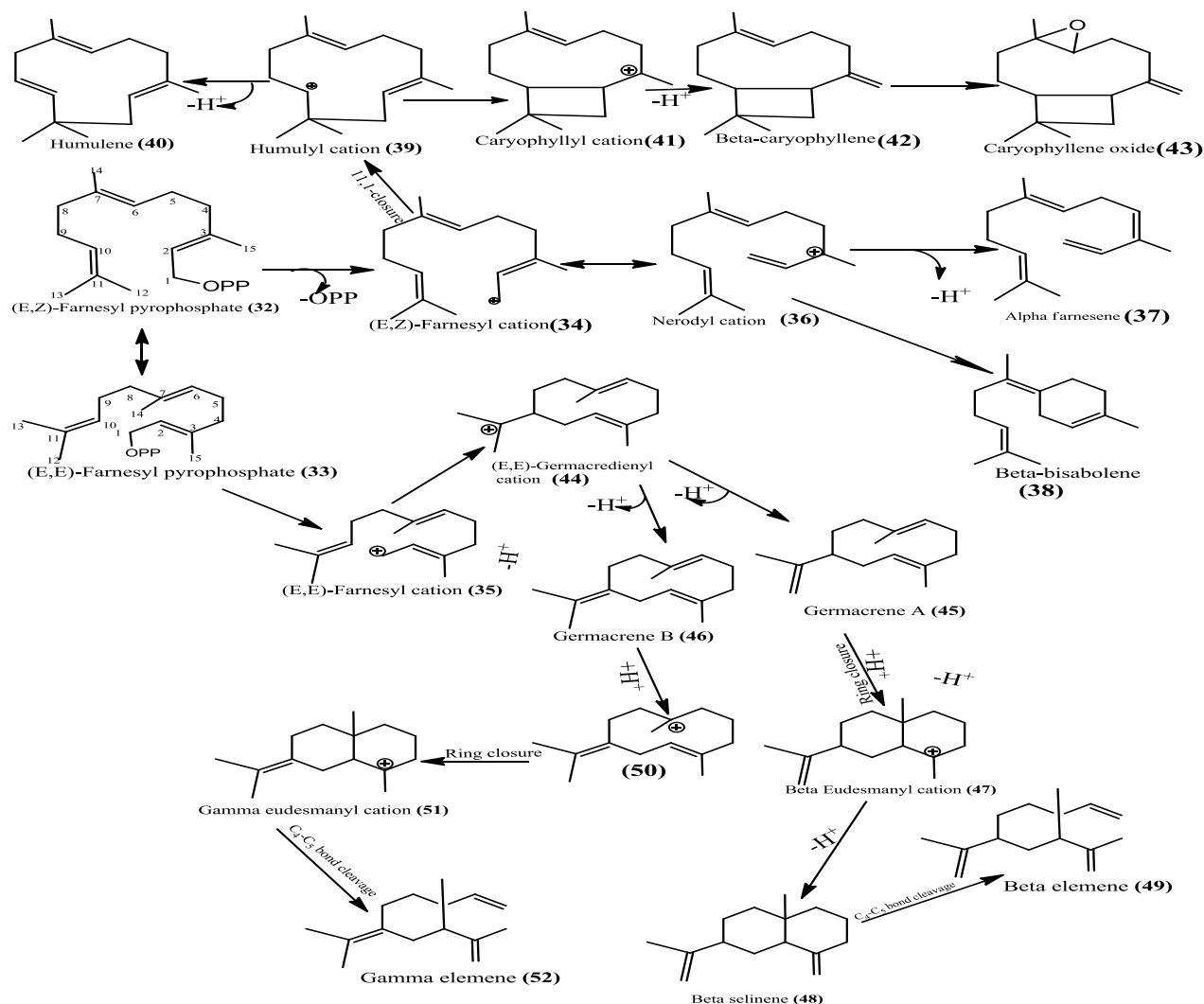
The predominance of limonene in the oils signifies that its synthase aided the formation of all monoterpenoids in the oils (**Reaction Schemes 1**).



Reaction Scheme 1: Biosynthesis of monoterpenoids catalyzed by D-limonene synthase

In the scheme, the limonene synthase facilitate the transformation of geranyl pyrophosphate (**1**) to geranyl (**2**) and linalyl cations (**3**). Hydration of geranyl cation followed by hydrogenation at C₂ & C₃ gives citronellol (**4**). Oxidation of the compound (**4**) produces citronellal (**5**). Nucleophilic attack of C₆-C₇ double bond on the carbonyl of citronellal, followed by deprotonation at C₈ forms isopulegol (**6**). Loss of proton by linalyl cations at C₄ (**3**) forms trans-β-ocimene (**7**) and cis-β-ocimene (**8**). Electrophilic attack of the ion (**3**) on C₆-C₇ double bond gives α-terpinyl cation (**10**). Deprotonation of the ion (**10**) at C₇ forms limonene (**17**) which undergoes 2,3-epoxidation to form limonene oxide (**18**). 6-7, 5-6 and 4-5 hydride shifts of the ion followed by C₄ hydration and C₈ dehydrogenation forms Carveol (**19**). Deprotonation of the ion (**10**) at C₅ and C₁ followed by electrophilic attack of the ion on the resulting carbanions give car-3-ene (**11**) and car-2-ene (**12**) respectively. Folding of the ion (**10**) towards C₂-C₃ double bond followed by Markovnikov addition of the ion on the double bond gives pinyll cation (**13**). Loss of proton by the ion (**13**) at C₄ and C₁₀ give α-pinene (**14**) and β-pinene (**15**) respectively. 1,7-hydride shift of α-terpinyl cation (**10**) gives phellandryl cation (**44**) which deprotonated at C₄ to give α-phellandrene (**21**). Hydration of terpinyl-4-yl cation (**16**) forms terpinen-4-ol (**22**). The ion (**16**) subsequently loses proton at C₇ and C₅ to give α-terpinolene (**20**) and γ-terpinene (**23**) respectively. Markovnikov's addition of the ion on C₂-C₃ double bond gives thujyl cation (**45**) which undergoes hydration at C₃ to give sabinene hydrate (**9**).

The abundance of β -caryophyllene in the oils indicates that its synthase facilitated the formation of all sesquiterpenoids in the oils (**Reaction Scheme 2**).



Reaction Scheme 2: Biosynthesis of sesquiterpenoids catalyzed by β -caryophyllene synthase

In the scheme, β -caryophyllene synthase facilitated the ionization of E,Z-farnesyl pyrophosphate (32) and E,E-farnesyl pyrophosphate (33) to E,Z-farnesyl cation (34) and E,E-farnesyl cation (35) respectively. The E,Z-farnesyl cation isomerised to nerodyl cation (36). Deprotonation of the ion (36) at C₁₅ formed α -farnesene (37) in the oil of dry season harvest. Markonikof's addition of the ion (36) on C₆-C₇ double bond followed by deprotonation at C₁₄ formed β -bisabollene (38) in the oils. Electrophillic attack of the ion (36) on C₁₀-C₁₁ double bond formed humulyl cation (39). Deprotonation of the ion (39) at C₉ formed humulene (40) in the oils. Similarly, electrophillic attack of the ion (39) on C₂-C₃ double bond produced caryophyllyl cation (41). The ion (41) deprotonated at C₁₅ and gave β -caryophyllene (42) in the oils. 5,7-epoxidation of β -caryophyllene formed caryophyllene oxide (43) in the oils. Electrophillic attack of the ion (35) on C₁₀-C₁₁ double bond produced germacreanyl cation (44). Deprotonation of the ion (44) at C₁₀ and C₁₂ formed germacrene A (45) and germacrene B (46) in the oils respectively. Protonation of germacrene A on C₆-C₇ double bond followed by electrophillic addition of the intermediate ion on C₂-C₃ double bond formed β -eudesmanyl cation (47). Deprotonation of the ion (47) at C₁₅ formed β -selinene (48). Cleavage of the C₄-C₅ sigma bond in the compound (48) formed β -elemene (49) in the oils. Markovnikov addition of the ion on C₆-C₇ double bond of germacrene B formed an intermediate ion (50). Electrophillic addition of the ion (50) on C₂-C₃ double bond formed γ -eudesmanyl cation (51). Deprotonation of the ion (51) at C₁₅ followed by cleavage of the C₄-C₅ sigma bond formed γ -elemene (52) in the oils.

Table 2 shows the percentage mean mortality caused by 0.1ml of oils from the leaves of *Citrus limonium* to adult *Callosobruchus maculatus* at 6 hours interval over a 42 hours exposure period. In Table 2, percentage mortality of adult *C. maculatus* was observed to increase with increase in time of exposure to the oils. At the end of 42 hours of exposure, oils obtained from the two harvests caused 100% mortality of adult *C. maculatus*. However, no mortality was observed in the control experiment. Oil from dry season harvest was observed to cause the highest percentage mean mortality within 36 hours of treatment. Meanwhile, there was no significant difference ($P > 0.05$) in mortality caused by oils at each treatment period. Limonene, citronellal, geraniol, β -pinene, and terpinen-4-ol, have been

reported to have insecticidal activities against many insect pests (Mary *et al.*, 1997, Waliwitiya *et al.*, 2012). Therefore the observed activity of oils against adult *C. maculatus* may be due to the synergistic actions of the compounds in the oils.

Conclusion

Although, the oils were of the same chemotype, the major compounds varied with the type of season. Despite the variations, the oils were still active against *Callosobruchus matriculatus*. Hence, the oils can be used as cheaper, less toxic and readily available alternative to the synthetic insecticides that are characterized with draw backs.

Table 2: Percentage mortality of *Callosobruchus maculatus* at 6hrs interval over 42hrs exposure period

TREATMENTS	% MEAN MORTALITY						
	6HRS	12HRS	18HRS	24HRS	30HRS	36HRS	42HRS
DRY	25.0a	35.0a	55.0a	65.0a	75.0a	90.0a	100.0a
RAINY	15.0ab	30.0a	45.0a	55.0a	65.0a	75.0a	100.0a
CONTROL	0.0c	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b

Values in the same column followed by the same letter(s) are not significantly different at $P=0.05$

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