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Behavioural Bioassays and Identification of Cashew Leaf and Stem Volatiles Mediating Attraction to the Stem Girdler, *Analeptes trifasciata* (Coleoptera: Cerambycidae)

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Abstract

The cashew stem girdler, *Analeptes trifasciata* Fabricius (Coleoptera: Cerambycidae), damages cashew by its girdling activities in the stem thereby causing huge economic losses. The stem girdler is managed through cultural practice of burning girdled stems and beetles, though this has drawbacks. The objective of this study was to explore the cues mediating attraction to the cashew host plant; hence the role of olfaction in host plant location by *A. trifasciata* underlying the semio-chemical option for controlling this insect pest. A diffusional Y-tube olfactometer was used to study the behavioural response of *A. trifasciata,* to freshly cut cashew stem and leaves odour sources. Methanol-extract of these plant tissues was subjected to the coupled gas chromatography-mass spectrometry (GC-MS) analysis. Y-tube olfactometric assays demonstrated that both sexes oriented towards and spent significantly more time in stem odour arm compared to the leaf odour arm in both male (male: $t = 2.228$, d.f = 11, P = 0.040) and female ($t = 2.341$, d.f = 11, P = 0.040). A combination of fatty acids, amino acids and carbohydrates were detected in cashew stems. Some of these fatty acids are attractants to other insect pests. It is suspected that these fatty acid blends may possibly be responsible for facilitating host plant location by both sexes. In conclusion, both sexes were independently and strongly attracted to the stem volatiles; this study opens the possibility of utilizing cashew stem volatiles as surveillance and control tools.

Keywords

Analeptes trifasciata, Behavioural Bioassay, Host Plant, Volatile Cues

1. Introduction

The cashew stem girdler, *Analeptes trifasciata* Fabricius (Coleoptera: Cerambycidae) is one of the key pest species of cashew. It has a broad distribution across Central, East and West Africa [1] [2] and is reported to attack more than twenty different tree species in the forest [3]-[5]. *Analeptes trifasciata* is widely distributed along the savannah and forest ecological zones in Nigeria. It causes up to 26% yield loss of raw cashew nut (Mokwunye, unpublished data). It is known as "cashew stem girdler" because of its girdling behaviour which leads to the hanging and eventual breaking off of such branches at the girdled portion. In some instances, where the stems are not completely damaged, the tree may recover but experience a delay in fruit maturation. The control method of *A. trifasciata* is typically cultural and inadequate, therefore, necessitating the exploration of other control options.

Plants produce a complex diversity of volatiles in varying qualities and quantities; and can alter ecological interactions with other organisms [6]. Most insects employ plant volatile cues to locate host plants, oviposition sites [7]. Consequently, these host plant volatiles have been exploited for use as lures to either attract or confuse the insect pests. Mendesil *et al.* [8] reported coffee berry borer *Hypothenemus hampei* adults to respond behaviourally to ripe and dry fruit volatiles of its host plant *Coffea arabica.* According to Kathleen *et al.* [9], differential responses to host plant volatiles occur in several phytophagous insects for various reasons. For instance, according to Piesik *et al.* [10], female sawflies were attracted to wheat plant volatiles for the purpose of oviposition, while the males were attracted to female produced sex pheromone. In another instance, female moths are highly attracted to pest infested fruit for oviposition [11]; while male fruit flies were attracted to the floral substance—methyl eugenol [12]. This process ensures successful courtship and mating. According to Hern and Dorn [11], females usually use host plant cues mainly to locate oviposition sites. Egonyu [13] observed that only adult male of *Pseudotheraptus wayi* were attracted to cashew leaf volatiles, thus suggesting that cashew volatiles serve as attractant to *P. wayi.* A similar incidence was reported by Fujiwara-Tsujii *et al.* [14], where volatile cues from citrus and salix host plants attracted only male *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae).

The use of pheromones, kairomones and baits has been developed to detect the Asian long horn beetle, *Anoplophora glabripennis*, a related species. Nehme *et al.* [15] reported some evidence of attraction of *A. glabripennis* to a combination of host plant volatiles and sex pheromone. Whereas the stem girdler feeds and oviposits on the stem, the other plant parts are not attacked. It becomes necessary to identify the plant part that produces the volatiles which mediate host location. From a management standpoint, these volatiles can be used as lures in surveillance and control programs. This offers a safe alternative strategy to chemical application particularly for most Nigerian cashew farmers, who rely solely on insecticides.

The major ecological benefits of semio-chemicals in crop protection are relatively non-toxicity, species-specificity and non-persistence [16]. There is no known direct adverse effect of semio-chemicals on predators or pollinators. The objective of this study was to explore the cues mediating attraction to the cashew host plant; hence the role of olfaction in host plant location by *A. trifasciata* underlying the semio-chemical option for controlling this insect pest.

2. Materials and Methods

2.1. Insect and Plant Tissue Collection

Adult *Analeptes trifasciata* insects (20 - 30/week) were collected directly from a cashew germplasm plot at the Cocoa Research Institute of Nigeria (CRIN) Headquarters, Ibadan-Nigeria, (latitude 7˚21'N and longitude 3˚86'E) during the pre-flowering season. They were maintained outdoors in wooden cages, at fluctuating temperature of 31˚C ± 2˚C and relative humidity of 65% ± 5%. They were fed with freshly cut cashew stems. Freshly cut cashew stems and leaves were randomly obtained from the germplasm plot using cutlass on the stem while leaves were plucked by hand.

2.2. Olfactometer Bioassays

The behavioural responses of adults *A. trifasciata* to freshly cut cashew stems and young leaves volatiles were investigated in a glass Y-tube olfactometer (measuring ID 6 cm, main tube 26 cm, arm length 22 cm, angle between arms 70˚), used as diffusional olfatometer in the absence of directional airflow. A glass quick-fit round bottom flask measuring 14 cm ID × 55 cm high was coupled to each arm of the Y-tube olfactometer. The olfactometer and accessories were built locally in the Glass blowing unit, Chemistry Department, University of Ibadan, Ibadan, Nigeria; following the description by Ginzel and Hanks [17]. The round bottom flasks served as the odour source chamber, one glass chamber was loaded with a particular type of plant tissue (either five cashew stems measuring 5 cm long or cashew leaves) as the treatment odour source and the other served as a blank air control.

A piece of wire guage was inserted between each chamber and each arm of the Y-tube to prevent the stem girdler (test insect) from reaching the plant tissue. The timing of the bioassay was early photophase (between 12 noon and 15 hours), which corresponded to the peak period of activity in this species (pers. observation). Adult stem girdlers were starved for 24 hours prior to commencement of experiment. Individual test females and males were gently released into base of the main tube of the olfactometer and observed for 15 min. A stem girdler was assumed to respond by moving from the point of release to the treatment odour arm or the blank control arm. The time spent in each zone was recorded using a stop watch. Any stem girdler that failed to respond during this time was assumed non-respondents and removed.

Treatments included five cashew stems versus blank air control and five cashew leaves versus blank air control. The experiment was laid out in a completely randomized design with 30 female and 30 male respondents as replicates. To minimize possible positional effects, the olfactometer was rotated 90˚ every 2 min in a clockwise direction, to control for any directional bias [18]. A fresh set of insects was used for each replicate. Insects used once were not used again for another experiment. Parameters measured included mean time spent in, and number of entries (visits) into the treatment odour and control odour arms of the olfactometer.

2.3. Extraction Preparation

Four chopped pieces of cashew stems (measuring 5 cm × 1 cm) and 10 pieces of leaves were air-dried for 5 days under laboratory conditions. Thereafter Solvent-assisted extraction method was used, with slight modification [19]. The dried samples were pulverized with an electric blender to increase their surface area. The coarse samples were weighed and macerated in methanol for 120 hr. The solution was filtered using Whatman's filter paper and the filtrate was concentrated en vacuo in a rotary evaporator (manufactured by Stuart, UK). The extract was stored in the freezer for subsequent analysis.

2.4. Sample Trimethylsilyl-Dithioacetal (TMSD) Derivatizations

Trimethylsilyl-dithioacetal derivatization (TMSD) of samples was performed using procedures described by Bonaduce *et al.* [20], with appropriate modifications. Briefly, a mixture of ethanethiol and trifloroacetic acid (TFA) (2:1) was added to standards or samples, and the reaction vessel was closed tightly with a screw cap. The residue in the vial was dissolved by swirling and the resulting solution was kept for 10 min at 25˚C. Naturally dried residues were added to 100 μL of pyridine, 68 μL of HMDS, and 22 μL of TMCS.

The resulting mixed solution was further heated for 30 min at 70˚C in a water bath. Additionally, appropriate amounts of both water and chloroform were added into this solution for liquid-liquid extractions. The resulting chloroform solution was directly filtered through a 0.22 μm membrane before further GC-MS analysis.

2.5. Gas Chromatography-Mass Spectrometry

Coupled gas chromatography-mass spectrometric (GC-MS) analyses of the solvent extracts derived from the filtrates were carried out at the Central Laboratory, University of Lagos, Lagos, Nigeria. We used an Agilent HP 7890 GC coupled to an HP 5975 mass spectrometer (EI, 70 eV, Agilent, Palo Alto, California, USA) equipped with an HP-5MS column (30 mm × 0.320 mm ID × 0.25 μl, Agilent, Palo Alto, California, USA) in the spitless mode. The oven temperature was programmed at 80˚C for 2 min and then increased by 12˚C·min^{-1} to 240˚C, and held at this temperature for 6 min. The interface temperature between GC injector and MS was 250˚C. The carrier gas was helium. An aliquot of 1 μl of the cashew stem and cashew leaf extracts were injected into the GC column. GC-MS identifications were made by comparison of retention time and spectra with mass spectral databases [21], and confirmed by peak enhancement on GC using authentic compounds.

2.6. Statistical Analyses

All the statistical tests were conducted using SPSS (version 23). Data on the mean time spent in either arm of the olfactometer were analysed using student *t-test* ($p > 0.05$); while data on the number of ntries (visits) into odour and control arms of the olfactometer were analysed using chi-squared (χ^2) test at $p > 0.05$. Individuals who were unresponsive were not included in the analyses.

3. Results

3.1. Behavioural Responses

In the Y-tube olfactometer bioassays, both male (male: $t = 2.228$, d.f = 11, p = 0.040) and female ($t = 2.341$, d.f = 11, p = 0.040) *A. trifasciata* spent significantly greater proportion of time in the arm with cut cashew stems compared to the control (Figure 1). Mean time spent by male and female was 448.25 seconds and 299.0 seconds, respectively, compared to 121.5 seconds and 30.22 seconds spent respectively in the control arm of the olfactometer. There was no significant difference between proportion of time spent by either male or female *A. trifasciata* in the olfactometer arm containing the cut cashew leaves and control arm (Figure 2). Also, there was no significant difference in the number of times male and female *A. trifasciata* entered either odour zones (cashew stems and leaves) (Figure 3 and Figure 4) compared to the control arm of the olfactometer.

Figure 1. Mean (±SE) response time (min) of male and female *Analeptes trifasciata* to odours from cut cashew stem tested against clean air (control) in a two-arm olfactometer assay.

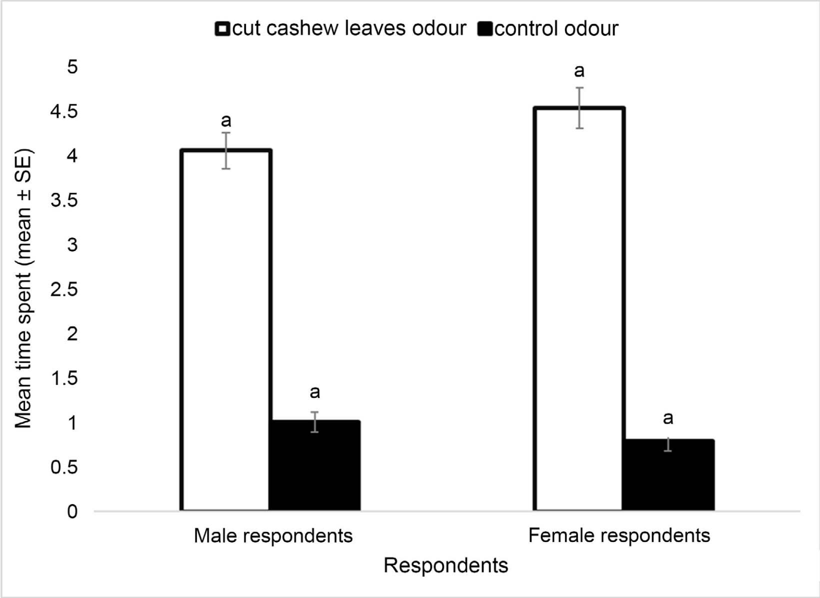

Figure 2. Mean (±SE) response time (min) of male and female *Analeptes trifasciata* to odours from cut cashew leaves tested against clean air (control) in a two-arm olfactometer assay.

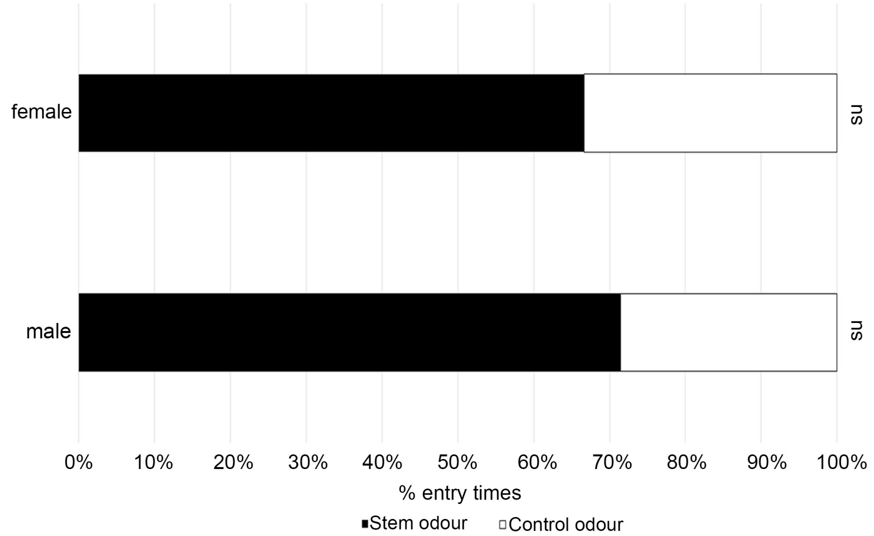

Figure 3. Responses of male and female *Analeptes trifasciata* within an olfactomete to cashew stems as expressed as mean number of entries into cashew stem and control odour arms.

3.2. Analytical Compounds in Stem Bark Extracts

The chemical composition profile of the extract is arranged according to their peak numbers in an ion chromatogram is presented in Table 1. Fourteen cashew stem compounds were detected, representing 94.76% of the extract (Figure 5). The most abundant copounds were 5-hydroxymethyl furfural (60.87%), Alanyl-β-alanine (10.63%), n-Hexadecanoic acid (5.25%) and 2,3-dihydro-3,5-dihydroxy-6-methyl 4H-pyran-4-one (4.70%). Also, trace amounts of carbohydrates such as

D-mannoheptulose (0.78%), D-allose (0.51%), 4-O-methylmannose (0.10%) and amino acids namely cycloserine (0.59%), L-asparagine (0.55%) were identified in the cashew stem extract (Table 1).

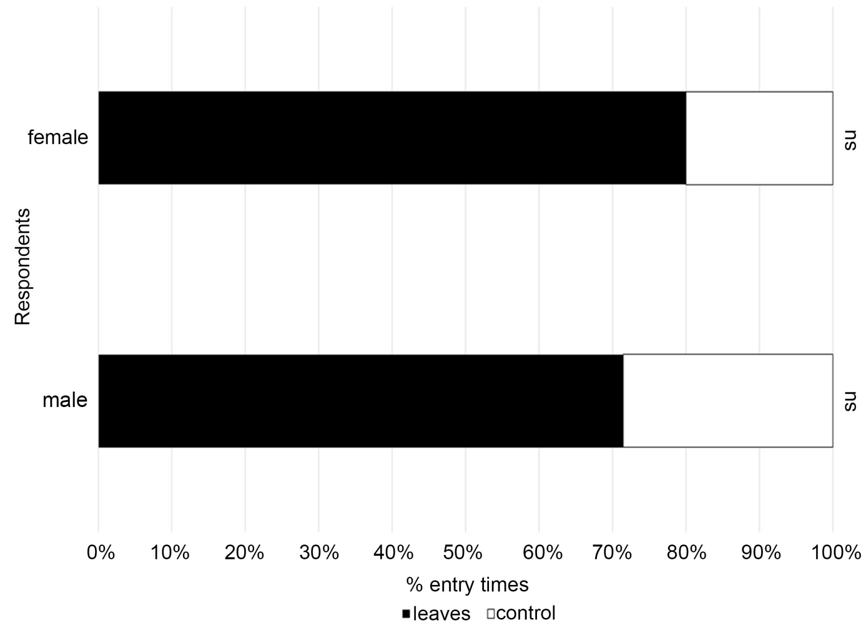

Figure 4. Responses of male and female *Analeptes trifasciata* within an olfactometer to cashew leaves as expressed as mean number of entries into cashew leaves and control odour arms.

Table 1. Volatile Compounds identified from GC-MS analysis of cashew stems.

Peak Number	Name of Compound	Molecular formula	Retention time (min)	% Composition	Nature of Compound
1	Cyanoacetylurea	$C_4H_5N_3O_2$	5.10	4.43	Amide
2	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	5.95	4.70	Ketone
3	5-Hydroxymethylfurfural	$C_6H_6O_3$	6.86	60.87	Alcohol
4	Alanyl-β-alanine	$C_9H_2ON_2O_3Si$	8.48	10.63	Amino acid
5	D-Allose	$C_6H_{12}O_6$	9.87	0.51	Carbohydrate
6	D-Mannoheptulose	$C_7H_{14}O_7$	9.95	0.78	Carbohydrate
7	Tetrahydro-4H-pyran-4-ol	C_4H_9O	11.12	2.00	Alcohol
8	L-Cystine	$C_3H_7NO_2S$	11.22	3.08	Amino acid
9	Cycloserine	$C_3H_6N_2O_2$	12.06	0.59	Amino acid
10	L-Asparagine	$C_4H_8N_2O_3$	12.20	0.55	Amino acid
11	4-O-Methylmannose	$C_7H_{14}O_6$	12.23	0.10	Carbohydrate
12	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	14.82	5.25	Fatty acid
13	9-Octadecenoic acid	$C_{18}H_{34}O_2$	16.25	2.28	Fatty acid
14.	4-Fluoroamphetamine	$C_9H_{12}FN$	20.77	4.22	Fluoro group

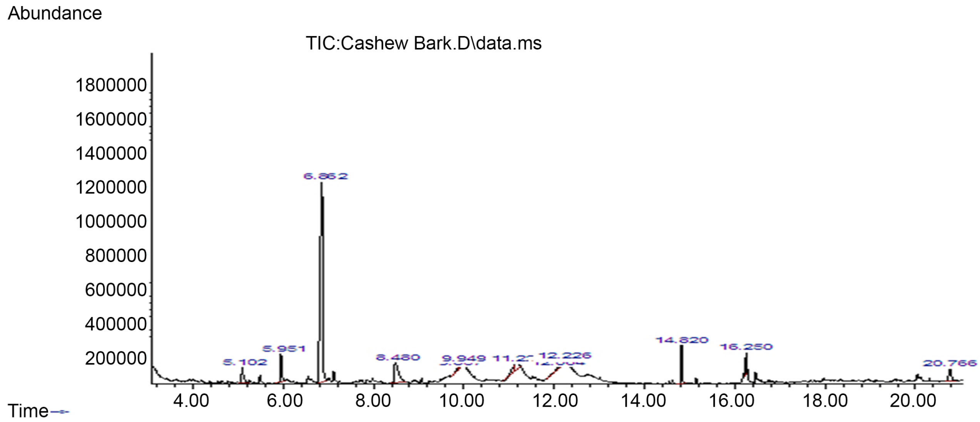

Figure 5. Chromatogram of intact cashew stem extract.

3.3. Analytical Compounds in Leaf Extracts

A total of twenty-four compounds were identified in the cashew leaf extract and they belonged to classes such as hydrocarbons, fatty acids, esters and terpenes (**Table 2**). Terpenes were predominantly present. The major compounds present in the leaf extract were 3β, 17β-dihydroxyestr-4-ene (10.89%), Hexadecanoic acid, ethyl ester (7.49%), I-Methylbicyclo[3.2.1] octane (7.28%) and 3α, 17β-dihydroxysterene (7.06%). The least abundant compounds were Methyl (*Z*)-5,11,14,17-eicosatetranoate (0.31%), Aromandendrene (1.42%) and 2,2,6-trimethyl-1-(2-methyl-cyclobut-2-enyl)-hepta-4,6-dien-3-one (1.60%) (**Figure 6**).

Table 2. Volatile compounds identified from GC-MS analysis of cashew leaf extract.

Peak Number	Name of Compound	Molecular Formula	Retention Time (min)	% Composition	Nature of Compound
1	Azelaic acid, monoethyle ester	$C_9H_{16}O_4$	11.89	2.25	Ester
2	Diethyl azelate	$C_{11}H_{20}O_4$	12.20	2.74	Carboxylic acid
3	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	14.85	3.45	Fatty acid
4	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	15.16	7.49	Ester
5	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	16.22	2.93	Fatty acid
6	9-Octadecanoic acid	$C_{36}H_{68}O_4$	16.29	3.41	Fatty acid
7	Linoleic acid, ethyle ester	$C_{20}H_{36}O_2$	16.47	6.90	Ester
8	Ethyl oleate	$C_{20}H_{38}O_2$	16.54	5.65	Fatty acid
9	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	16.76	3.05	Ester
10	3*α*,17*β*-dihydroxy-Esterene	$C_{18}H_{28}O_2$	18.27	7.06	Alcohol
11	Aromandendrene	$C_{15}H_{24}$	18.44	1.42	Sesquiterpenoid
12	Thunbergol	$C_{20}H_{32}O_2$	18.75	3.13	Diterpenes
13	Arachidonic acid	$C_{20}H_{32}O_2$	18.97	2.77	Fatty acid
14	Caryophyllene oxide	$C_{15}H_{24}O$	19.10	2.41	Sesquiterpenoid
15	1(3H)-Isobenzo-furanone,3,3-dimethyl	$C_{10}H_{10}O_2$	19.16	4.21	

Continued

16	*β*-santalol	$C_{15}H_{24}O$	19.34	5.29	Alcohol
17	Bicyclo [5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl	$C_{15}H_{24}$	19.40	6.27	Hydrocarbon
18	1-Methylbicyclo [3.2.1]	C_9H_{16}	19.62	7.28	Hydrocarbon
19	3*β*,17*β*-dihydroxyestr-4-ene	$C_{18}H_{28}O_2$	19.71	10.89	
20	7-Oxabicyclo [4.1.0]heptanes,	$C_{10}H_{16}O_2$	19.81	2.96	Hydrocarbon
21	Cyclohexane,6-butyl-1-nitro3-Oxatricyclo [3.2.1.0(2,4)]octane	$C_{10}H_{17}NO_2$	19.99	3.62	Hydrocarbon
22	cis-Z-*α*.-Bisabolene epoxide	$C_{15}H_{24}O$	20.04	2.91	Sesquiterpenoid
23	2,2,6-Trimethyl-1-(2-methyl-cyclobut-2-enyl)-hepta-4, 6-dien-3-one	$C_{15}H_{22}O$	20.07	1.60	
24	Methyl (Z)-5,11,14,17-Eicosatetraenoate	$C_{21}H_{34}O_2$	20.30	0.31	Fatty acid

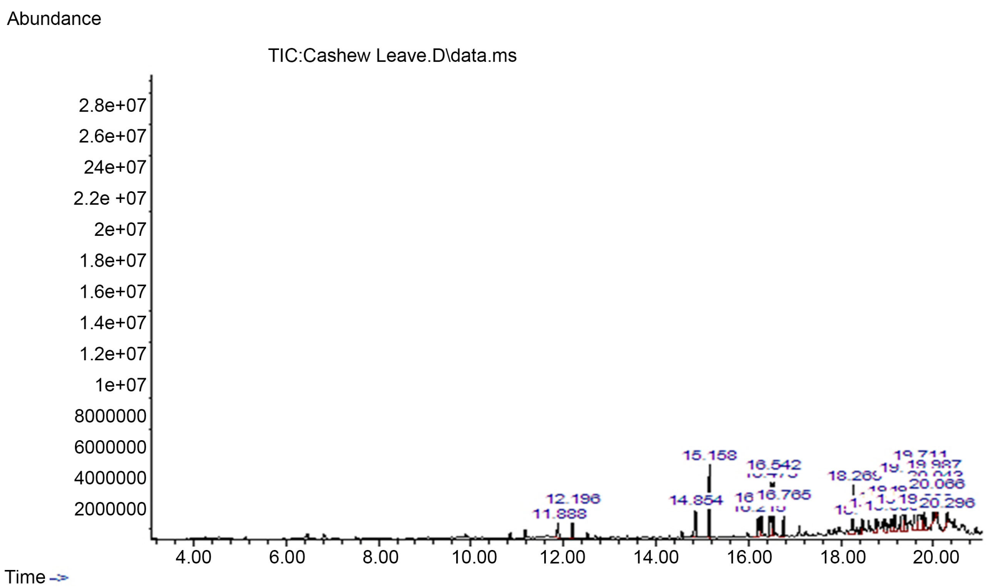

Figure 6. Chromatogram of cashew leaf extract.

4. Discussion

Both sexes spent more time in the stem odour zone compared to leaves and control odour zones, thus demonstrating their preference for the stem cues. This underscores the presence of volatile cues in the stem playing a significant role in mediating attraction. It is instructive to note that the blend of volatile cues in the two plant parts was qualitatively and quantitatively dissimilar, hence the observed results. Phytophagous insects including the stem girdler are under selection pressure to find quality host plants, more so suitable plant parts to maximize their survival and fitness.

Host plant chemicals can influence insect behavior and sex pheromone communication; regulate reproduction to coincide with periods of food availability and suitable oviposition sites [22]. For most cerambycid species, plant volatiles are attractive to both sexes, thus playing a role in mate location. The result of this study has provided support for this assertion. Both sexes were independently attracted to cashew stems; they did not show significant attraction to the cashew leaves. Thus, this signifies that the cashew stem volatiles play a role in host location and maybe mate location as well. It has been established that both sexes of cerambycidae such as *Semanotus japonicas* Lacordairo [23], *Monochamus alternatus* Hope [24] are attracted to host plant volatiles. The ability of female, *A. trifasciata* to independently find host plant is crucial for oviposition purpose and larval development. Hanks and Wang [25] reported that the chances of mate-finding are enhanced when both sexes can independently locate the host plant volatiles. Both sexes of *Xylotrechus colonus* (F.), *Megacyllene caryae* (Gahan) and *Neoclytusmucronatus mucronatus* (F.) were attracted to their host plant volatiles in Y-tube assays [17]. Apple volatiles were attractive to apple maggot fly, *Rhagoletis pomonella* in the laboratory and field trials [26]. In another study, adults of the subfamily lamiine, *A. malasiaca* were attracted to volatiles released from host plants fed by conspecifics [27]. Adults of the cerambycine subfamily, *A. subfasciatus* feed on nectar and pollen and locate their host through these volatiles [28]. Barata and Araujo [29] demonstrated that another cerambycidine, *Phoracanthase mipunctata* (F) responded to odour blends from their host plant, Eucalyptus.

Plant odours are a complex blend of many volatile chemical components [30]. Cashew stem contained fatty acids, carbohydrate and amino acid required for growth and development of the stem girdler. Both stem and leaf extracts contained fatty acids and esters. Terpenes were predominant in the cashew leaf profile. Earlier studies have established that hydrocarbons such as sesquiterpenes, monoterpenes, alkanes and fatty acids function as olfactory cues for host finding among insect species [31] [32]. Fatty acids such as Hexadecanoic acid (palmitic acid), cis, cis-9, 12-Octadecadienoic acid (linoleic acid), Octadecenoic acid and Octadecanoic acid (stearic acid) are used as whitefly attractants [33]. These can be used in combination for synergistic effects. In addition, synthesized hexadecanoic acid ester derivatives have been used as attractants to monitor and trap *Caryedon serratus,* an insect pest of groundnut [34]. Some fatty acids compounds such as n-Hexadecanoic acid (found in cashew stem and leaf extracts) and 9-Octadecenoic acid (found in stem) could possibly serve as attractants to both sexes.

5. Conclusion

In conclusion, male and female, *A. trifasciata* were independently attracted to cashew stems, thereby providing further evidence that adult cerambycidae utilize chemical cues to locate host plants for feeding and possibly mating. The behavioural response of adult *A. trifasciata* to the cashew stem tissue underlines the significance of specific blends of compounds which guide the insect to the host and host plant tissue. It is suspected that the fatty acid blends may possibly be responsible for facilitating host plant location by both sexes. However, further study should be conducted on the behavioural response of *A. trifasciata* to this blend of compounds using Electroantennography (EAG) technique. The attractant effect of cashew stem is a new finding that may be incorporated into existing management measures.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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