

## REPELLANT PROPERTIES FROM ESSENTIAL OIL OF *ALPINIA CALCARATA* ROSC. AGAINST THE AMERICAN COCKROACH, *PERIPLANATA AMERICANA*

PRIYANI A. PARANAGAMA<sup>1\*</sup> and E.M.D. SUJANTHA EKANAYAKE<sup>1</sup>

*Department of Chemistry, University of Kelaniya, Kelaniya.*

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**Abstract:** A steam distillate from freshly cut rhizomes of *Alpinia calcarata* was analyzed by gas chromatography, combined gas chromatography-electroantennographic detection (GC-EAD) and gas chromatography-mass spectrometry (GC-MS) to detect bio-active components of the essential oil against the American cockroach, *Periplanata americana*, a common household pest in Sri Lanka. Six gas chromatographic peaks representing major component were found to possess electroantennogram (EAG) activity. The peaks were identified as  $\alpha$ -pinene, camphene,  $\beta$ -pinene, 1,8-cineole, camphor and fenchyl acetate. EAG assay of essential oil of *A. calcarata* did not elicit a significant response from antenna of the nymphs-male of *P. americana*, whereas the female nymphs showed EAG amplitudes similar to that of the adult female. The electrophysiological responses of essential oil of *A. calcarata* also showed the highest activity compared to known attractants and known repellants. In the behavioral bioassay, the essential oil elicited significantly higher repellent properties in *P. americana* than in the control.

**Key words:** *Alpinia calcarata*, essential oil, household pest, *Periplanata americana*, repellent.

### INTRODUCTION

*Periplanata americana* L. (Dictyoptera: Blattidae), commonly known as the American cockroach is a common household pest in Sri Lanka and one of the most disagreeable pests of human habitations. Although there are several domestic cockroach species in Sri Lanka, *P. americana* is the most common species. Large amounts of synthetic insecticides such as cypermethrin, cyhalothrin, chlorpyrifos and pyrethroid are applied annually to control this insect.<sup>1</sup> However, the use of these insecticides leads to development of resistance, a high cost and a potential health risk for human beings.<sup>1</sup> Therefore, there is an urgent need to develop ecologically safe and sound pest control agents, which have the potential to replace toxic synthetic insecticides.

It is an age-old practice of local people in developing countries to use plant materials, which are indigenous to their countries to repel insects. There is a wide range of repellent substances present in plants with biological potency and diversity, resulting in a formidable range of barriers to feeding of insects.<sup>2</sup>

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\* Corresponding author

Essential oils are plant secondary metabolites, which contain volatile components of monoterpenes, sesquiterpenes and phenolic compounds.<sup>3</sup> Sri Lanka cultivates essential oil bearing plants and has developed an essential oil industry mainly as a means of earning foreign exchange. In recent years, the essential oil bearing plants have been considered as a source of potentially bioactive compounds due to their antimicrobial, antitumour and insecticidal properties.<sup>4,5</sup>

Essential oils of leaves and flowers of *Ocimum basilicum* (S. Maduruthala) plants, have been studied as repellants against mosquitoes. It was reported that the smoke given off when these leaves were added to burning charcoal was used locally to repel mosquitoes.<sup>6</sup> Freshly harvested paddy mixed with leaves of *Vitex negundo*, *Ocimum sanctum*, *Eucalyptus terreticornis*, *Curcuma longa*, *Citrus* species and *Azadirachta indica* at the rate of 1% by weight and stored for a period of 6 months showed that primary and secondary pests of stored paddy were appreciably reduced.<sup>7</sup>

*Alpinia calcarata* Rosc. (Zingiberaceae), locally known as Heenaraththa, is an important rhizomatous medicinal herb which grows in the wet zone of Sri Lanka. The rhizomes of *A. calcarata* have long been used in the indigenous system of medicine.<sup>8</sup> The extract from the rhizome is used as an expectorant for curing bronchitis and asthma. It stimulates digestion, purifies blood and improves the voice<sup>9</sup> and also shows minor anti-inflammatory activity.<sup>10</sup> It is a common practice in rural areas of Sri Lanka to use pieces of *A. calcarata* rhizomes as a cockroach repellant. *A. calcarata* is known to contain pungent odoriferous oils and the chief components of the essential oil are 1,8-cineole (42%), camphene (7.6%),  $\alpha$  &  $\beta$ - pinene (11.3%),  $\alpha$ - fenchyl acetate (14.7%), camphor (5%), borneol (2.5%).<sup>11</sup> Although the chemical constituents of the essential oils are well documented, a literature search revealed that the insecticidal activity of *A. calcarata* has not been studied extensively against the household pest, *P. americana*. We report here for the first time results of laboratory bioassays conducted to determine the repellency of essential oil of *A. calcarata* derived from rhizomes against the American cockroach *P. americana* and the identification of the bio-active compounds by GC-EAD and GC-MS.

## METHODS AND MATERIALS

**Steam Distillation:** Rhizomes (2 kg) of *A. calcarata* were cut into small pieces and steam distilled for 4 hours. The essential oil was collected on condensing into a glass apparatus (500 ml). The condensed solution (400 ml) was saturated with NaCl and subsequently extracted with diethyl ether (Analar grade 3 x 100 ml). The diethyl ether phase was dried with anhydrous sodium sulphate and concentrated using a flash evaporator. A stream of nitrogen gas was passed through the concentrated sample to remove any remaining diethyl ether and this essential oil sample was used for the bioassays, EAG analysis and GC analysis.

*Insects:* Cultures of *P. americana* were maintained at  $30 \pm 2$  °C and  $75 \% \pm 5$  R and photoperiod of 12:12 (L:D) in the insectary of the Department of Chemistry, University of Kelaniya, Sri Lanka. They were fed with breadcrumbs and tap water.<sup>2</sup>

*Gas Chromatography:* Gas chromatography (GC) was performed on a Hewlett Packard 5890 series II chromatograph with a splitless injector, flame ionization detector (FID), and a fused silica capillary column (30 mm x 0.25 mm, DB Wax or DB-5), 5 min at 35 °C, 35-100 °C at 4 °C/min, 100-250 °C at 10 °C/min, hold 10 min at 250 °C. The carrier gas was He.

*Gas Chromatography - Mass Spectrometry (GC-MS):* Hewlett Packard 5890 series II chromatograph fitted with a split-splitless injector, coupled with a Hewlett Packard 5970 series mass spectrophotometer, was used. GC conditions were the same as the above.

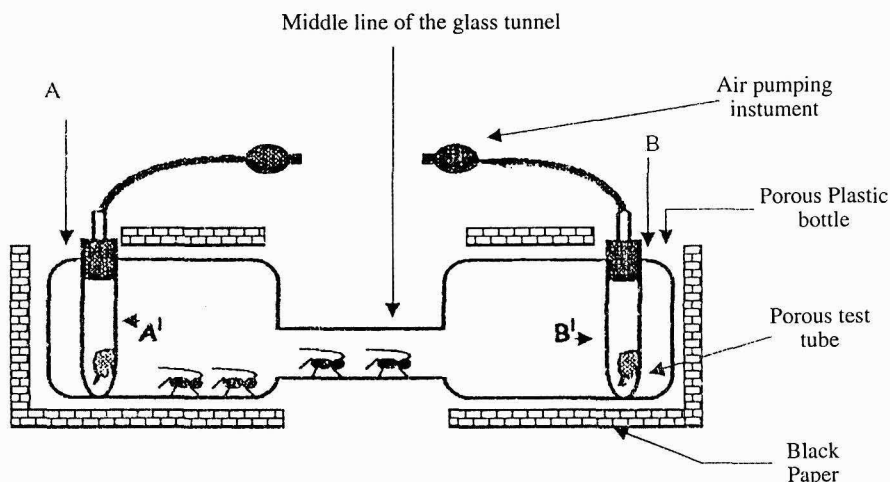
*Electroantennography (EAG) of known samples:* Male and female *P. americana* adults (5-10 days old) and nymphs (3-4 days old) were used for the experiments. The antenna of the cockroach was cut off from the head as close as possible to its base. The distal end of the antenna (1.5 cm) was fixed on to two gold electrodes filled with insect ringer solution (insect saline was used to provide a physiological medium for the antenna). The recording electrode and the reference electrode were positioned at the tip and the base of the antenna respectively. The recording electrode was connected to a high impedance amplifier. The test sample was directly applied (20 µg) on to filter paper strips (3 mm x 2 cm) and the solvent was allowed to evaporate for 10 min. The filter paper strip was placed inside the pasteur pipette and the pasteur pipette was then connected to a plastic syringe, which already contained 1 ml of air locked inside. The test sample with 1ml of air was then directly exposed to the antenna. Each antenna was exposed to 20 µg of the following samples: (Figure 2) (i) Extract of fried coconut (A), as a known attractant<sup>2</sup> of cockroaches, (ii) Extract of a faecal matter (B) as a source of the aggregation pheromones<sup>12</sup>, (iii) Phenol (C), as a known repellent<sup>13</sup>, (iv) Camphor (D), as a known repellent<sup>13</sup>, (v) Diethyl ether (E), the solvent used, (vi) Essential oil of *A. calcarata* in diethyl ether (F) (1 mg/ml) and each bioassay was replicated seven times.

*EAG Recording of the essential oil samples:* EAG responses were recorded for the essential oil of *A. calcarata*. Male and female cockroaches (adults and nymphs) were used for the above analysis. Eight doses (1, 5, 10, 20, 40, 50, 75 and 100 µg) of the test oil were tested against male and female cockroaches separately using the same method as above and each bioassay was replicated seven times.

*Combined gas chromatography-Electro Antennography of the essential oil of A. calcarata:* Coupled gas chromatography-electroantennographic detection (GC-EAD) was performed according to the same GC conditions as above with a DB-wax column, (30 mm x 0.25 mm ID, 0.25 µm film thickness, J and W Scientific, California).

The column effluent was split between the FID and the antennal mounting in the ratio of 20:80 respectively. The effluent was passed through a heated transfer line (200 °C) into a glass tube (200 mm length, 6 mm ID) in order to prevent any condensation of the effluent. The most suitable distance for the antenna preparation was determined to be 5 mm away from the GC effluent and each bioassay was replicated seven times.

*Behavioral bioassay of the essential oil:* Behavioral bioassay was carried out using the method described by Contreras *et al.*<sup>12</sup> (Figure 1). The whole apparatus was covered with a black paper during the experiment in order to simulate a habitat similar to the natural environment of cockroaches. The baits were prepared by introducing appropriate amounts of the essential oil from the stock solution (1 mg/ml) on to cotton wool and keeping for 10 min for the residual solvent diethyl ether to evaporate. Control assays were carried out using diethyl ether (3  $\mu$ l) and distilled water (3  $\mu$ l) separately. Cockroaches were placed in one container (e.g. A), baits were placed in the porous tube A<sup>1</sup> (Figure 1) and a slow stream of air was pumped through the sample. After 10 min, the number of cockroaches that crossed the glass tunnel was counted. This sequence was interchanged randomly in the subsequent replicates. All bioassays were conducted using eight adult (5-10 days old) and nymph (3-4 days old) cockroaches of the same sex separately. Bioassays were carried out during 8.00 a.m. to 4.00 p.m. in a dark room and each bioassay was replicated seven times.



**Figure 1: Experimental apparatus used for behavioral bioassay.**

A and B - two transparent plastic containers

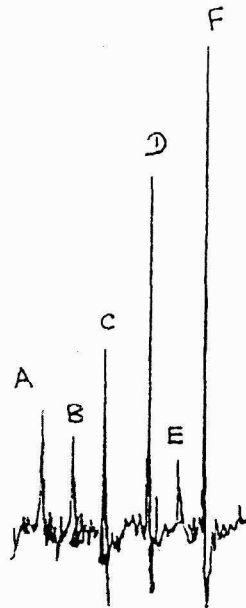
A<sup>1</sup> and B<sup>1</sup> - two porous test tubes

*Statistical analysis:* Data obtained during behavioral assay was statistically analyzed using chi-square test and the results of the EAG assay were statistically analyzed using one way ANOVA and Tukeys Pairwise comparison Test.

## RESULTS

### Gas Chromatography analysis of essential oil of *A. calcarata*

The yield of the essential oil isolated from the steam distillation of 2 kg of the rhizomes was 0.2 %. The GC study of the volatile constituents of the essential oil revealed the presence of more than 43 compounds. The confirmation of the identity of the chemical constituents of the essential oil was based on the GC-MS data, relative retention times and the injection of authentic compounds. The major constituents identified were 1,8-cineol (45.5%),  $\alpha$ -pinene (4.6%),  $\beta$ -pinene (7.6%), camphene (5.2%), limonene (4.3%),  $\alpha$ -terpineol (4.6%), camphor (5.6%), and fenchyl acetate (6.6%).



**Figure 2: Electroantennogram detector (EAD) responses of antennae of *P. americana* for known attractants, known repellants and essential oil of *A. calcarata*.**

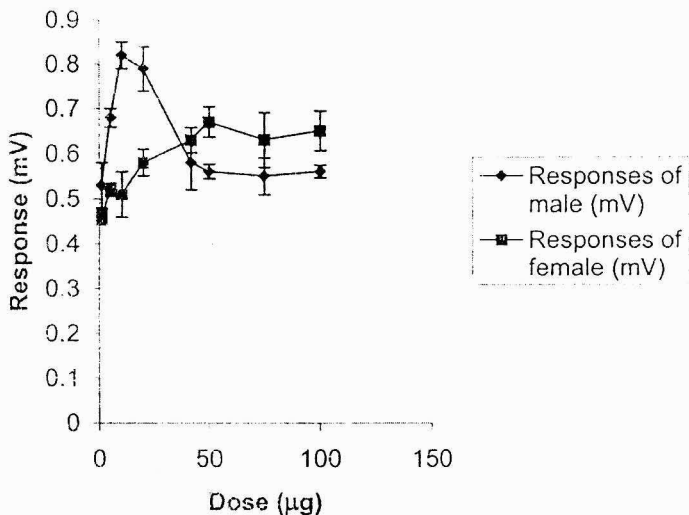
Cockroach antenna was stimulated by 20  $\mu$ g of the test sample;  
A-Extract of fried coconut, B- Extract of faecal matter, C- Phenol,  
D- Camphor, E- Diethyl ether, F- Essential oil of *A. calcarata*.

### EAG assay of known attractants and repellants

In order to compare the activity of the essential oil, EAG was performed for known sensorially active compounds and for the test essential oil. Figure 2 shows the EAG responses of adult *P. americana* antennae for known repellants, attractants and the essential oil. Although the results showed a prominent antennal response for all test samples, the electrophysiological response of the essential oil was significantly higher than those of equal amounts of the known repellants and attractants.

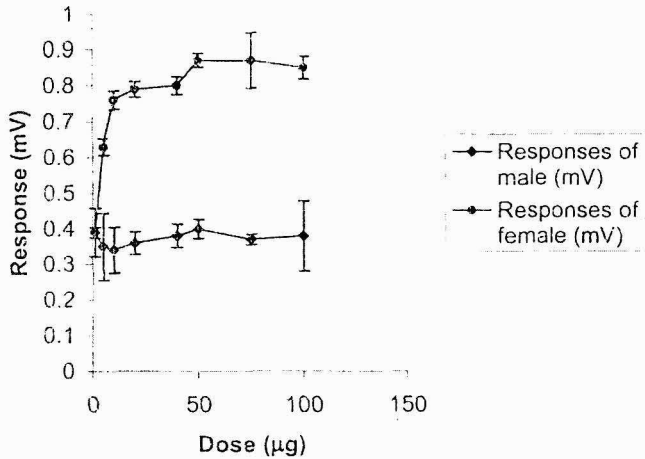
### EAG assay of the essential oil

A series of doses of the essential oil (1-100  $\mu\text{g}$ ) was used for the electroantennogram assay (Figure 3 and 4). In the adult male cockroaches, the highest EAG amplitude was observed at 10  $\mu\text{g}$  dose of the essential oil and showed a significant decrease in activity for doses ranging from 4-100  $\mu\text{g}$  (Figure 3). In the adult female cockroaches, the essential oil however showed significant EAG amplitudes and revealed an increase of the electrophysiological activity at higher doses (Figure 3). In an EAG assay of nymphs, males did not show significant electrophysiological responses compared with females in a dose range from 1-100  $\mu\text{g}$  (Figure 4). The electrophysiological responses of nymph-females were even higher than that of adult-female cockroaches at equal doses of the essential oil.



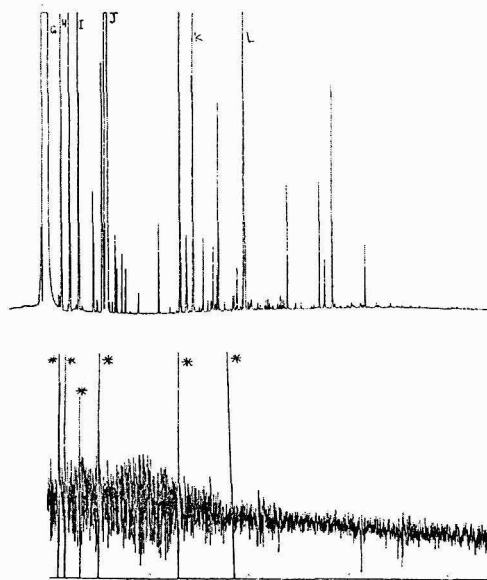
**Figure 3: Dose response curves of relative electroantennogram responses of adult male and female cockroaches, *P. americana* to essential oil of *A. calcarata*.**

Doses were prepared from a 1mg/ml solution of the essential oil. The results were analyzed using one way ANOVA and Tukey's pair wise comparison test  $p < 0.05$ .



**Figure 4: Dose response curves of relative electroantennogram responses of nymph male and female cockroaches, *P. americana* to essential oil of *A. calcarata*.**

Doses \* were prepared from a 1mg/ml solution of the essential oil. The results were analyzed using one way ANOVA and Tukey's pair wise comparison test  $p < 0.05$ .



**Figure 5: Gas chromatography-Electroantennogram detector (GC-EAD) responses of antennae of *P. americana* to essential oil of *A. calcarata*.**

(a) G-H shows the EAD active peaks in the FID chromatogram of the essential oil; G-  $\alpha$ -pinene, H- camphene, I-  $\beta$ -pinene, J- 1,8-cineol, K- camphor , L- Fenchyl acetate.

(b) \* marks show the EAG amplitudes of antennae of *P. americana* for the essential oil.

## GC-EAD analysis

Figure 5 represents the GC-EAD response profile of *P. americana* to the essential oil of *A. calcarata* under the above mentioned GC conditions. Analysis of the essential oil by GC-EAD on DB wax column revealed more than six compounds that elicited strong antennal responses against cockroaches. Both male and female (adults and nymphs) antennae produced identical responses to the essential oil. Those peaks of the FID trace denoted by letter G to L coincided with EAG and were considered as EAG active and marked with asterisks (Figure 5). Identical mass spectra as well as relative retention times on two different columns (DB Wax and DB-5 in methods and materials) confirmed the identity of bio-active components and they were identified as  $\alpha$ -pinene, camphene,  $\beta$ -pinene, 1,8-cineole, camphor and fenchyl acetate.

**Table 1: Behavioral bioassay results of essential oil of *A. calcarata* with adults *P. americana*\***

Bait	Number of cockroaches that crossed the glass tunnel (Mean $\pm$ SE)				
	N	Adults		Nymphs	
		Male	Female	Male	Female
Distilled water (30 $\mu$ l)	07	0.90 $\pm$ 0.08 <sup>a</sup>	1.01 $\pm$ 0.06 <sup>a</sup>	0.95 $\pm$ 0.23 <sup>a</sup>	0.91 $\pm$ 0.11 <sup>a</sup>
Diethyl ether (30 $\mu$ l)	07	1.13 $\pm$ 0.31 <sup>a</sup>	1.25 $\pm$ 0.09 <sup>a</sup>	0.98 $\pm$ 0.22 <sup>a</sup>	1.03 $\pm$ 0.33 <sup>a</sup>
Essential oil of <i>A. calcarata</i>					
20 $\mu$ g	07	7.17 $\pm$ 0.98 <sup>b</sup>	6.83 $\pm$ 0.73 <sup>b</sup>	7.71 $\pm$ 0.04 <sup>b</sup>	7.43 $\pm$ 0.05 <sup>b</sup>
40 $\mu$ g	07	6.94 $\pm$ 0.54 <sup>b</sup>	7.56 $\pm$ 0.43 <sup>b</sup>	7.76 $\pm$ 0.23 <sup>b</sup>	7.11 $\pm$ 0.42 <sup>b</sup>
75 $\mu$ g	07	7.46 $\pm$ 0.81 <sup>b</sup>	6.89 $\pm$ 0.32 <sup>b</sup>	7.67 $\pm$ 0.41 <sup>b</sup>	7.33 $\pm$ 0.25 <sup>b</sup>
Camphor (20 $\mu$ g)	07	7.10 $\pm$ 0.45 <sup>b</sup>	7.53 $\pm$ 0.09 <sup>b</sup>	7.44 $\pm$ 0.61 <sup>b</sup>	7.40 $\pm$ 0.12 <sup>b</sup>
Extract of aggregation pheromone (20 $\mu$ g)	07	0.97 $\pm$ 0.15 <sup>a</sup>	1.45 $\pm$ 0.21 <sup>a</sup>	0.92 $\pm$ 0.04 <sup>a</sup>	1.21 $\pm$ 0.07 <sup>a</sup>

\* Except in the cases of distilled water, diethyl ether and extract of aggregation pheromone, the mean numbers of insects in baited and non-baited arms were significantly different ( $p < 0.001$ , chi-square test).

\* Eight insects were used in each experiment. Insects in each container were counted 10 min after introducing the bait to the container. Mean numbers of insects responding followed by similar letters are not significantly different ( $p < 0.05$ , ANOVA, Tukeys Pairwise comparison test).

## Behavioral bioassay of the essential oil

The repellent activity of the essential oil was compared with camphor (known repellent), extract of faecal matter (known attractant) and distilled water (control) (Table 1). The rapid movements of the cockroaches towards the bottle B (figure 1) were considered as positive responses. The results obtained for male and female cockroaches (nymphs and adults) were not significantly different from each other ( $p < 0.05$ ). More than six cockroaches moved to the non-baited container when the essential oil (20 - 75  $\mu\text{g}$ ) was used as the treatment. The results obtained for camphor were not significantly different from that of the essential oil. However when distilled water and extract of faecal matter (extract of aggregation pheromone) were used, only a minimum number of cockroaches moved from container A to container B.

## DISCUSSION

The composition of the essential oil of *A. calcarata* has already been reported by Pant *et al.*<sup>11</sup> The essential oil extracted from the rhizome of *A. calcarata* in Sri Lanka was analyzed by GC and characterized by GC-MS and relative retention times. The present results revealed that the major constituent of the essential oil is 1,8-cineol and the composition of the Sri Lankan variety, *A. calcarata* ROSC F. is similar to the composition of the Indian variety.<sup>11</sup> The presence of aroma compounds such as camphor and 1,8-cineol explains the characteristic odour of the essential oil.

The use of plant extracts, including allelochemical compounds such as essential oils, with known effects on insects, could be a useful complementary or alternative method to the heavy use of classical insecticides.<sup>6</sup> Commonly, essential oil can be inhaled, ingested or skin absorbed by insects. Thus, these oils may show repellent as well as toxic effects on insects.<sup>5</sup>

The EAG and GC-EAD assays of neem seed volatiles were tested against *P. americana*.<sup>2</sup> More than five compounds in the neem seed volatiles were identified as repellent compounds and the major compound 2-methyl-2-pentanal was also identified as a bioactive constituent in neem seed volatiles.<sup>14</sup>

The present investigation was carried out to identify the bio-active components of rhizome of *A. calcarata* as a repellent for the common household pest, *P. americana*. In the behavioral bioassay, the crude essential oil showed repellent properties when compared with the known attractants and the known repellents (Table 1). In the case of the control, more than 6 test insects remained in the bottle 'A' whereas the insects in the bottle 'A' was reduced to 1-2 in the essential oil treated assays. When the EAG responses of the essential oil were compared with the known attractants and known repellents, the highest EAG amplitudes were shown by the essential oil (Figure 2) under the laboratory conditions used. Furthermore, it is likely that the number of olfactory receptors for the essential oil is much higher than the number

for the aggregation pheromone. As a consequence, the receptor responses of the essential oil were significantly higher than that of the other test samples ( $p < 0.05$ ).

Margosan-O, a commercial preparation of neem seed extract was tested for its effects as a toxicant, growth inhibitor or repellent against *P. americana* and the neem extract strongly repelled both male and female *P. americana* adults for the first week but not during the second week.<sup>14</sup>

The present study demonstrated the potency of essential oil of *A. calcarata* as a repellent for *P. americana*. In the EAG assay selective stimulation of a particular zone of antenna of *P. americana* showed positive peaks. These peak patterns were reproducible and characteristic for each compound. Further, the EAG assay results indicate that all groups of cockroaches (male and female) displayed a similar pattern of repellency. This could be attributed to the fact that general odour receptors are equally distributed on the antennae of male and female cockroaches and those receptors are stimulated by the volatile components.<sup>2</sup> The present study also revealed the lowest EAG amplitudes of the nymph male cockroaches indicating the chemoreceptors responsible for EAG responses of *P. americana* were minimum in nymph males. EAG responses of *P. americana* for neem seed volatiles were compared and the results indicate that both adults and nymphs showed higher EAG amplitudes for neem seed volatiles than that of the essential oil of *A. calcarata*.<sup>2</sup>

Bioassays carried out with the same concentrations of the test essential oil on EAG correlated fairly with the pattern of EAG. In the EAG assay, maximum antennal response was recorded at the dose of 20  $\mu\text{g}$  of the essential oil. In the behavioral bioassay, which was carried out using a whole insect providing natural environment showed maximum repellency at a range of 20-40  $\mu\text{g}$  of the essential oil. Similar results were reported for the behavioral bioassay carried out to test the repellent effect of neem seed volatiles against *P. americana*.<sup>2</sup> These correlations provided experimental evidence for the existence of a relationship between the results obtained by the entire insect on bioassay and parts of removed antennae on EAG. The coupling of these results of EAG and behavioral bioassay experiments should be studied further to know more about the types of sensillae distributed along the antenna and individual responses.

All the physiologically active compounds showed antennal responses when analyzed by GC-EAD. The EAG assays were followed by GC-EAD (Figure 5). There were six compounds in the essential oil that could be identified as bioactive compounds. It has also been reported that the high sensitivity to plant odours could also be explained by the fact that many of these compounds are closely related to pheromones and attractants. Hence they may stimulate some pheromone receptors and odour receptors on the antennae of the insects.<sup>2</sup>

In conclusion, the botanical used in this study can be found as a supporting crop widely in the tropics. Rhizome of *A. calcarata* is used for medicinal purposes in indigenous medicine and offers a cheap and easy control method against *P. americana*. More development research is necessary on the feasibility of introducing the essential oil as a repellent for commercial application.

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