# CHAPTER IV

# BIOLOGICAL ACTIVITY OF BEE MATERIAL AGAINST ANTS


## Summary

A bioassay was designed to test the response of the predatory ant species, Asian weaver ants, *Oecophylla smaragdina*, and European red wood ants, *Formica polyctena,* toward bee material from three species in the genus *Apis* (*A. florea*, *A. andreniformis* and *A. mellifera*) and six species in the genus *Trigona* (*T. apicalis*, *T. collina*, *T. terminata*, *T. melanocephala*, *T. laeviceps* and *T. minor*). The raw bee materials were found to be highly repellent against weaver ants. Interestingly, pentane extracts of these bee materials exhibited repellent activity toward Asian weaver ants whereas the acetone and methanol extracts did not. However, in contrast, all the tested bee materials showed very low repellent activity against European red wood ants. Those extracts revealing active repellent activity against Asian weaver ants were analysed by GC-MS with a view to characterization of the active components. Four chemical groups were indentified in active samples; namely terpenoids, phenol derivatives, long chained hydrocarbons and naphthalene derivatives.

## 4.1 Introduction

The resionous material that bees collect from various plants is used in various purposes. This also refer to "propolis" which is the general name for the resinous material gathered by honeybees, *Apis mellifera*, from various plants (Burdock, 1998). A few foragers from a colony visit the buds or wounds of these trees and collect the material for the flight home. The European honey bee, *A. mellifera* use the propolis to plug cracks and holes in their nest's wall to reduce the nest entrance which is easier for defense and to embalm or mummy of invaders which they killed but could not carry away. Propolis also serves in colonial defense against fungi, bacteria and viruses (Lokvan and Braddock, 1999). The open nest honeybee, the dwarf honeybees, *A. florea* and *A. andreniformis*, use plant resins rely on the nesting branches. This is a kind of sticky plant resin called "sticky band or sticky ring". Sticky bands serves as the barrier separating the nest from the other crawling insect especially ants.

Stingless bees prepare their nests either in aerial situations or in hollow cavities, both above - and underground. The habitats of many stingless bees are confined to forests or aboreal habitats. The importance of propolis in stingless bees should be stressed. Besides being admixed into wax for preparation of cerumen, propolis is used to isolate the nest cavity from the substrate. Propolis lining is thin or virtually absent on the intact inner walls of cavity, but all the open cracks are tightly closed with propolis. Lining is especially thick near the entrance, where the surface is often sticky by constant addition of fresh propolis (Michener, 1974; Roubik, 2006).

Several publications reported that the bee material had the activities against virus, fungi and bacteria, but none of them had mentioned about direct activities against insect predators especially ants. We designed a bioassay to discover whether or not the bee materials posses the repellent compounds used for defense against weaver ant, *Oecophylla smaragdina*, and red wood ant, *Formica polyctena.* The European bee material, propolis from *A. mellifera* and the Asian bee materials, sticky band from *A. florea* and *A. andreniformis*, and nest entrances of *Trigona* spp. were studied.

## 4.2 The Bioassay, Repellent Index and Statistical Analysis

### 4.2.1 The Bioassay

The bioassay was based on the same concept for *O. smaragdina* and *F. polyctena*. Ants were trained to forage on a feeding dish and – after a short training period – the connecting bridge to the feeding dish was replaced either by a neutral or by a bridge applied by the test material.

To install the bioassay, a natural ant trail was chosen on which there were sufficient ants passing per minute. The natural ant trail was then connected to one end of a wooden stick (0.5 meter long). At the other end this stick was attached to the feeding arena. The feeding arena consisted of feeder in the middle of platform (7.62 × 7.62 $cm^2$). At the edge, the bridge was attached to the stick was the bridge. This bridge was made from binary wooden fork. The binary fork could be opened so that the bridge could be replaced with the new bridge for the next shuffle experiment (fig 4.1).

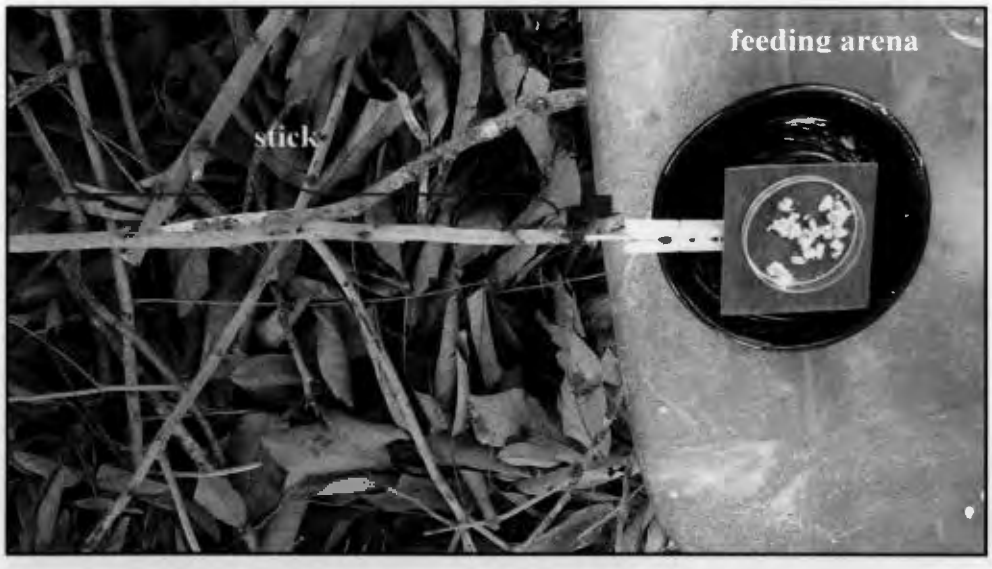


**Figure 4.1** The tests ants were trained to the stick and cross over the bridge to collect food offered on feeding arena. Photo by O. Duangphakdee.

For training, some scout ants were collected and gently put on the feeding arena. There the ants fed and went back over the stick to their trail and back to the

colony. After the successful return foragers labeled their path by trail pheromones. The feeding dish was connected to colony's trail system and the first newly recruited ants arrived at feeding dish shortly afterward. Then the number of ants increased exponentially until it had reached a nearly constant level similar to model suggested by Beekman *et al.* (2001). At that point the feeding arena was replaced by the control arena. This control arena was similar to feeding arena except the control arena has bridge made from control material and no food in the feeding arena. After the control arena was placed, the numbers of ants crossing the bridge to the arena was counted for 5 minutes. Later, the feeding arena was replaced again and ants started foraging. We waited until the number of ants in the trail riased to the same number as before the control experiment (feeding period 2). Then, the treatment arena, which consisted of the platform and the bridge applied with samples and the empty feeder, was replaced (treatment group). The number of ant crossing the treatment bridges to the feeder within five minutes was counted. The experimental sequences were shown in figure 4.2.

The size of the bridge was approximately 0.5 cm × 3 cm. The trial was performed continuously when the number of ants on the ant road coming up at least 15 ants (with *O. smaragdina*) and 30 ants (with *F. polyctena*). We used canned tuna fish as the food for the weaver ants and honey water as the food for the red wood ants. The weaver ants were able to arrange themselves in multiple chains to bridge a gap. Therefore, in the assay for weaver ants, *O. smaragdina*, we made a 1.5 cm. gap between the bridge and stick (fig. 4.3) to block the weaver ants from building the chain over our test sample bridge. The duration of feeding period was adjusted to the number of ants in the ant road. To reduce any possible effect of the previous trial, we waited for at least 30 min before the next test was started. For each sample, the tests were repeated seven times using the new materials from the same colonies with the same procedure. All trials were conducted between 8 -18 hours. The ant's response to the bee material was observed and recorded.

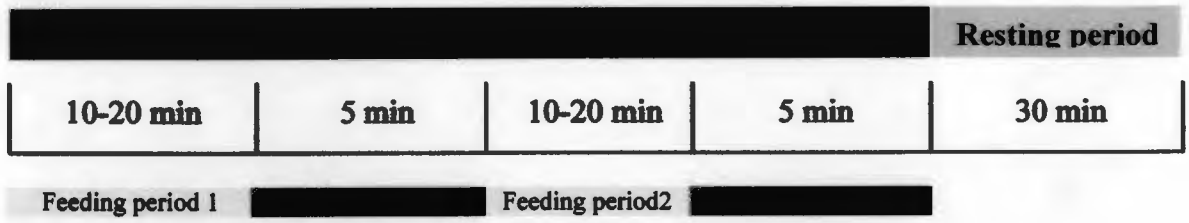


**Fig 4.2** Sequences of the assay.

The assay for identification of the repellent activity presented in fig. 4.2 The resting period was performed before conducting the next experiments in both ant species. The number of ants in control group and treatment group was counted and later the repellent indices (R) were calculated.

The assay with weaver ants was done with the nest in natural habitat (the locations were described in 4.3). Conversely, we maintain the colonies of red wood ants for experiments in the laboratory.

**Figure 4.3** The 1.5 cm gap between the stick and the bridge was made in purpose to prevent the *O. smaragdina* ant from forming multiple chains over the material bridge. Photo by O. Duangphakdee.

### 4.2.2 The General Procedure to Maintain the Colony of Red Wood Ants, *F. polyctena*

The ant colony was collected from the large mounds of fir needle in natural forest of Germany. We gently dug up the needles containing several thousand ants. Then, the colony was transported to the laboratory in plastic containers. The paraffin oil was applied around the apical of the plastic container to prevent the ants from

escaping. The plastic bands was set up on the apical of container to apply the paraffin oil (K21827062, Merck). The colony was maintained at Institut für Bienenkunde in the container (67 cm x 46 cm x 31cm) connected to the ant arena (74cm x 48cm x 24cm) with the 0.50 meter tubular path. We maintained an ambient temperature in the laboratory at 25 $^{0}$ C.

The ants were fed daily with 20 ml of honey water, 10 ml of water and 3-5 bee larvae. To prevent colony and arena from drying out, water was daily sprayed into the ant arena (5 ml) and ant nest (15-20 ml). Everyday, the ants were heated using the heating lamp (Elstein 10T, 150 W 220/230 V) on the arena for 1.5 hr from 18.00 – 19.30 o'clock.

### 4.2.3 The Index

To compare and quantitate the effect of the test material, we calculated a repellent index based on the following equation (Koeniger and Duangphakdee, unpublished data):

$$R = 1 - \left[ \frac{N2}{N1 + N2} \right]$$

$R$ = Repellent Index
$N1$ = number of ant crossed control bridge
$N2$ = number of ant crossed treatment bridge

R is the repellent index which can range between 0-1. The unit $\left[ \frac{N2}{N1+N2} \right]$ comes directly from the data of counted the numbers of ants crossed over the control and treatment bridges (fig. 4.2) in five minute. N1 represented the number of ants crossing the control bridge whereas N2 represented the number of ant crossing the treatment bridge. So when we considered the unit $\left[ \frac{N2}{N1+N2} \right]$, if no ants crossed the treatment bridge (100% repellent), the unit $\left[ \frac{N2}{N1+N2} \right]$ would be 0 which defined the R

equal to 1. If the ants crossed the treatment bridge in the same number as the control group (no repellent activity, no attractiveness), then the unit $\left[\frac{N2}{N1+N2}\right]$ equal to 0.5 which defined the R equal to 0.5. In the case that the number of ants crossing the treatment bridge was more than that of the control bridge (attractiveness), then R would be less then 0.5. The summary of the significance of R values is revealed in Table 4.1.

**Table 4.1** The definition of repellent index (R) value.

| Number of ants crossing the bridge | R value | Significance |
|---|---|---|
| control > treatment | $0.5 > R \leq 1$ | repellent |
| control = treatment | R=0.5 | no repellent, no attractiveness |
| control < treatment | $0 \geq R < 0.5$ | acttractiveness |

### 4.2.4 Statistical Analysis

SPSS software was used for statistical tests. The R was applied first to boxplot to compare the distribution within species. We compared the R values within and among species or extract types by various statistical procedures. Non parametric test, Kruskal Wallis-test and Wilcoxon rang test were used to determine whether R values are significantly different within species and between each species respectively. A Bonferroni correction was also applied to correct in type II error: thus $\alpha$ will be 0.05 or 0.1.

## 4.3 Materials and Methods

### 4.3.1 The Ants and Locations of Experiments:

**Weaver ant, *O. smaragdina***

**Locations of experiments**

The experiments were carried out in Thailand and Sabah, Malaysia. They were conducted in four different locations in these two geographic regions. Weaver ants from natural nests in the area were used for the experiments (table 4.2).

**Location 1: Faculty of Science, Chulalongkorn University, Bangkok, Thailand**

The ant nest used in this study was from the *Ficus benjanina* tree (fig 4.4: A). The nest used for the experiments was 20×30 cm, 3 meters above the ground. This tree grows near a physic building, faculty of science. The ants established trails along a wall besides the building. The trials were extended to a small tree near their nest where ants were trained from the trail nearby. The distance to the nearest nest was approximately 5 meters.

**Location 2: Agricultural Research Station, Chantaburi, Thailand**

This station is located in the Chantaburi province (fig 4.4: B). There were two colonies used for research in this area. The nest of the first colony was on the *Sandoricum koetjape* tree, 6 meters above the ground. The second colony nested on *Nepelium lappaceum*, 5 meteres above the ground. Both ant roads branched from the main trail to the host trees. These host trees were surrounding pepper plantation.

**Location 3: Coconut plantation at Maklong, Samut Songkarm, Thailand**

The ants nested on *Mangifera indica*. This tree grows in the area of a coconut plantation. This plantation was established by the local. For many years, they practiced the "biological way of life for sustainable development" and no chemical pesticides have not been applied. Furthermore, the coconut trees provide very rich food sources for bees in form of nectar and pollen. For that reason, this area is the natural habitat of honeybees as well as the weaver ants.

**Location 4: Agricultural Research Station, Tenom, Sabah, Malaysia**

The Agricultural Research Station is located at Lagud Seberang, Tenom which is administered and owned by the Agriculture Department of Sabah, Malaysia. This area is an agriculture research station with a seed production centre for cash crops, a farmer training center, a honey bee center and an agricultural park, Taman Pertanian Sabah. Our experiments were conducted in the honeybee centre. The ants nested on *Nephelium lappaceum* L. beside the guest house. The colony used for the experiment had a size of approximately 20-30 centimeter width and 20-40 length. We performed the experiments during February- March in 2004 and 2005.

**Table 4.2** Test ant nest description and experimental locations.

| Number | Locality | Ant nest descriptions | | |
|---|---|---|---|---|
| | | Nest size W×L (cm) | Hight (m) | Host plant |
| Colony 1 | Bangkok, Thailand | 20×30 | 3 | *Ficus benjamina* L |
| Colony 2 | Chantaburi, Thailand | 30×35 | 6 | *Sandoricum koetjape* Merr. |
| Colony 3 | Chantaburi, Thailand | 25×20 | 5 | *Nephelium lappaceum* L. |
| Colony 4 | Samut Songkarm, Thailand | 30×40 | 3 | *Mangifera indica* L |
| Colony 5 | Tenom, Sabah, Malaysia | 20×30 | 3.5 | *Nephelium lappaceum* L. |
| Colony 6 | Tenom, Sabah, Malaysia | 25×40 | 3.5 | *Nephelium lappaceum* L. |

**Figure 4.4** The locations of tested weaver ant nests in Thailand A. Faculty of Science, Chulalongkorn University and B. Agricultural Research Station, Chantaburi. Photo by O. Duangphakdee.

**Red wood ant, *F. polyctena***

**Locations of experiments**

The experiments were performed in the Institut für Bienenkunde (Polytechnische Gesellschaft), Fachbereich Biowissenschaften der J.W.Goethe-Universitaet Frankfurt am Main, Oberursel, Germany. The ant colonies were collected from the natural habitat and were maintained in the laboratory for experiments (See 4.2.2). Two different colonies were tested during May-August 2004 and May-September 2006.

### 4.3.2 Samples Collected from Bee Nests

The bee material (propolis and sticky bands from honey bees and nest entrances from stingless bees) were collected during 2004-2006 from the different locations due to the bee nest habitats. The methods of conservation of the material depended on the quantity of samples.

**Samples from nests of genus *Apis***

**Sticky band from colonies of *A. florea* and *A. andreniformis***

The dwarf honeybees, *A. florea* and *A. andreniformis*, are open nesting bees. They apply the plant resin on the nesting branches called "sticky band" to be a barrier and to protect the nest from the ants and other insects (fig 4.5 A). We chose bee colonies which had prominent sticky bands. Because this band was very thin, we could not remove it from the bark to use as a pure propolis. So sticky bands of *A. florea* and *A. andreniformis* were collected by cutting those parts of branches that contained the sticky band area (fig 4.5 B). For control, the parts of the branches without the sticky bands were used as a bridge for the ants to reach the food. Sticky bands are not commonly found on every branch with a dwarf honeybee nest. Accordingly, we set up the discriminating standard that sticky band areas have to form a complete ring around the nesting branch area with a width of at least 1.5 cm. In total, the sticky bands of 47 colonies were collected from *A. florea* in different

geographic regions and of 10 colonies from *A. andreniformis* in three different geographic regions.

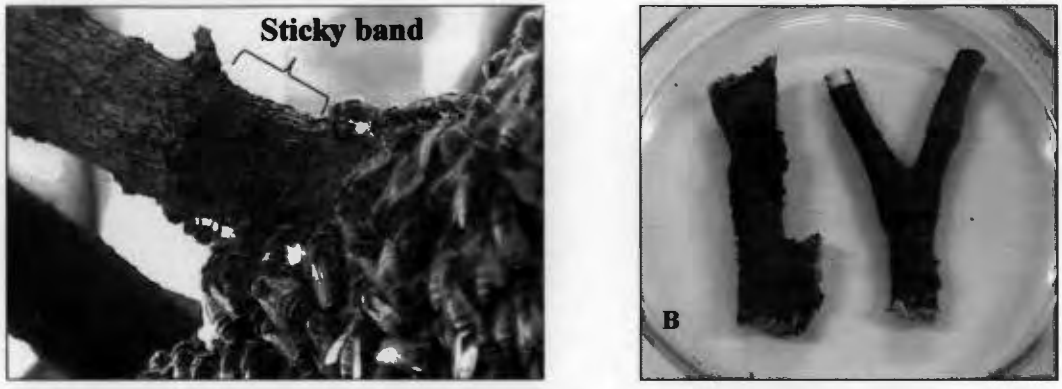


**Figure 4.5** The sticky band of *A. florea* **A.** The branches with sticky band in natural nest, **B.** the sticky band prepared for biassay. Photo by O. Duangphakdee.

**Propolis from *A. mellifera***

The propolis of *A. mellifera* was obtained from the scabs of the frames from beehives. The samples were collected in Oberursel, Germany from three different apiaries belonging to the Institut für Bienenkunde (Polytechnische Gesellschaft), Fachbereich Biowissenschaften of J.W.Goethe Universität Frankfurt am Main. In total propolis of 19 colonies in Germany and 17 colonies (fig. 4.6 A)from three different apiaries in Thailand (fig. 4.6 B), Chulalongkorn University, Nan and Chantaburi. The propolis was used as a bridge to the feeding dish in experimental groups (see detail of preparation in 4.3.3).


**Figure 4.6** The propolis from *A. mellifera*, **A.** Propolis collected from the apiary at Oberursel, Germany **B.** Propolis collected from Nan province, Thailand. Photo by O. Duangphakdee.

**Nest entrance of *Trigona***

**Collection and identification methods**

We collected the nest entrances of stingless bees by cutting the basal of entrances and placing them into plastic bags. Ten to 20 bees from the same colony were also collected for identification of the species. The samples were transferred into amber bottles to protect the samples from light. Then they were kept in the refrigerator at 4 $^{0}$C. The stingless bees genus *Trigona* consists of many species which are very divers. Several species were identified but a few species still need to encompass more details of identification. Consequently, we recorded the following data based on the study of Eltz and colleagues (2003).

**A: Bee species**: The 20-30 bees from each colony were collected and preserved in 75% ethanol. For identification I used the key given by Sakagami *et al.* (1990) and compared with the reference collection in Center of Excellence in Entomology: Bee Biology, Biodiversity of Insects and Mites, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok , Thailand. Some species could be identified by visual inspection of typical characteristics and the shape of the nest entrance tube. However, the unconfident specimens were sent to Charles Michener, Department of Ecology and Evolutionary Biology, University of Kansas, United State for confirmation of identification.

**B: Nest types**: There are two general types of nest locations in stingless bees, cavity in hollow stems (including trees) or underground, in crevices of rocks or on tree trunks. Often underground nests are located under or at the base of the tree. The entrance tube of this type is mostly emerging from soil surface or sometimes curves up into the lower section of trunk.

**C: Nest entrance material characteristics:** The stingless bee's nest entrance tubes have high variety of material. The texture may be sticky, soft, hard, or bristle. The material is composed of different materials like: wax, plant resin, mud, plant leaves and sticks. The color differs from light yellow to blackish. The shape may be tubular (longer than wider), or like a funnel (very wide at the entrance and narrower at

the base), bellshaped (narrower than tubular in the shape of bell). The length varies from few centimeter up to 50 cm.

**D: Nest trees**: Trees were identified with different methods. Agricultural plants were identified by specialized gardeners. Or leaves and flowers were collected and identified using the literature *Families of Flowering Plants* (1926, 1934) by Hutchinson.

**Bee species**

***T. apicalis***

*T. apicalis* colonies nest in hollow tree trunks. The nest entrances have a brownish color at the base and are blackish on the apical rim. The texture is hard and bristle with a rough surface. The mouth is expanded into funnel shape. The length of nest entrance tube varies from 10 to 40 cm from the substrate to the opening. In this study, the 23 nest entrance tubes from 8 different locations were collected (fig 4.7 ).

***T. collina***

*T. collina* colonies nest in the lower section of tree trunks or holes in the ground. The orifice is not expended upwards. The surface of nest entrance is relatively smooth, hard, very brittle and thin-walled. The color varies from light amber to grayish. The lengths vary from 15-45 cm above the ground (fig 4.8). I collected 21 nest entrances of this species.

***T. terminata***

*T. terminata* (collected from Thailand) colonies nest preferentially in hollow trees. The protruding entrance tube is attached to the bark and has a length of about 8-10 cm. It is cylindrical in shape and has a funnel-like orifice. This entire tube is constructed of a light brown or reddish resinous material, which is so soft that removal of the tube resulted in destruction of the tube (figure 4.9). I collected 20 nest entrances of this species from 6 locations.

In Tenom, Malaysia, *T. terminata* commonly nest in hollow tree trunks. Their nests are found abundance frequently in the area of Agricultural Research Stations, Tenom, Sabah Malaysia. The color of the nest entrance tubes was approximately that of coffee with milk. The surface was smooth, soft, thin-walled and sticky. The opening is bending downwards and can be passed by many bees (fig 4.10). Nineteen nest entrance tubes of this species were collected.

**Figure 4.7** Nest entrance of *T. apicalis* in Uthaitani, Thailand.
Photo by O. Duangphakdee.

**Figure 4.8** Nest entrance of *T. collina* in Kanchanaburi, Thailand.
Photo by O. Duangphakdee.

**Figure 4.9** Nest entrance of *T. terminata* in Chantaburi, Thailand. Photo by O. Duangphakdee.

**Figure 4.10** Nest entrance of *T. terminata* in Tenom, Malaysia. Photo by O. Duangphakdee.

***T. melanocephala***

Colonies of this *Trigona* species build their nest cavities in crevices of walls or rocks. We often found this species in cracks of building, of plastic pipe and in the ground. The nest entrance is about 10-15 cm long with light yellowish color. The entrance material is sticky on the apical part and becomes hard and bristle gradually to the basal part (fig 4.11).

**Figure 4.11** Nest entrance of *T. melanocephala* in Tenom, Malaysia.
Photo by O. Duangphakdee.

**Figure 4.12** Nest entrance of *T. laeviceps* in Tenom, Malaysia.
Photo by O. Duangphakdee.

***T. laeviceps***

This *Trigona* species has a small nest entrance tube which is 3-5 cm long extruding from the tree trunk. The material is hard and very bristle. The color of the entrances is dark brownish (fig 4.12). This color is good camouflage to the surrounding which is the bark of the host tree.

***T. minor***

This *Trigona* species builds the nest entrance tube similar to *T. laeviceps*. The length of tube is about 5-15 cm. The apical part of entrance is covered with sticky material. Like other species, *T. minor* nests in cavities in the soil or in tree trunks. The nest entrance tube has a blackish color, the texture is hard and slightly bristle. The surface is rough and sometime includes pieces of dried leaves. The mouth is slightly expanded like a funnel with yellowish color at the apical part. The tube length is approximately 5-10 cm above the nest. The margin is expanding (fig 4.13). The experiments with the material of this species were performed with *O.smaragdina* at Tenom and with *F. polyctena* at Germany.

In summary, bee material was collected from 3 species from genus *Apis*, *A. mellifera*, *A. florea* and *A. andreniformis* and 6 species from genus *Trigona*, *T. apicalis*. *T. collina*, *T. terminata*, *T. melanocephala*, *T. laeviceps* and *T. minor* from three different locations (table 4.3 ) in total of 183 colonies.

**Figure 4.13** Nest entrance of *T. minor* in Chantaburi, Thailand.
Photo by O. Duangphakdee.

**Table 4.3** The list of bee material in experiments. THA = sample was collected from Thailand, MAS = sample was collected from Tenom, Malaysia and GER = sample was collected from Germany.

| Genus and species | Code | Ant species/Location of experiment (number of colony) | | |
|---|---|---|---|---|
| | | *O. smaragdina* | | *F. polyctena* |
| **Genus *Apis*** | | **Thailand** | **Malaysia** | **Gemany** |
| *A. florea* | THA | 40 | - | 7 |
| *A. andreniformis* | MAS | 5 | 5 | - |
| *A. mellifera* | THA | 12 | - | 7 |
| *A. mellifera* | THA | 12 | - | 5 |
| **Genus *Trigona*** | | | | |
| *T. apicalis* | THA | 14 | - | 9 |
| *T. collina* | THA | 14 | - | 7 |
| *T. terminata* | THA | 13 | - | 7 |
| *T. terminata* | MAS | 8 | 5 | 6 |
| *T. melanocephala* | MAS | - | 3 | - |
| *T. laeviceps* | MAS | - | 1 | - |
| *T. minor* | THA | - | - | 2 |
| *T. minor* | MAS | - | 1 | - |

### 4.3.3 Preparation of the Experimental Samples

**The slightly sticky and soft material**

In case of the sticky nest entrance tubes of *T. terminata*, we could attach it directly around small sticks which were used in the experiment as bridges to the feeding dish (fig. 4.14 A and B).

**Figure 4.14**: The bridges covered with the resinous material from *T. terminata* collected from Thailand (**A**) and *T. terminata* collected from Malaysia. Photo by O. Duangphakdee.

**The slightly hard and bristle material**

The nest entrance tubes of *T. apicalis, T. collina, T. melanocephala, T. laeviceps* and *T. minor* are slightly hard and bristle. So we had to carefully cut it into a 3.5×0.5 cm piece and used it as such directly as an experimental bridge. This technique was also used for *A. mellifera* (fig 4.15 A, B and C).

As a control, we used the normal stick 0.5 cm×3.5 (diameter ×long) as a bridge.

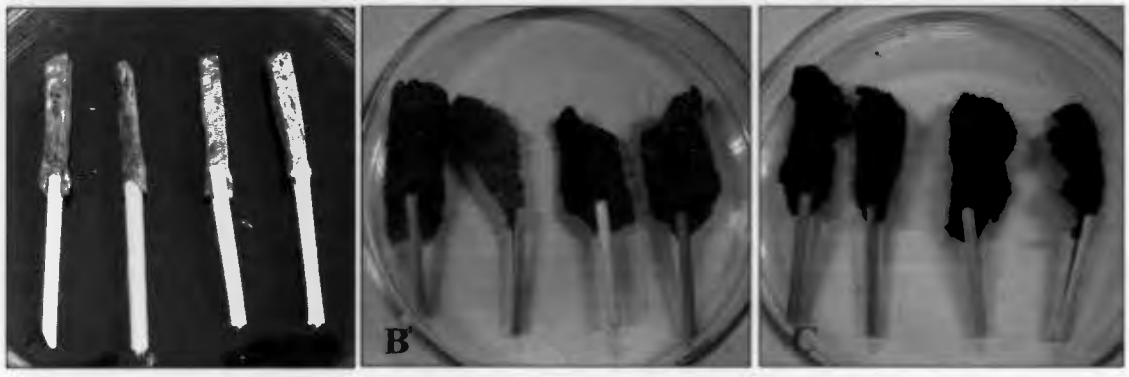


**Figure 4.15** The bridges covered with resinous material from *A. mellifera* **(A)**, *T. collina* **(B)** and *T. minor* **(C)**. Photo by O. Duangphakdee.

### 4.3.4 Isolation of Active Fraction

**Figure 4.16** Soxhlet extractor
Photo by O. Duangphakdee.

The bioassay using sticks provided with test substances as bridges to the feeding dish enabled to test fractions of the raw material to isolate the active compounds. The isolation of fractions started by extracting the raw material with a Soxhlet extractor (fig 4.16) using solvents with different polarity. Afterwards the active fractions were further separated by column chromatography. In every step, the fractions were tested in the bioassay to determine the active fractions.

**A: Extract preparation from Soxhlet Extractor**

The raw material was grounded and first extracted with pentane (Soxhlet, 2 hrs, 3 g). An acetone extraction with the same protocol was added. In the case of the sticky band of *A. florea*, the bark provided with sticky material was peeled off and used for extraction. For control, I extracted the bark from the same tree without sticky ring. The final step was the extraction in 95% methanol. The solvents with propolis fractions were put through a series of filters to remove any remaining particles.

The suspension was evaporated in rotary evaporator below 40 °C. The weight was calculated with a micro balance. Since the test solution had to be calibrated to the same concentration, new solvent was added according to the weight of fractions. For the test 0.3 ml of each calibrated extract was transferred to 3 cm long sticks with a diameter of 3 mm. At room temperature, 25 °C, the solvents with low boiling points were evaporated while the compounds of propolis which have a higher boiling point than room temperature remained on the stick. The control sticks were contaminated with the same quantity of pure solvents. The sticks were used as the bridge in the bioassay test. A total of seven replicates were performed per treatment in each colony. The suspension were used in the same concentration as in the raw material to minimize the effect of different concentration on experiments. For that purpose the weight of the raw material was calculated before and after the extraction. The different weights were used to estimate the concentration of the extracted compounds in the raw material.

**B: Chromatographic methods**

Various chromatographic methods were applied to isolate an active fraction from a more complex natural mixture. The chromatographic methods used during the present work are briefly described.

In principle the solute progresses through the chromatographic system, albeit through a column or along a plate, only while it is in the mobile phase. This process, whereby the substances are moved through the chromatographic system, is called chromatographic development. There are three types of chromatographic

development, *elution development*, *displacement development* and *frontal analysis*. Elution development is now virtually the only development technique employed in both Gas Chromatography (GC) and Liquid Chromatography (LC) although some displacement development is occasionally used in preparative LC.

Displacement development is only effective with a solid stationary phase where the solutes are adsorbed on its surface. The sample mixture is placed on the front of the distribution system, and the individual solutes compete for the immediately available adsorption sites. Initially, all the nearby adsorbent sites will be saturated with the most strongly held component. As the sample band moves through the system the next available adsorption sites will become saturated with the next most strongly adsorbed component. Thus, the components array themselves along the distribution system in order of their decreasing adsorption strength. The sample components are usually held on the stationary phase so strongly that they are eluted very slowly or even not at all. Consequently, the solute must be displaced by a substance more strongly held than any of the solutes (called the displacer). The displacer, contained at a low concentration in the mobile phase, first displaces the most strongly held component. In turn this component will displace the one next to it. Thus, the displacer forces the adsorbed components progressively through the distribution system, each component displacing the one in front until they are all pass through the system. The solutes will be characterized by the order in which they elute and the amount of each solute present will be proportional to the length of each band.

**Thin Layer Chromatography (TLC)**

TLC involves the use of a particular adsorbent spread on an inert sheet of glass, plastic, or metal as a stationary phase. The mobile phase is allowed to travel up the plate carrying the sample that was initially spotted on the adsorbent just above the solvent. Depending on the nature of the stationary phase, the separation can be either partition or adsorption chromatography. The advantage of TLC is that the samples do not have to undergo extensive cleanup steps, and the ability to detect a wide range of compounds. In our separation, the Thin Layer Chromatography (Alumina, Merck, 1.05554.0001) was used to monitor the optimum condition for column

chromatography and to compare the constituents of propolis in each fraction. For detection, UV light was used or TLC plate was dipped in 10% $H_2SO_4$ in ethanol and heated afterwards at 40 °C for five minutes.

**Column Chromatography (CC)**

In overview, CC consists of a column of particular material such as silica or alumina (like on the TLC) and a solvent passes through it at atmospheric, medium or low pressure. The separation can be liquid/solid (adsorption) or liquid/liquid (partition). The columns are usually glass or plastic with sinter frits to hold the packing. This system often relies on gravity to push the solvent through or medium pressure pumps are commonly used in quick CC. The sample is dissolved in solvent and applied to the front of the column (wet packing), or alternatively adsorbed on a coarse silica gel (dry packing). The solvent elutes the sample through the column, allowing the components to separate according to different absorbance characteristics. Normally, the solvent is non polar and the surface polar, although there is a wide range of packing material including chemically bound phase systems. Bounded phase systems usually utilize partition mechanisms. The solvent is usually changed stepwise, and fractions are collected according to the separation time required. The eluted products are usually monitored by TLC. The technique is not efficient, with relatively large volumes of solvent being used, and particle size is constrained by the need to have a flow of several ml/min. The advantage is that no expensive equipment is required, and the technique can be scaled up to handle sample sizes approaching gram amounts.

In our research, the fraction was first separated using quick column chromatography followed by column chromatography until the repellent activity is lower than in the raw materials.

**Quick Column Chromatography**

The active suspension of Soxhlet extract was evaporated with the rotary evaporator to eliminate the solvents. The solvent free material was weighed by the balance (Precisa XT 220A, accuracy: 0.0001 grams). The solvent system was

determined for column by using TLC. Because the mixture is relative oily, the mixture was applied to the column by first getting the compounds absorbed onto Silica gel. First, using a beaker, the mixture was dissolved in dichloromethane and/or pentane. Then silica gel was added in about double the weight of the compound. The solution was concentrated on the rotary evaporator-assuming nothing in the test mixture is volatile. After the mixture is totally dried on the silica gel, the silica gel pellets can now be added to the top of the column that was already packed before. For packing column, the appropriate ratio of silica gel to the contaminated gel is 30 : 1 (by weight). The appropriate column was chosen from the amount of silica gel contaminated with the test material. The compressed air was used to pack the column which pressed the silica gel in the column to about half of its original height. Cautiously, I checked to make sure that the top of the column is flat. Carefully, silica gel contaminated with the test substances was added to the top of the column. Then, the filter paper and cotton were added on top as a protective layer to prevent the destruction of the silica layer by the solvent. Then, the gradient elution column was started using hexane as an initial solvent system, followed by 100% hexane, 5% ethyl acetate in hexane, 15% ethyl acetate in hexane, 20% ethyl acetate in hexane, 40% ethyl acetate in hexane, 60% ethyl acetate in hexane, 80% ethyl acetate in hexane, 100% ethyl acetate, and 10% methanol in ethyl acetate, respectively (table 4.4). Each fraction approximately 250 ml was collected and concentrated to small volume and then checked by TLC. After all compounds of interest had been eluted from the column, the air were allowed to push all of the remaining solvent out of the column.

**Column Chromatography**

The active fractions were further purified to follow the group of active compounds. In cases of solvent extraction, hexane or dichloromethane or ethyl acetate were used. This resulted in several mainly mixtures of compounds. The composition of the crude extracts was inspected by using TLC (aluminium sheets (1.0554) 25 DC 20×20, Merck). The following part is mainly a repetition from TLC chapter. The visualizations were aided by either observing the TLC under an UV lamp or by dipping with visualized reagents (10%$H_2SO_4$ in ethanol) followed by heating. The TLC was repeatedly improved by changing the solvent systems until a system that

gave the best separation was obtained. The fractions were chromatographed using columns packed with silica-gel (Silica gel, 1.07734 (0.063-0.200 nm), Merck) until the active repellent fractions are no remains. The repellent activity was tested using the same bioassay described before.

**Table 4.4** The eluent of the separation in each species (MeOH=methanol, $CH_2CL_2$= dichloromethane, EtOAc= ethyl acetate).

| Species/Type of bee materials | Eluent | | |
|---|---|---|---|
| | Soxhlet | Quick column chromatography | Column chromatography |
| *A. florea* | Pentane/Acetone/MeOH | - | - |
| *A. andreniformis* | - | - | - |
| *A. mellifera* (THA) | Pentane/Acetone/MeOH | Hexane/EtOAc | Hexane/$CH_2CL_2$/ EtOAc |
| *A. mellifera* (GER) | Pentane/Acetone/MeOH | Hexane/ EtOAc | Hexane/$CH_2CL_2$/ EtOAc |
| *T. apicalis* | Pentane/Acetone/MeOH | Hexane/ EtOAc | Hexane/$CH_2CL_2$/ EtOAc |
| *T. collina* | Pentane/Acetone/MeOHl | Hexane/ EtOAc | Hexane/$CH_2CL_2$/ EtOAc |
| *T. terminata* (THA) | Pentane/Acetone/MeOH | Hexane/ EtOAc | Hexane/$CH_2CL_2$/ EtOAc |
| *T. terminata.* (MAS) | Pentane/Acetone/MeOH | Hexane/ EtOAc | Hexane/$CH_2CL_2$/ EtOAc |

### 4.3.5 Characterization of Compounds by Gas chromatography–mass spectrometry (GC-MS)

**The fractions**

Active fractions were selected according to the biological activity (screening by the bioassay). Active fractions of each species were selected by comparison of the repellent activity to the raw material. We chose fractions which had repellent indices on a higher or similar level. The results of the fractions were given in table 4.5.

**Instruments**

Gas chromatography was performed on an Agilent Technologies 6890N GC equipped with a fused silica capillary column (30 m 0.25 mm, 0.25 μm, Restek: Rxi-5MS, USA). The GC oven was programmed at 100 $^0$C for 1 min then increased 15 $^0$C/min up to 300 $^0$C. Helium was used as carrier gas at a linear velocity of 1 ml s$^{-1}$. The sample volume injected was 1μl in splitless mode. MS analyses were carried out on a Agilent Technologies 5975 mass selective detector, under electron impact ionization (70 eV). Mass scan range was 50 to 600 amu, solvent delay was 3.8 min.

**Compound characterization**

The characterization of the components was based on comparison with mass spectra Wiley 7N library data. A quantification of substances was not performed. In the software routine, each chromatogram produced a "hit list" of library spectra, which was ordered by similarity to the target spectrum according to a computed "match factor". Target spectra were finally matched visually to the best library hit. The variety of modifications and calculations of match factors could further improve the reliability. Consequently, our comparison to mass spectra from the Wiley database characterizes at least the chemical nature (class) of the GC peaks.

**Table 4.5.** The active fractions identified in this study (√ indicate the fraction which the GC-MS identification was proceeded).

| Eluant | Species | | | | | |
|---|---|---|---|---|---|---|
| | *A. mellifera* (THA) | *A. mellifera* (GER) | *T. apicalis* | *T. collina* | *T. terminata* (MAS) | *A. florea* |
| Pentane | - | - | - | - | - | √ |
| 1st Quick column chromatography | | | | | | |
| Hexane | - | - | - | - | √ | - |
| 5% Hexane in Ethyl acetate | - | √ | - | - | - | - |
| 15% Hexane in Ethyl acetate | - | - | - | - | - | - |
| 20% Hexane in Ethyl acetate | √ | - | - | √ | - | - |
| 40% Hexane in Ethyl acetate | √ | √ | √ | - | √ | - |
| 80% Hexane in Ethyl acetate | - | - | - | - | - | - |
| Ethyl acetate | - | - | - | - | - | - |
| 10% Methanol in Ethyl acetate | - | - | - | - | - | - |
| 2nd column chromatography | | | | | | |
| Fraction 1 | √ | - | - | - | - | - |
| Fraction 2 | - | - | - | - | - | - |
| Fraction 3 | √ | - | √ | - | - | - |
| Fraction 4 | √ | √ | - | - | - | - |
| Fraction 5 | - | - | - | - | - | - |
| Fraction 6 | - | - | - | - | - | - |
| Fraction 7 | - | - | - | - | - | - |
| Fraction 8 | - | - | - | - | - | - |
| Fraction 9 | - | - | - | - | - | - |
| Fraction 10 | √ | - | - | - | - | - |
| Fraction 11 | - | - | - | - | - | - |
| Fraction 12 | - | - | - | - | - | - |
| Fraction 13 | - | - | - | - | - | - |

## 4.4 Results

### 4.4.1 Effect of material used by bees at the nest on *O. smaragdina*

**The biological activity of raw material**

According to the repellent index (1= 100% repellent activity; 0.5= no repellent activity, no attraction, 0.00= 100% attraction), the activity of raw materials showed a significant variability. The repellent indices range from 0.67 ±0.08 in the entrance tubes of *T. collina* to 0.93 ±0.11 in sticky band materials from *A. andreniformis* nests (fig. 4.37, table 4.7 ).

Under the headline of the same species, the results are given for two geographic regions where we performed the experiments on, in Thailand and Tenom, Malaysia. The tested samples were collected from the same or different geographic region with the experimental ants. Some samples were tested in one geographic region only.

*A. florea*

The samples collected from Thailand from 40 colonies were tested in Thailand. Because the sticky ring appear only intensively on the nesting branches connected to the tree stem, we used one sample per colony. The mean of repellent indices was 0.92 ± 0.08 with a range from 0 .74 to 1.00 (fig. 4.17).

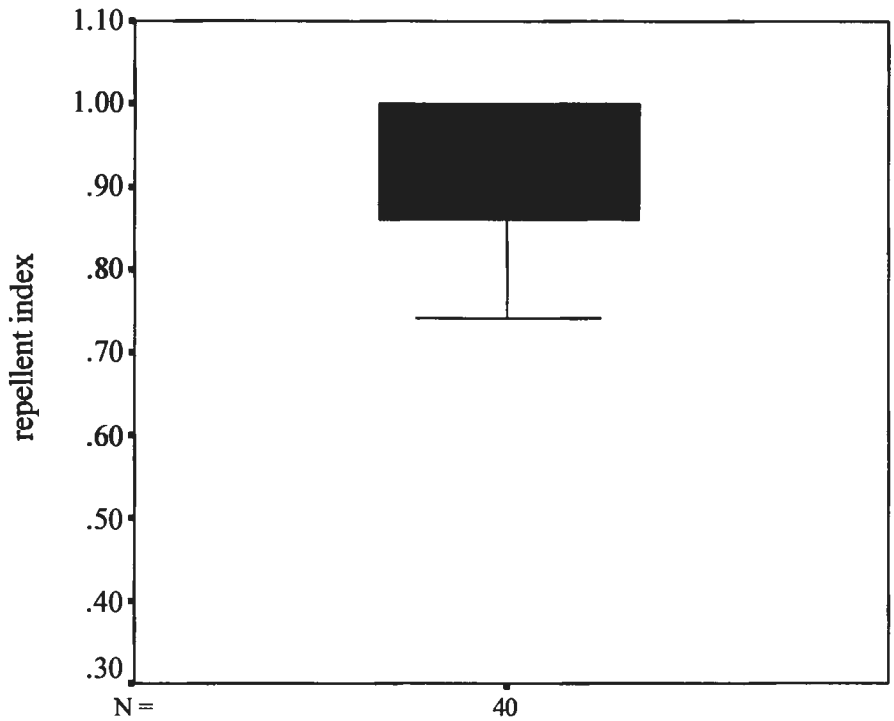


**Figure 4.17** The repellent indices of sticky band raw materials of 40 colonies of *A. florea.* Samples collected in Thailand. N = number of tests. The box (interquartile range, IQR) contains 50% of the values. The horizontal line represents the median. The vertical whiskers show the highest and lowest values.

*A. andreniformis*

All samples, in total 10 colonies (5 colonies from Thailand and 5 colonies from Tenom, Malaysia), were tested in Thailand. The mean of repellent indices was 0.93 ± 0.11 which range from 0.70 to 1.00 (fig. 4.18).

Additional experiments were done also in Tenom, Malaysia using the samples collected from Tenom, Malaysia. The mean of repellent indices was 0.88 ± 0.11, ranging from 0.73 to 1.00 (fig. 4.19).

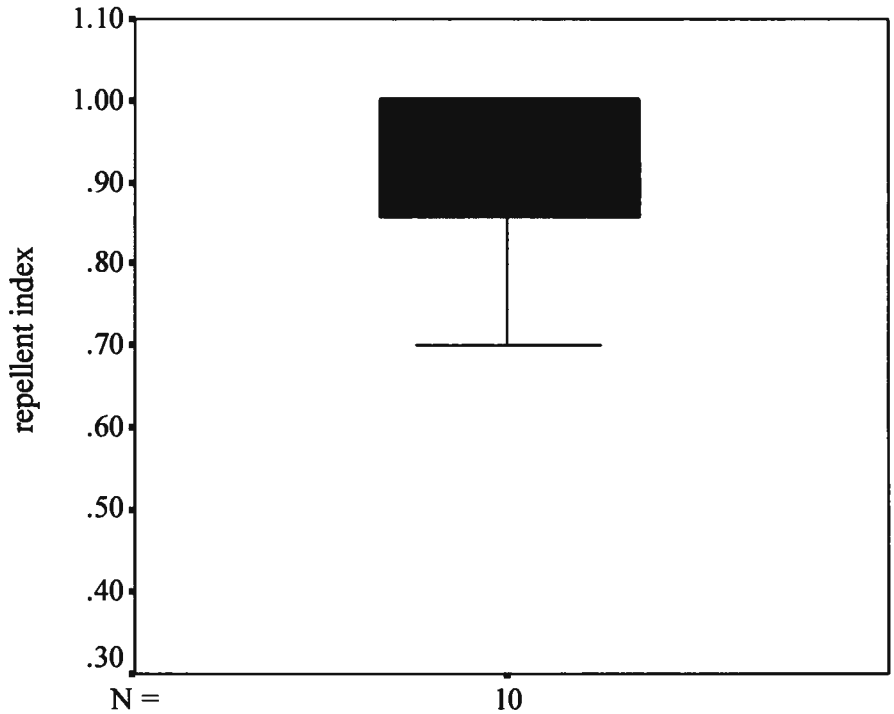


**Figure 4.18** The repellent indices of sticky band raw material from 10 colonies of *A. andreniformis*. Samples collected from Tenom, Malaysia and Thailand. N = number of tests (tested in Thailand). See Figure 4.17 for explanation of boxplots.

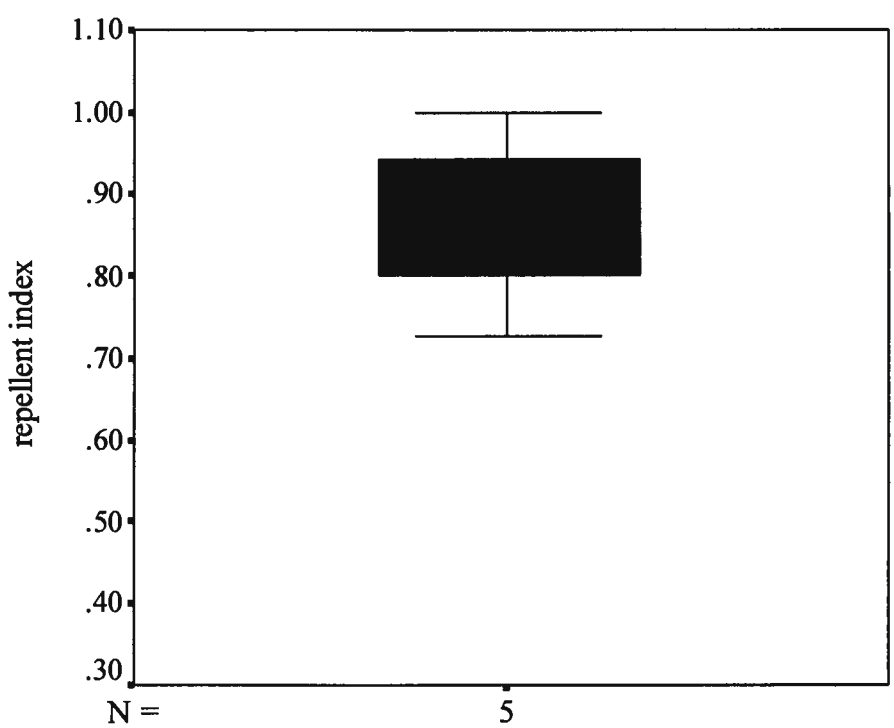


**Figure 4.19** The repellent indices of sticky band raw material from 5 colonies of *A. andreniformis*. Samples collected from Tenom, Malaysia. N = number of tests (tested in Tenom). See Figure 4.17 for explanation of boxplots.

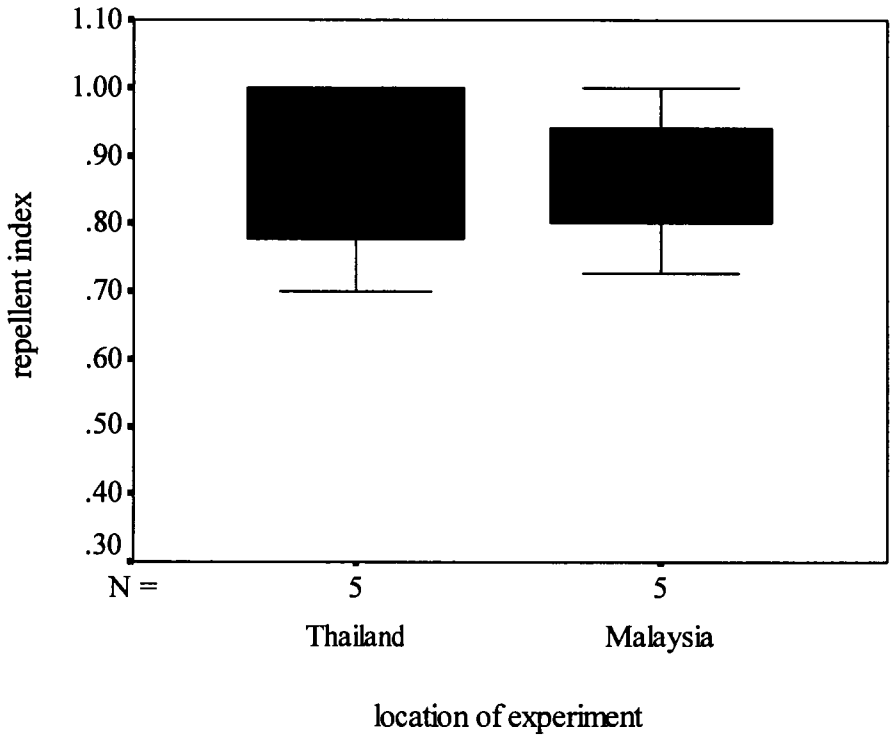


**Figure 4.20** The repellent indices of sticky band raw material of *A. andreniformis*. Experiments were conducted in Thailand and Tenom, Malaysia. N = number of tests. See Figure 4.17 for explanation of boxplots.

The box plots showed a similar distribution of repellent indices (fig. 4.20). Statistical analysis (Kruskal-Wallis) confirmed that weaver ants from both geographic regions showed similar reactions to sticky bands of *A. andreniformis* (Wilcoxon rank test, $p < 0.292$).

### *A. mellifera* (THA)

Propolis samples from 12 colonies, collected in Thailand, were tested in Thailand. The mean of repellent indices was 0.91 ± 0.06 ranging from 0.84 ± 0.05 in colony no. 10 to 0.95 ± 0.07 in colony no. 6. (fig. 4.21). Within these samples , the repellent indices were not significantly different ( Kruskal Wallis test $p < 0.353$, table 4.6 ).

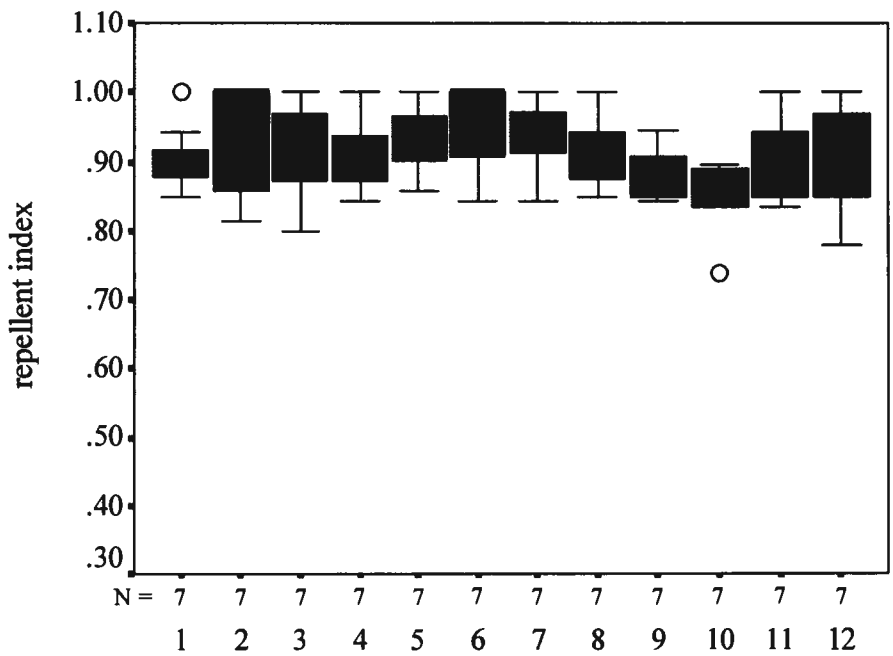


**Figure 4.21** The repellent indices of raw material from 12 colonies of *A. mellifera.* Samples collected in Thailand. N = number of tests. The box (IQR = Q3-Q1) contains 50% of the values. The horizontal line represents the median, the vertical whiskers show the highest and lowest values excluding outliers. O (the outlier) represents the value which more than Q3+1.5IQR or less than Q1-1.5IQR. IQR =Interquatile range, Q1= 25$^{th}$ percentile, Q3= 75$^{th}$ percentile.

### *A. mellifera* (GER)

Samples, collected from 12 colonies in Oberursel, Frankfurt am Main, Germany, were tested in Thailand. The mean of repellent indices was 0.77 ± 0.11 ranging from 0.67 ± 0.06 in colony number 9 to 0.90 ± 0.03 in colony number 5 (fig. 4.22). The repellent indices of these samples from Oberursel were significantly different (Kruskal Wallis-test, $p < 0.0005$ table: 4.6)

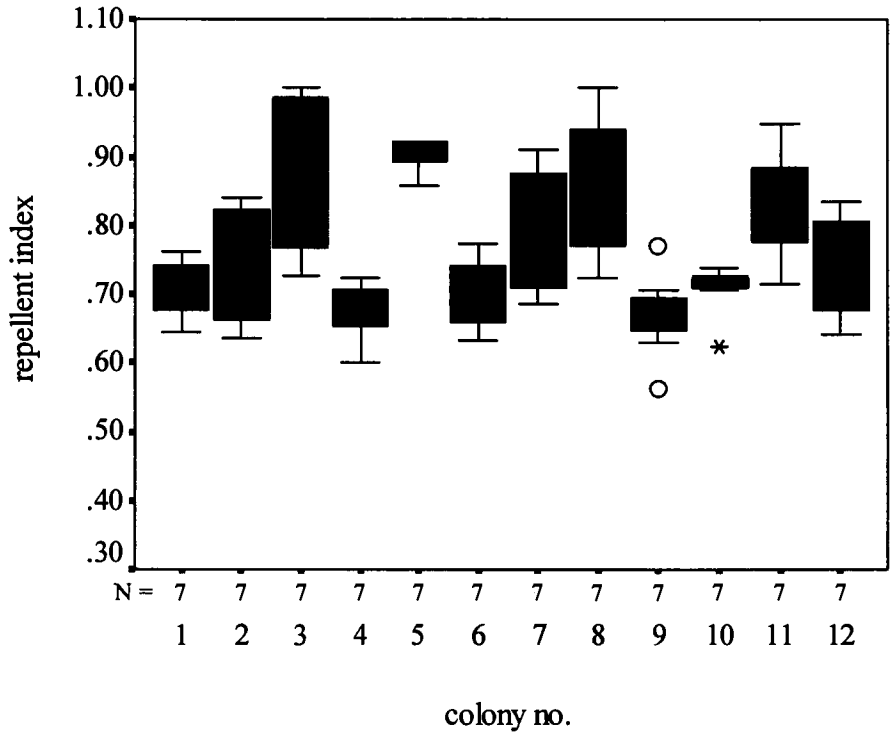


**Figure 4.22** The repellent indices of raw materials from 12 colonies of *A. mellifera.* Samples collected in Germany. N=number of tests. The box (IQR = Q3-Q1) contains 50% of the values. The horizontal line represents the median, the vertical whiskers show the highest and lowest values excluding outliers. O (the outlier) represents the value which more than Q3+1.5IQR or less than Q1-1.5IQR. * (the extreme) represents the value which more then Q3+3IQR or Q1-3QIR. IQR =Interquatile range, Q1= 25$^{th}$ percentile, Q3= 75$^{th}$ percentile.

When we compared the repellent indices from the samples collected from Thailand and Germany (fig. 4.23), the result showed a significant difference (Wilcoxon rank test, $p< 0.0005$)

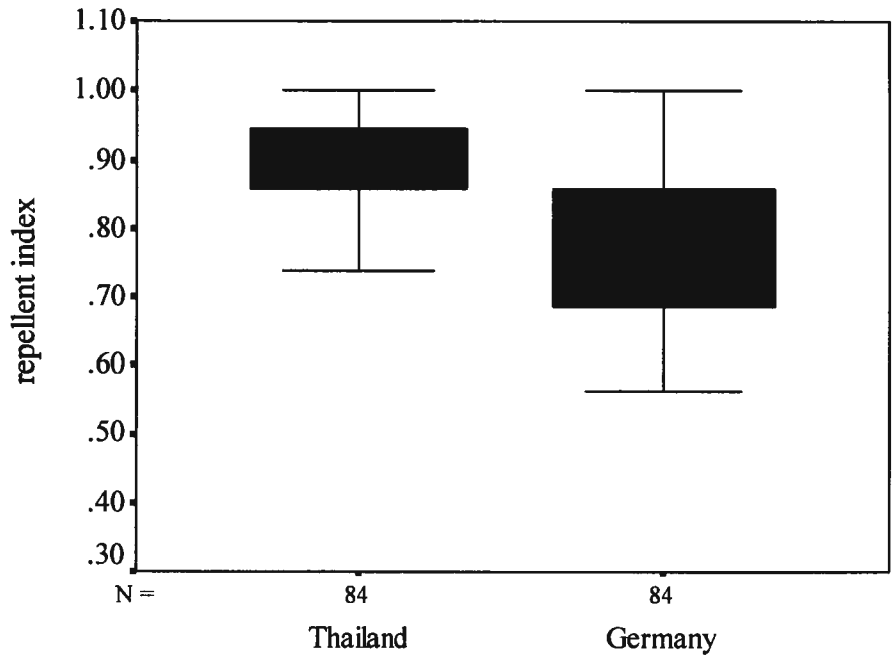


**Figure 4.23** The repellent indices of raw material of *A. mellifera.* Samples collected in Thailand (12 colonies) and Germany (12 colonies). N=number of tests. See Figure 4.22 for explanation of boxplots.

### *T. apicalis*

The samples of 14 colonies, collected from Thailand, were tested in Thailand. The mean of repellent indices was 0.82 ± 0.09. The repellent indices had a wide range from 0.71 ± 0.05 in colony no. 12 to 0.95 ± 0.06 in colony no. 2. (fig.4.24, 4.25). When we had a closer look within each colony we found that the repellent activity fluctuated. The repellent indices show significant differences between colonies ($p <$ 0.0005, Kruskal Wallis-tested, table 4.6).

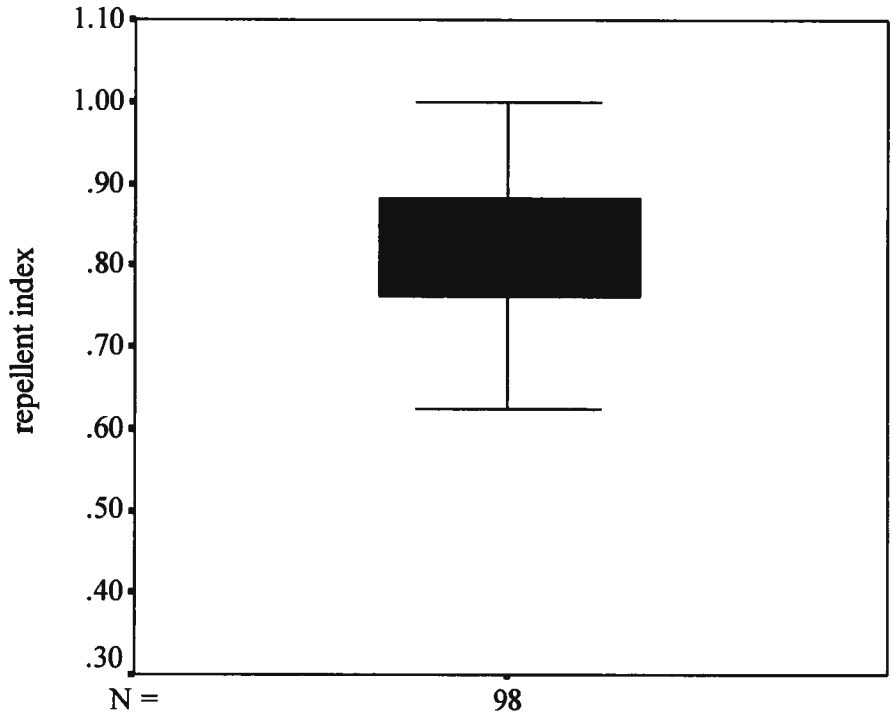


**Figure 4.24** The repellent indices of raw materials from 14 colonies of *T. apicalis*. Experiments conducted in Thailand. N= number of tests. See Figure 4.22 for explanation of boxplots.

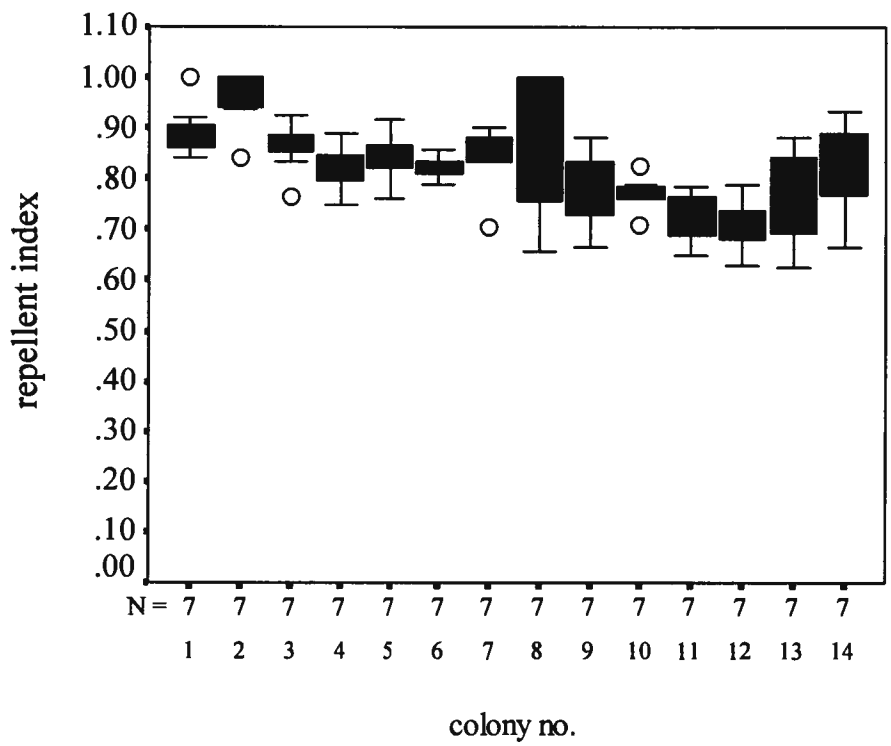


**Figure 4.25** The repellent indices of raw material from 14 colonies of *T. apicalis*. Samples were collected in Thailand. N=number of test. See Figure 4.22 for explanation of boxplots.

***T. collina***

The 14 colonies of samples, collected from Thailand, were tested in Thailand. The mean of repellent indices was 0.63 ± 0.09 (fig. 4.26) which widely range from 0.60 ± 0.03 in colony no. 10 to 0.81 ± 0.14 in colony no. 1 (fig. 4.27). The repellent indices were significantly different between samples collected from different colonies ($p$<0.028, Kruskal Wallis-tested, table 4.6 ).

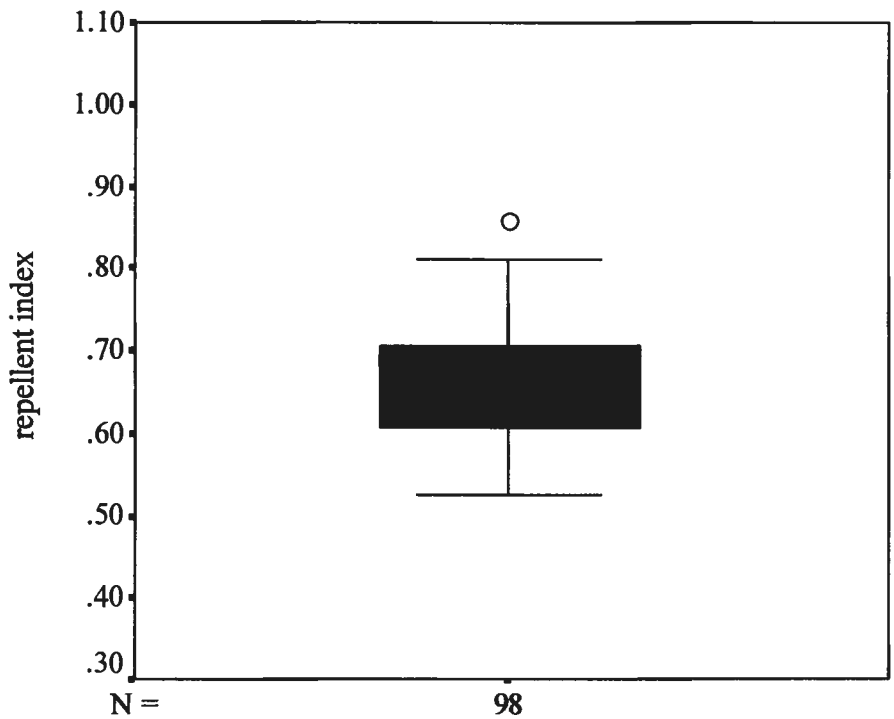


**Figure 4.26** The repellent indices of raw materials from 14 colonies of *T. collina*. N = number of test. See Figure 4.22 for explanation of boxplots.

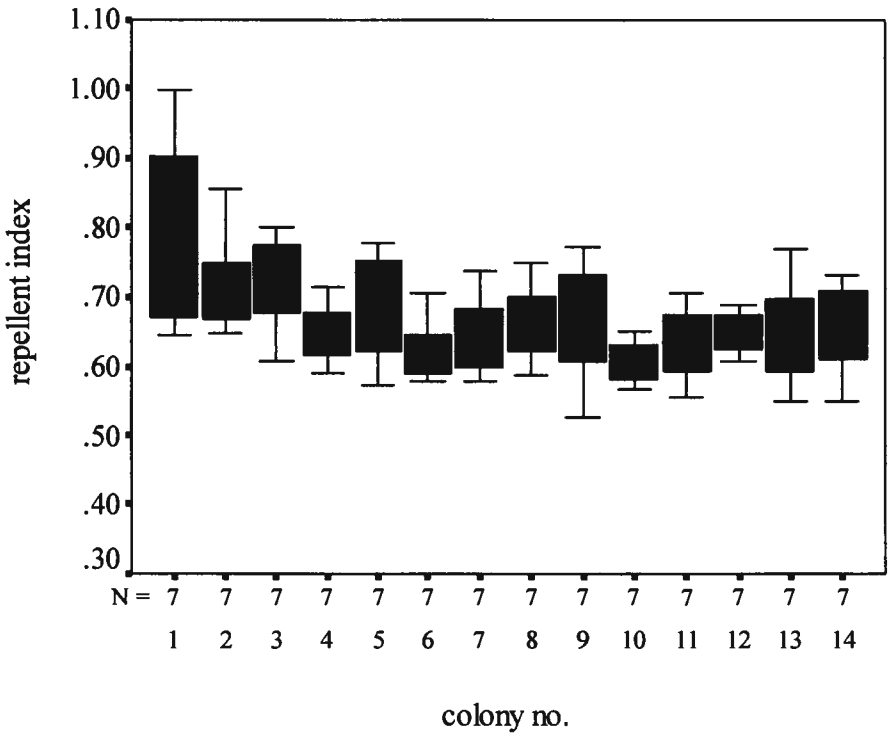


**Figure 4.27** The repellent indices of raw material from 14 colonies of *T. collina.* Samples collected in Thailand. N= number of tests. See Figure 4.22 for explanation of boxplots.

### *T. terminata* (THA)

The samples of 13 colonies, collected from Thailand, were tested in Thailand. The mean of repellent indices was 0.86 ± 0.09 (fig. 4.28 ) The figure shows that the repellent indices have broad range from 0.69 ±0.02 in colony no. 11 to 0.98 ± 0.03 in colony no. 12 (fig. 4.29) because of the bioactivities were vary each colony. The comparisons between samples collected from different locations were significantly different ($p < 0.0005$, Kruskal Wallis-tested, table 4.6).

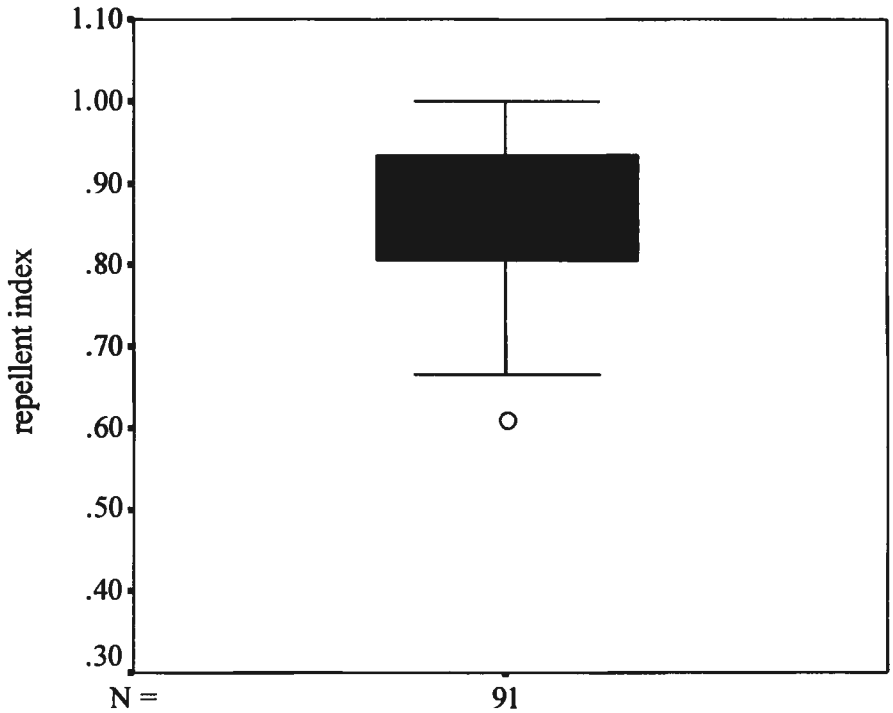


**Figure 4.28** The repellent indices of raw material from 13 colonies of *T. terminata* (test in Thailand). N=number of tests. See Figure 4.22 for explanation of boxplots.

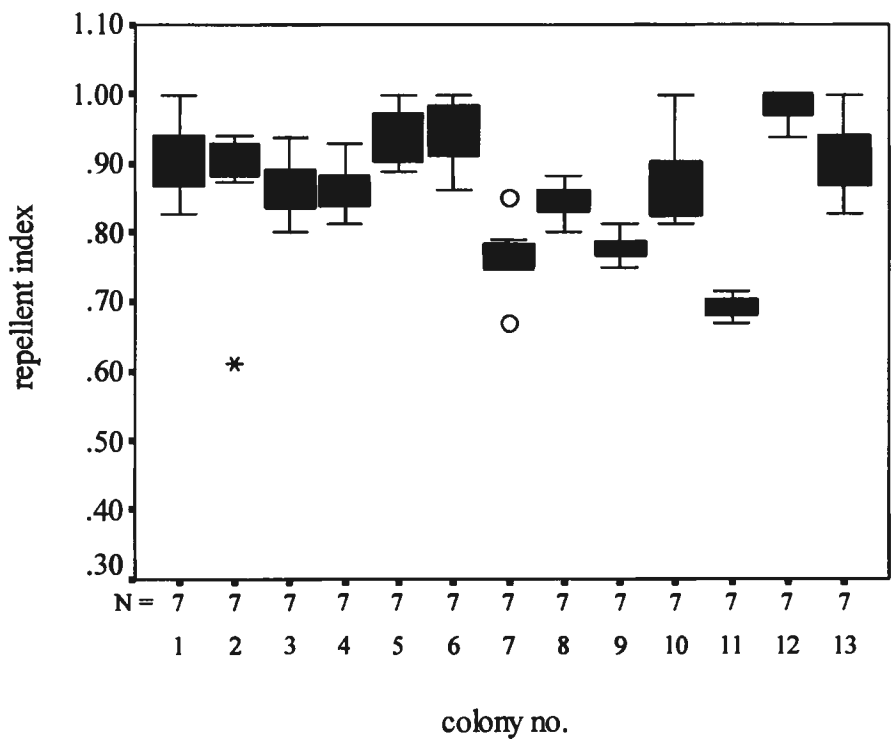


**Figure 4.29** The repellent indices of raw material from 13 colonies of *T. terminata* (test in Thailand). Samples collected in Thailand. N= number of tests. See Figure 4.22 for explanation of boxplots.

***T. terminata* (MAS)**

The samples of 8 colonies, collected from Tenom, Malaysia, were tested in Thailand. The mean of repellent indices was 0.71 ± 0.09. The repellent indices show wide range from 0.63 ± 0.03 in colony no. 2 to 0.81 ± 0.18 in colony no. 4. (fig. 4.30). Explanatorily, the repellent indices between colonies were significantly different ($p$<0.005, Kruskal Wallis-tested, table 4.6 ).

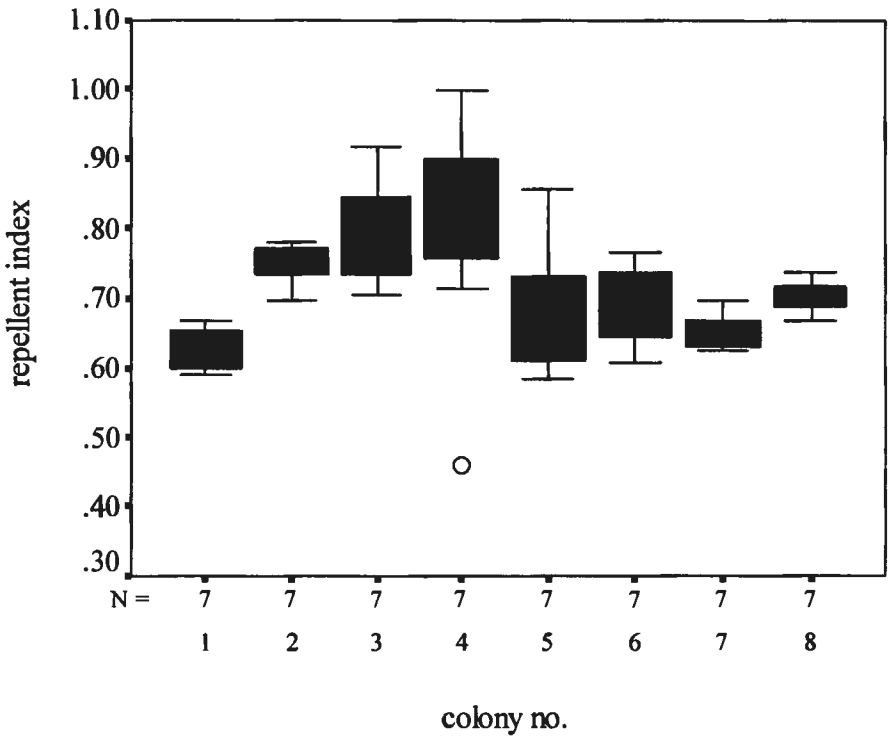


**Figure 4.30** The repellent indices of raw material of 8 colonies of *T. terminata* (test in Thailand). Samples collected in Tenom, Malaysia. N=number of tests. See Figure 4.22 for explanation of boxplots.

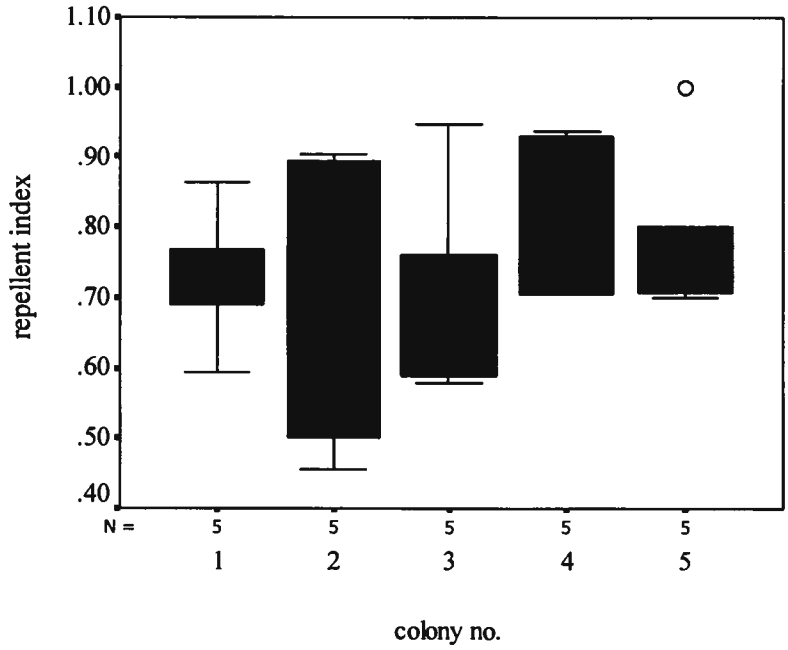


**Figure 4.31** The repellent indices of raw material from 5 colonies of *T. terminata* (test in Tenom, Malaysia). Samples collected in Tenom, Malaysia. N=number of tests. See Figure 4.22 for explanation of boxplots.

The experiments were also done with weaver ants in Tenom, Malaysia. The mean of repellent indices was 0.72 ± 0.11. The indices range from 0.68 ± 0.21 in colony no. 2 to 0.81 ± 0.12 in colony no. 4. (fig. 4.31). The repellent indices were not significantly different within the same geographic region ($p$ <0.629, Kruskal Wallis-tested, table 4.6 ). The repellent indices from the samples collected from Thailand and Tenom, Malaysia also show no significant difference (Wilcoxon rank test, $p$<0.307).

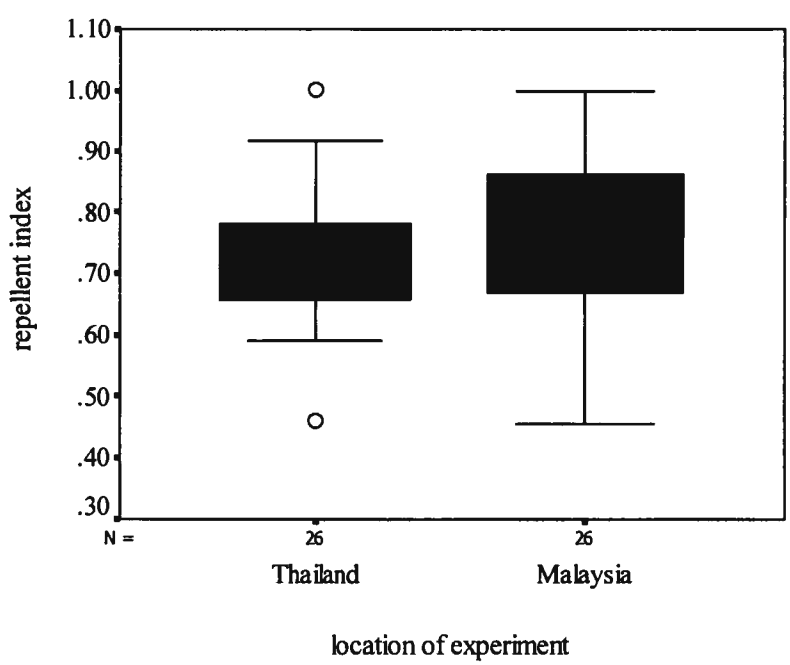


**Figure 4.32** The repellent indices of raw material of *T. terminata*. Experiments were conducted in Thailand (8 colonies) and Tenom, Malaysia (5 colonies). N=number of tests. See Figure 4.22 for explanation of boxplots.

***T. melanocephala***

The samples of 3 colonies, collected from Tenom, Malaysia, were tested in Tenom, Malaysia. The mean of repellent indices was 0.8937 ± 0.1124 (fig. 4.33) which range from 0.82 ± 0.13 in colony no. 3 to 0.99 ± 0.02 in colony no. 1 (fig. 4.34). The repellent indices within species were significantly different ($p$<0.028, Kruskal Wallis-tested, table 4.6).

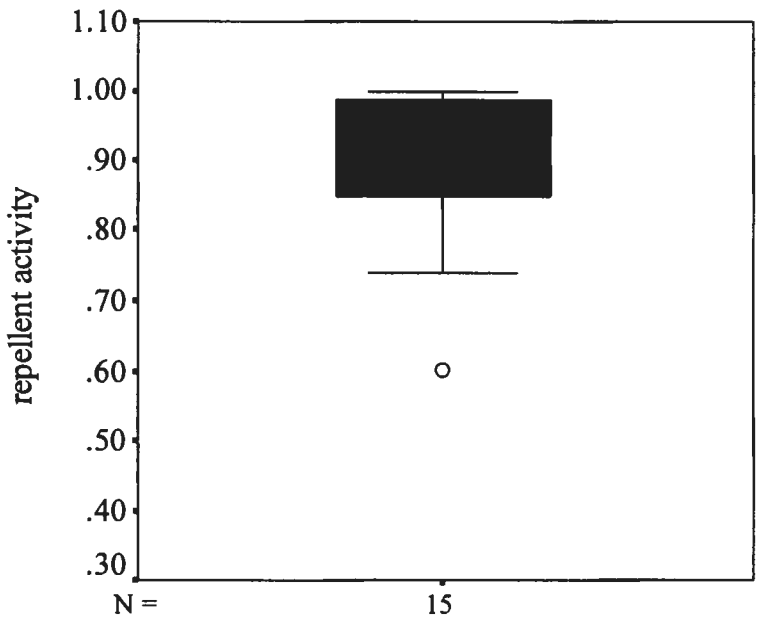


**Figure 4.33** The repellent indices of raw material from 3 colonies of *T. melanocephala*. Samples collected in Tenom, Malaysia. N=number of tests. See Figure 4.22 for explanation of boxplots.

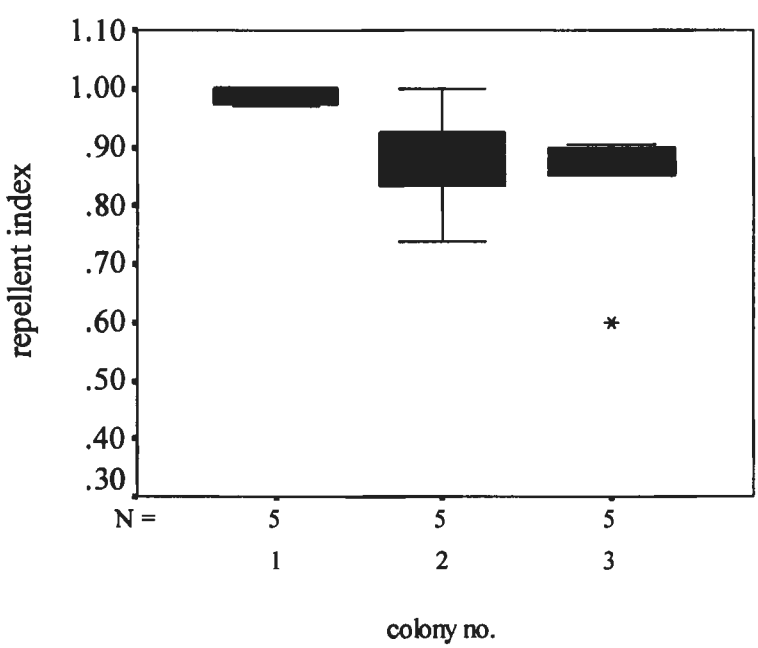


**Figure 4.34** The repellent indices of raw material from 3 colonies of *T. melanocephala*. Samples collected in Tenom, Malaysia. N=number of tests. See Figure 4.22 for explanation of boxplots.

***T. laeviceps***

The sample collected from Tenom, Malaysia was tested in Tenom, Malaysia. The mean of repellent indices was 0.92 ± 0.11 which range from 0.74 to 1.00 (fig. 4.35).

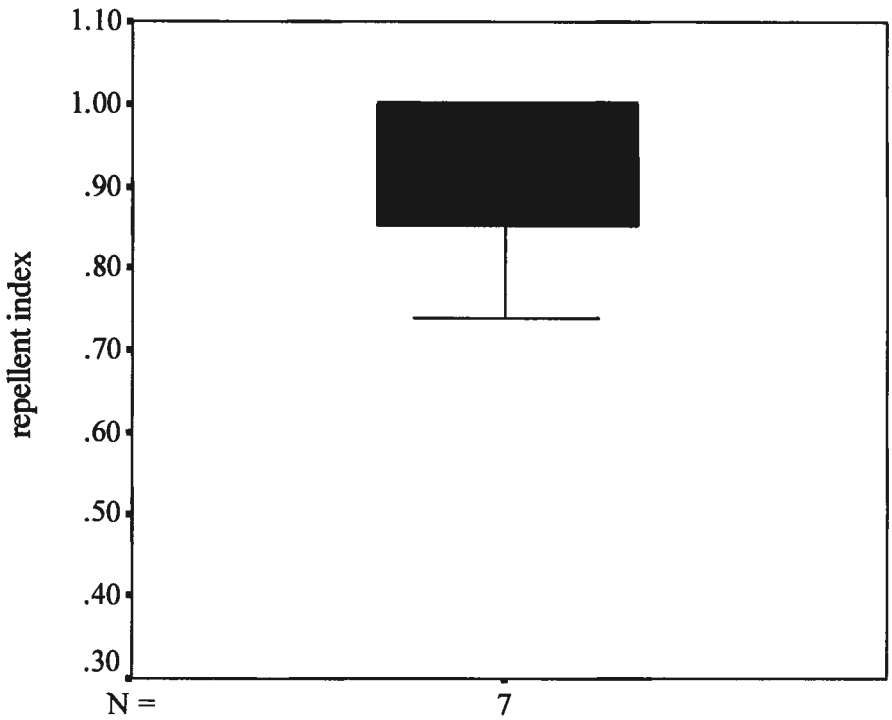


**Figure 4.35** The repellent indices of raw material of *T. laeviceps*. Sample collected in Tenom, Malaysia. N= number of tests. See Figure 4.22 for explanation of boxplots.

### *T. minor*

The sample collected from Tenom, Malaysia was tested in Tenom, Malaysia. The mean of repellent indices was 0.78 ± 0.12 which range from 0.67 to 1.00 (fig. 4.36).

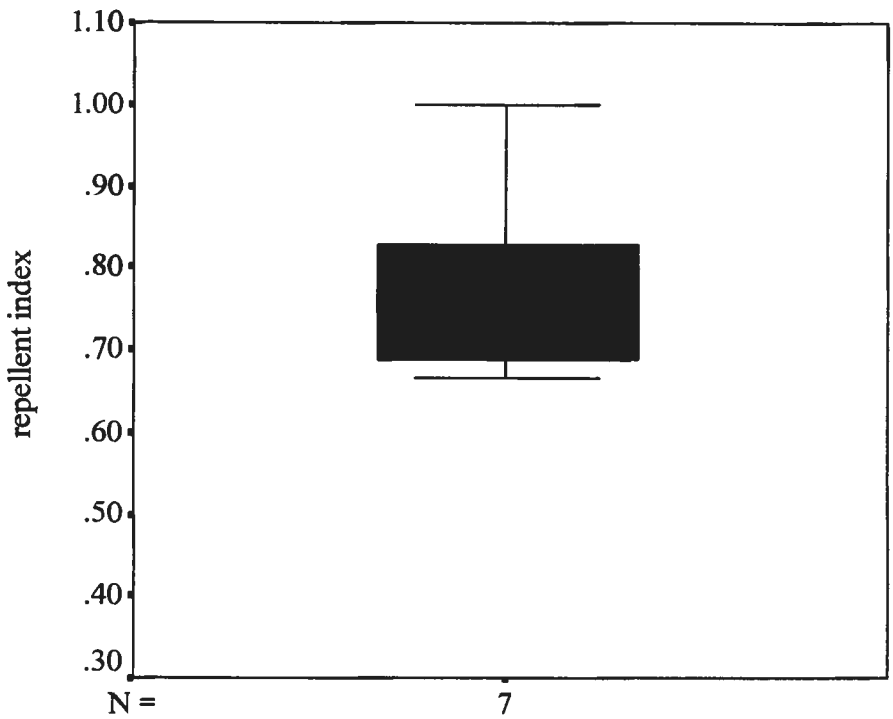


**Figure 4.36** The repellent indices of raw material from *T. minor*. Sample collected from Tenom, Malaysia. N=number of tests. See Figure 4.22 for explanation of boxplots.

**Table 4.6** The comparison of repellent indices within species and geographic region (Thailand, Malaysia) (Kruskal Wallis-tested). ** = significant difference, $p \leq 0.01$.

| Species | no of colony | Location of experiment | *p* (Kruskal Wallis-tested) |
|---|---|---|---|
| *A. florea* | 40 | Thailand | - |
| *A. andreniformis* | 10 | Thailand | - |
| *A. mellifera* (THA) | 12 | Thailand | < 0.0005** |
| *T. apicalis* | 14 | Thailand | < 0.0005** |
| *T. terminata* (THA) | 13 | Thailand | < 0.0005** |
| *T. terminata* (MAS) | 8 | Thailand | <0.0005** |
| *A. andreniformis* | 10 | Malaysia | - |
| *T. terminata* (MAS) | 5 | Malaysia | < 0.2190 |
| *T. melanocephala* | 3 | Malaysia | < 0.0280* |
| *T. laeviceps* | 1 | Malaysia | - |
| *T. minor* | 1 | Malaysia | - |

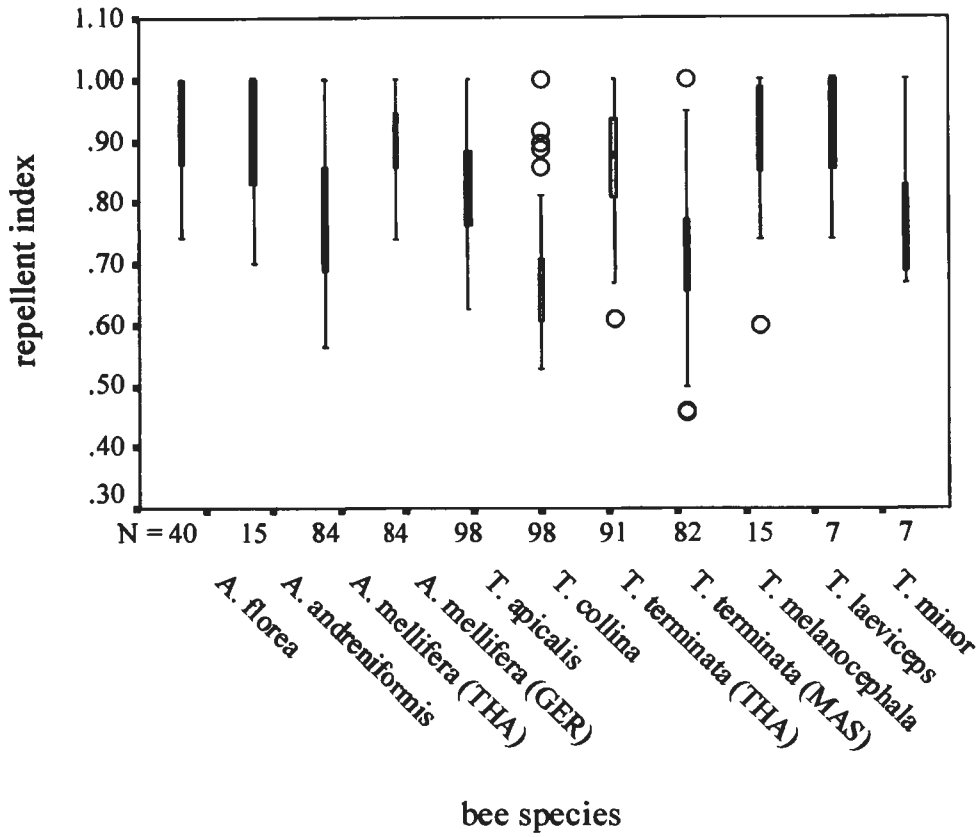


**Figure 4.37** The repellent indices of raw material. Samples from 183 colonies of 9 species of honeybees and stingless bees. N = number of tests. See Figure 4.22 for explanation of boxplots.

**Tests of fractions on repellent activity**

**A. The extract from Soxhlet**

In all pentane extracts the repellent index (R) showed a high activity in every tested sample. The values of R range from 0.74 ± 0.07 in fractions from *T. collina* and *T. terminata* to 1.00 ± 0.00 in the fraction from *A. florea* (table 4.7, fig 4.38).

The repellent indices in acetone and methanol extracts were approximately 0.5, which indicated no or little activity. The values of R in acetone extracts ranged from 0.49 ± 0.05 in extracts from *T. terminata*, samples collected from Tenom, Malaysia, to 0.62 ± 0.08 in extracts from *T. melanocephala* , samples collected from Tenom (table 4.7, fig 4.39). In methanol extract, R ranged from 0.47 ± 0.04 in extracts of *T. terminata* samples from Tenom, to 0.50 ± 0.02 in extracts from *A. mellifera* samples from Thailand (table 4.7, fig. 4.40). The comparison of repellent indices

between pentane – acetone and pentane – methanol extracts revealed significant differences in all species ( $p< 0.001$ and $p< 0.001$, respectively, Kruskal Wallis-test).

In pentane extracts the repellent activity was relatively higher compared to the raw material indicated by R (table 4.8). The repellent efficacy was also higher in the pentane fractions compared to the fractions from acetone and methanol extracts (table 4.7, figure 4.41). Most pigments were dissolved in the acetone fractions. A high repellent activity was found in the pentane extract from *A. florea* with R = 1.00±0.00. Comparing geographic regions, the repellent activity was similar in tests conducted in Thailand and Tenom. The acetone extracts from *T. melanocephala* sample showed a slight repellent activity (R= 0.62 ± 0.08). For samples from *A. andreniformis* nests we could not get the fractions because the amount of samples was too low.

**Table 4.7** The repellent indices of bee material. The Location of experiments were conducted at Thailand (THA) and Malaysia (MAS).

| Species/bee material | Location of experiment | Repellent index (mean ± SD) | | | |
|---|---|---|---|---|---|
| | | Raw material | Pentane | Acetone | Methanol |
| *A. florea* | THA | 0.9247±0.0827 | 1.0000±0.0000 | 0.5221±0.0354 | 0.5007±0.0218 |
| *A. andreniformis*(THA) | THA | 0.9334±0.1133 | - | - | - |
| *A. andreniformis*(MAS) | MAS | 0.8783± 0.1115 | - | - | - |
| *A. mellifera* (THA) | THA | 0.9098± 0.0647 | 0.9658± 0.0430 | 0.5538± 0.0520 | 0.5024± 0.0211 |
| *A. mellifera* (GER) | THA | 0.7692±0.1067 | 0.8807±0.0398 | 0.5502±0.0454 | 0.4774±0.0321 |
| *T. apicalis* | THA | 0.8191± 0.0919 | 0.8895± 0.0819 | 0.5316± 0.0492 | 0.4993± 0.0251 |
| *T. collina* | THA | 0.6695±0.0864 | 0.7453±0.0685 | 0.5168±0.0750 | 0.4897±0.0169 |
| *T. terminata* (THA) | THA | 0.8636± 0.0933 | 0.7453± 0.0685 | 0.5053± 0.0227 | 0.4996± 0.0192 |
| *T. terminata* (MAS) | THA | 0.7137±0.0976 | 0.6780±0.0274 | 0.4971±0.0467 | 0.4685±0.0378 |
| *T. terminata* (MAS) | MAS | 0.7228± 0.1128 | 0.8966± 0.0633 | 0.5460± 0.0271 | - |
| *T. melanocephala* (MAS) | MAS | 0.8937± 0.1124 | 0.8872± 0.0722 | 0.6193± 0.0799 | - |
| *T. laeviceps* (MAS) | MAS | 0.9206± 0.1103 | 0.9781± 0.0275 | 0.5868± 0.0258 | - |
| *T. minor* (MAS) | MAS | 0.7820± 0.1184 | 0.9252± 0.0466 | 0.5482± 0.0222 | - |

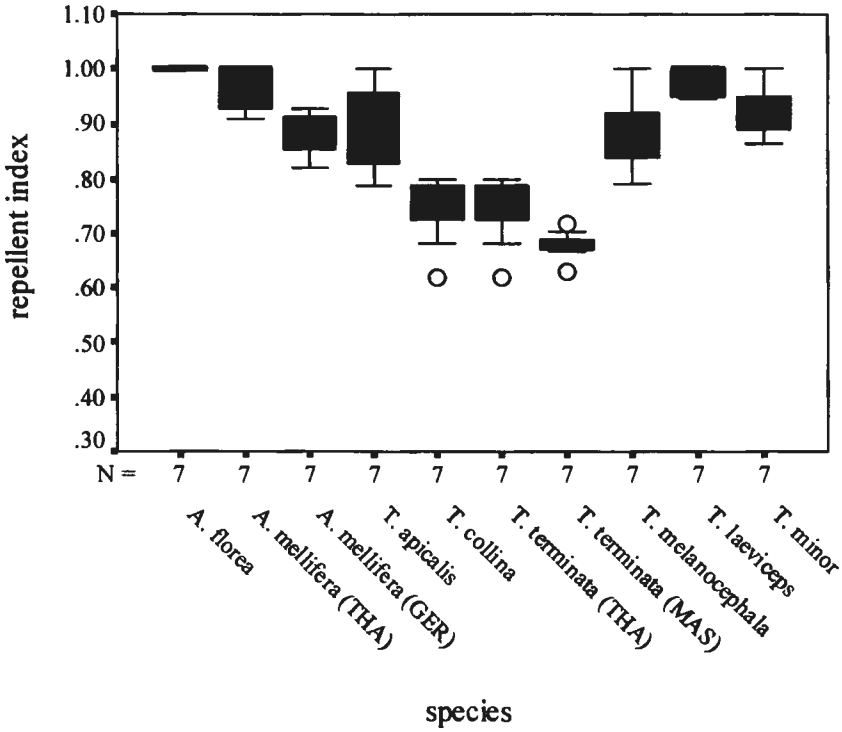


**Figure 4.38** The repellent indices of pentane extracts. Samples were collected from 9 species of social bees. N=number of tests. See Figure 4.22 for explanation of boxplots.

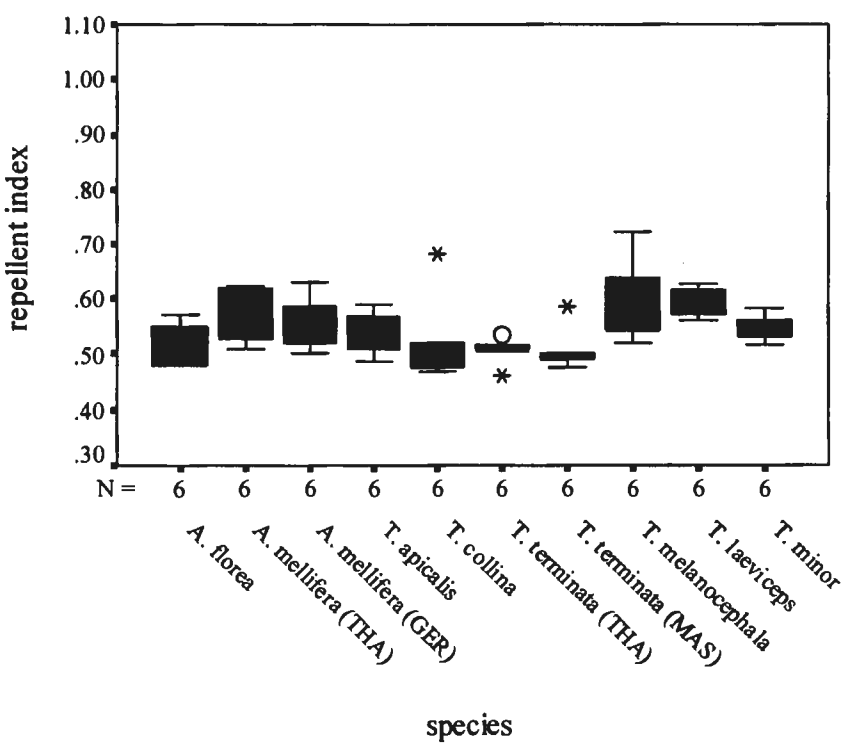


**Figure 4.39** The repellent indices of acetone extracts. Samples were collected from 9 species of social bees. N=number of tests. See Figure 4.22 for explanation of boxplots.

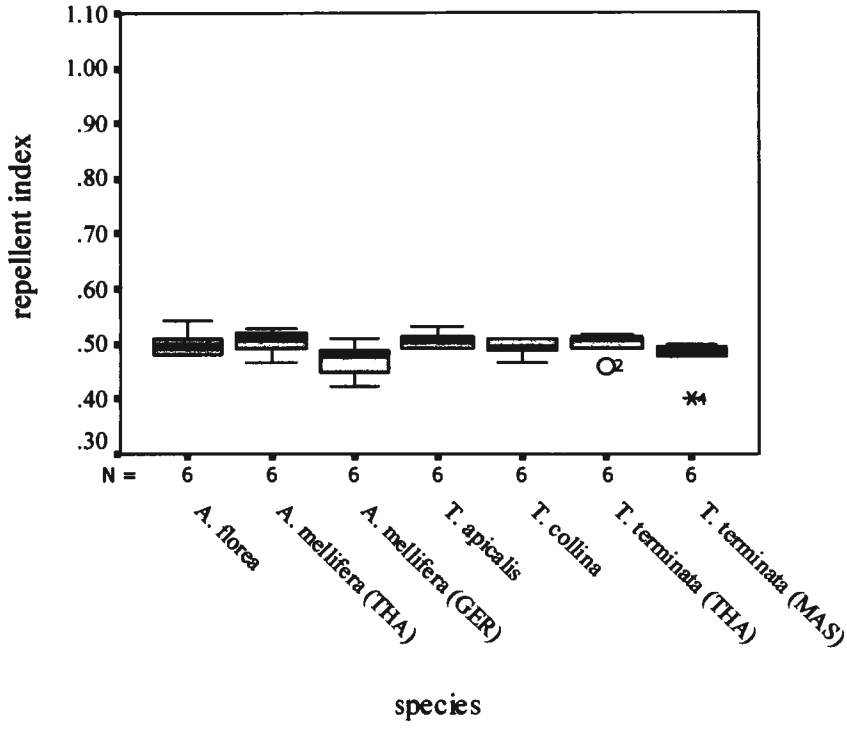


**Figure 4.40** The repellent indices of methanol extracts. The experiments were done in Thailand. Samples were collected from 7 species of social bees. N=number of tests. See Figure 4.22 for explanation of boxplots.

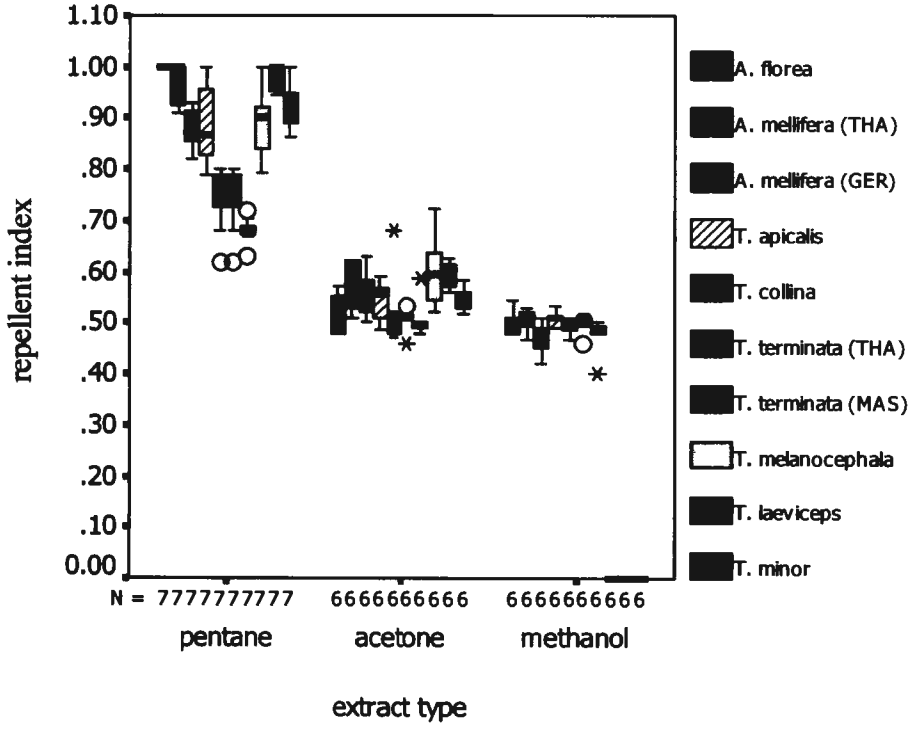


**Figure 4.41** The repellent indices of samples collected from nests of 10 species of bees. The results show in different extract types from soxhlet extraction. N=number of tests. See Figure 4.22 for explanation of boxplots.

**Table 4.8** The comparison of repellent indices from different extracts within species (Kruskal Wallis-tested). ** = significant difference, $p \leq 0.01$.

| species | pair of test | |
|---|---|---|
| | **pentane -acetone** | **pentane-methanol** |
| *A. florea* | $p < 0.001$** | $p < 0.001$** |
| *A. andreniformis* | - | - |
| *A. mellifera* (THA) | $p < 0.002$** | $p < 0.001$** |
| *A. mellifera* (GER) | $p < 0.003$** | $p < 0.000$** |
| *T. apicalis* | $p < 0.003$** | $p < 0.001$** |
| *T. collina* | $p < 0.005$** | $p < 0.002$** |
| *T. terminata* (THA) | $p < 0.003$** | $p < 0.002$** |
| *T. terminata* (MAS) | $p < 0.003$** | $p < 0.001$** |
| *T. melanocephala* | $p < 0.003$** | $p < 0.000$** |
| *T. laeviceps* | $p < 0.002$** | $p < 0.000$** |
| *T. minor* | $p < 0.003$** | $p < 0.000$** |

**B. Chromatographed fractions**

After fragmentation of the pentane extract into fractions from different species and geographic regions, various fractions showed repellent effects. We demonstrated the results in the ranking order of the chromatographic separation. Firstly, fractions separated by quick column chromatography and later by column chromatography (table. 4.9).

***A. mellifera* (THA)**

The repellent activity of the pentane extract of propolis from *A. mellifera* collected from Thailand apiary was found in the fractions eluted with 20% and 40% ethyl acetate in hexane (fig. 4.42). In the TLC chromatogram, too, there was an overlapping range of active compounds in these two fractions. Therefore, it seems that the active compounds of the two fractions belonged to the same group but were only partly eluted in 20% ethyl acetate in hexane whereas the remaining active compounds were eluted with 40% ethyl acetate in hexane. Accordingly, the 20% ethyl acetate in hexane fractions showed a lower ($R=0.76 \pm 0.04$) and 40% ethyl acetate/hexane showed a higher activity ($R = 0.95 \pm 0.03$).

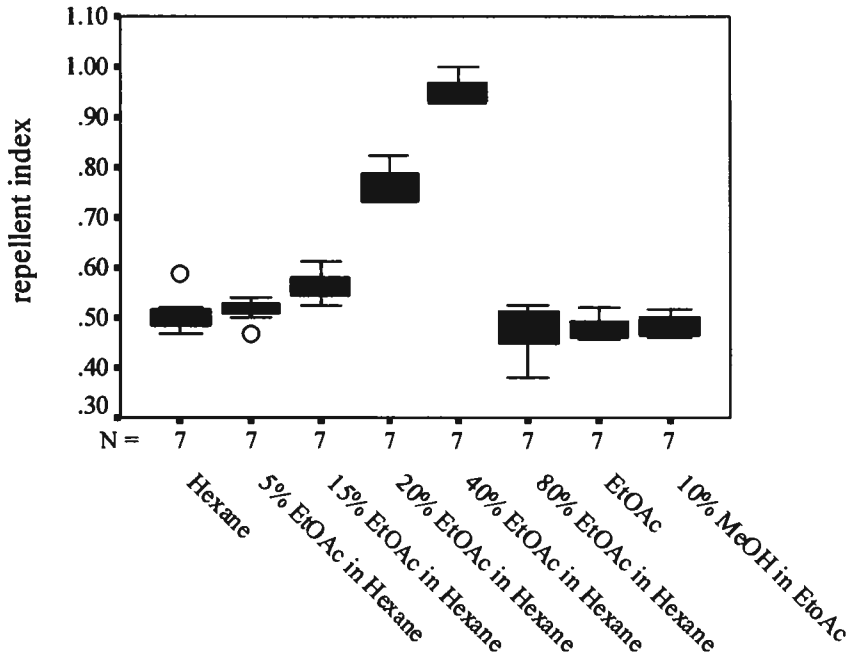


**Figure 4.42** The repellent indices of the pentane extract of *A. mellifera* collected from Thailand after quick column chromatography (solvent system: hexane/ethyl acetate). The last fraction was rinsed up with 10% methanol in ethyl acetate. N=number of tests. See Figure 4.22 for explanation of boxplots.

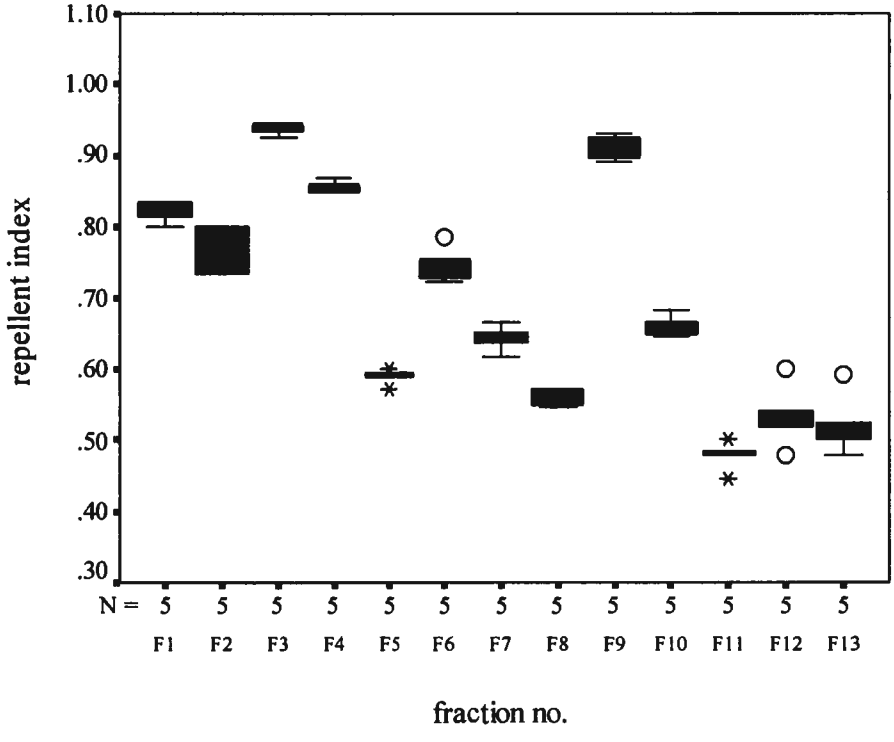


**Figure 4.43** The repellent indices of the second chromatographed fraction of pentane extract (1st chromatographed fractions were the mixture of 20% and 40% ethyl acetate in hexane) of *A. mellifera* collected from Thailand. The last fraction was rinsed up with 10% methanol in ethyl acetate. F = fraction, solvent system: hexane/dichloromethane/ethyl acetate, N=number of tests. See Figure 4.22 for explanation of boxplots.

Because the TLC results displayed the repellent activity in both fractions, we mixed the 20% and 40% hexane in ethyl acetate fractions. Then we purified this mixture again by column chromatography using a gradient system of hexane/dichloromethane/ethyl acetate as eluants. As a result, a high repellent activity was found in four fractions: Fraction 1: ~100% hexane (R=0.82 ± 0.01), Fraction3: ~20% dichloromethane/hexane (R=0.94 ± 0.0086), Fraction 4: ~30% dichloromethane/hexane (R=0.85 ± 0.01), Fraction 9: ~80% dichloromethane/hexane (R=0.91± 0.02). Furthermore, the other two fractions, Fraction 2 (~100% hexane) and fraction 6 (~60 %dichloromethane/hexane) showed only a slight repellent activity with R = 0.7663 ± 0.03 and R = 0.75 ± 0.02 respectively (fig.4.43). Using a gradient system the actual composition which eluted the fractions could only be a roughly estimate.

***A. mellifera* (GER)**

There were two active fractions in the pentane extract from *A. mellifera* collected from Germany: in 5% ethyl acetate in hexane and 40% ethyl acetate in hexane with R = 0.86 ±0.03 and R = 0.88 ± 0.03, respectively (fig. 4.44). The yield from 5% ethyl acetate in hexane fraction was very low compared to the yield from 40% ethyl acetate in hexane fraction. Therefore, for further separation the eluant with 40% ethyl acetate in hexane was used. The eluates from the 2$^{nd}$ separation step showed different activities. The repellent indices were high in fraction 1, 2 and 4 with indices of 0.87 ± 0.08, 0.83 ± 0.04 and 0.89 ± 0.05, respectively. There was a slight repellent activity in fraction 5 (R = 0.73 ± 0.05).

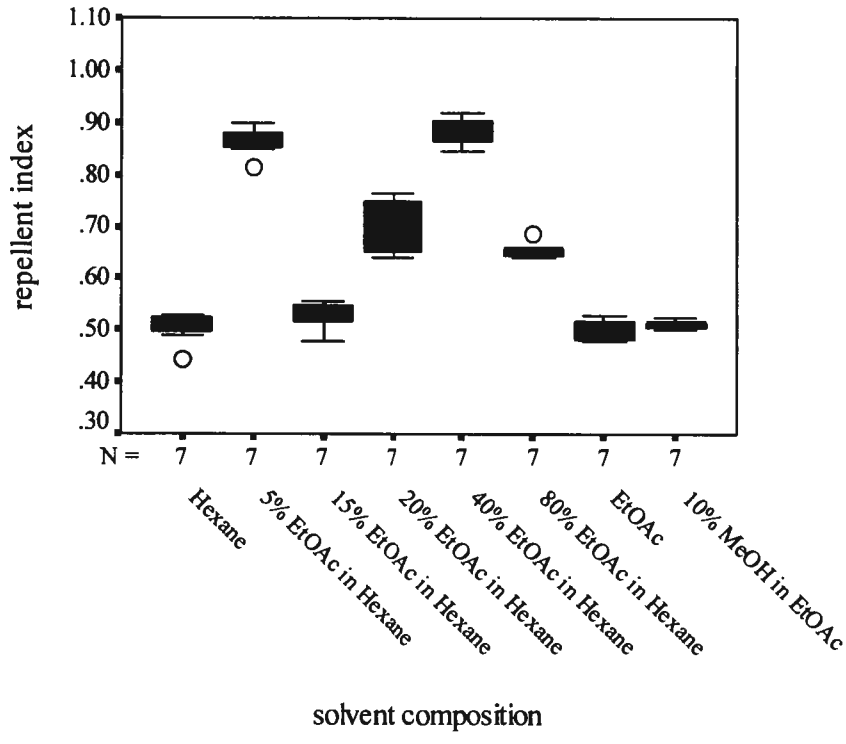


**Figure 4.44** The repellent indices of the pentane extract of *A. mellifera* collected from Germany after quick column chromatography (solvent system: hexane/ethyl acetate). N=number of tests. See Figure 4.22 for explanation of boxplots.

***T. apicalis***

The repellent activity was found in two fractions comparable to *A. mellifera* from Thailand. The active fractions were eluted with 20% and 40% ethyl acetate in hexane with R = 0.75 ± 0.06 and R = 0.84 ± 0.09, respectively (fig. 4.45). The TLC results showed the overlap of active compounds in these two fractions. Therefore, we combined these fractions and performed the second chromatograph method with a gradient system of hexane/dichloromethane/ethyl acetate. The high repellent activity was found in fraction 2 and fraction 3 (~60% and ~80 % dichloromethane/hexane with R = 0.74 ± 0.019 and R = 0.77 ± 0.15 (fig. 4.46). Compared to the data of the raw material, these efficiencies were slightly lower. Some amount of this fraction was also eluted with 60% dichloromethane/hexane. This fraction had the activity as well.

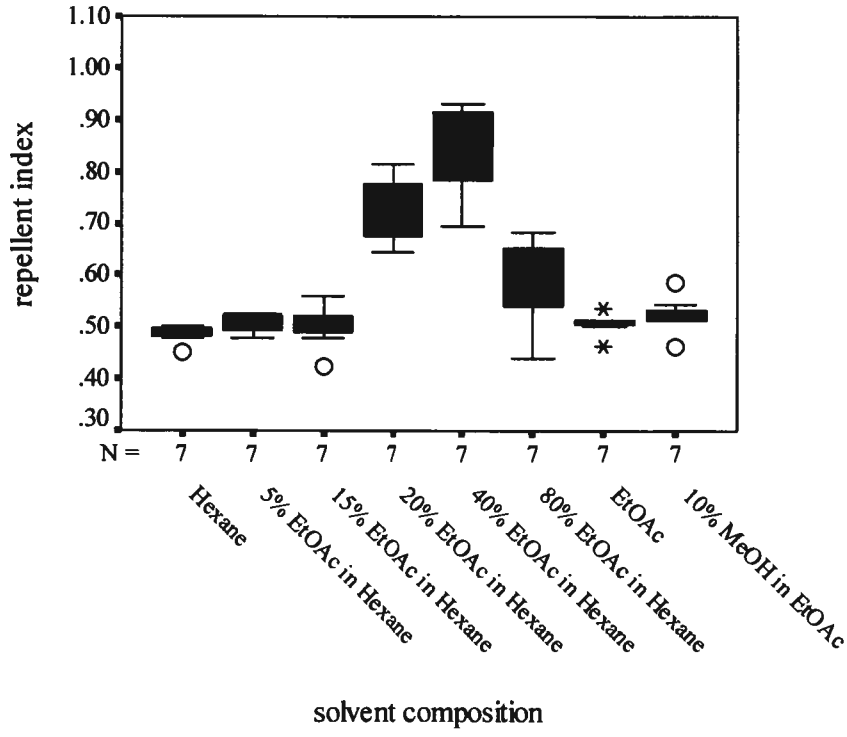


**Figure 4.45** The repellent indices of the pentane extract of *T. apicalis* collected from Tenom after quick column chromatography (solvent system: hexane/ethyl acetate). The last fraction eluted with 10% methanol in ethyl acetate. N=number of tests. See Figure 4.22 for explanation of boxplots.

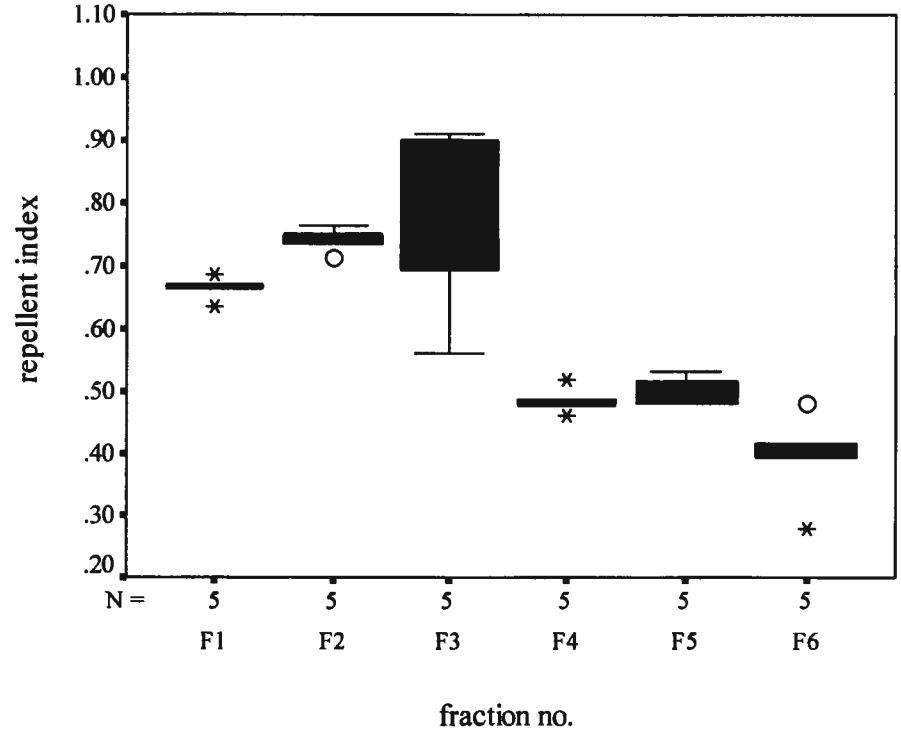


**Figure 4.46** The repellent indices of the second step of separation of fractions of pentane extract (1st step of elution: 20% and 40% ethyl acetate in hexane) of *T. apicalis* collected from Thailand (F = fraction, solvent system: hexane/dichloromethane/ethyl acetate). The last fraction was rinsed up with 10% methanol in ethyl acetate. N=number of tests. See Figure 4.22 for explanation of boxplots.

*T. collina*

As found in the other bee samples, the active fractions were eluted in the first chromatography by 40% ethyl acetate with R = 0.76 ± 0.07. The fractions from 2nd chromatography showed an uneven pattern with a slightly lower repellent activity compared to the fraction of the 1st step. The repellent activity was only marginal in all fractions (fig. 4.47).

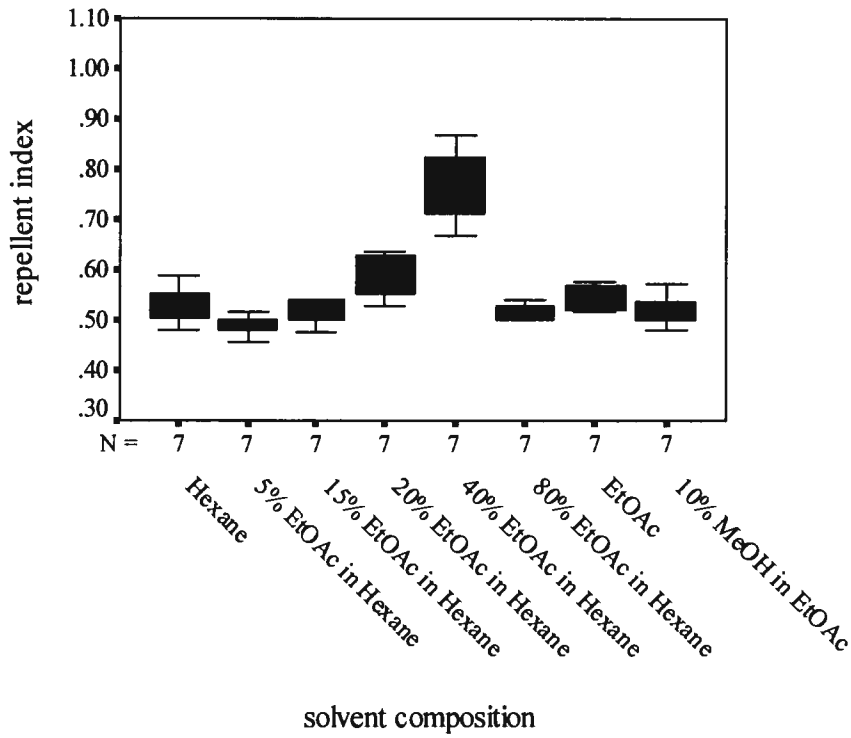


**Figure 4.47** The repellent indices of the pentane extract of *T. collina* collected from Tenom after quick column chromatography (solvent system: hexane/ethyl acetate). The last fraction was rinsed up with 10% methanol in ethyl acetate. N=number of tests. See Figure 4.22 for explanation of boxplots.

### *T. terminata* from Tenom, Malaysia

The active fractions in this species were eluted by 5% and 40% ethyl acetate in hexane (R= 0.69 ± 0.08)(fig. 4.48). We did not further separate the 5% ethly acetate in hexane because the amount was too low. Therefore, the further step of separation was done using 40% ethyl acetate in hexane fraction.

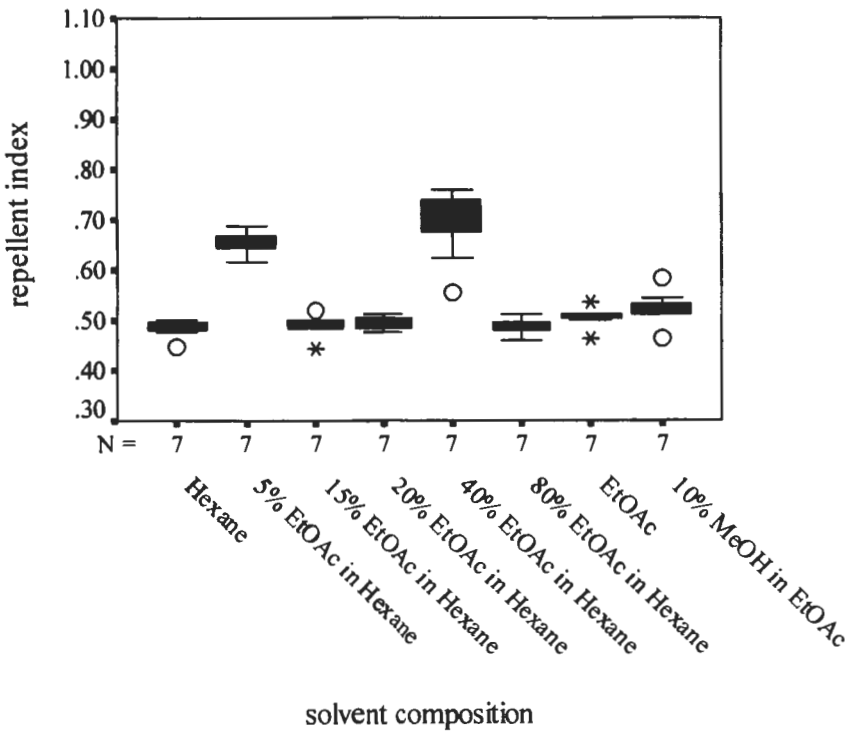


**Figure 4.48** The repellent indices of the pentane extract of *T. terminata* collected from Tenom after quick column chromatography (solvent system: hexane/ethyl acetate). The last fraction was rinsed up with 10% methanol in ethyl acetate. N=number of tests. See Figure 4.22 for explanation of boxplots.

**Table 4.9** The repellent indices resulted from the different chromatographed fractions

| Eluent | Species | | | | | | |
|---|---|---|---|---|---|---|---|
| | *A. mellifera* (THA) | *A. mellifera* (GER) | *T. apicalis* | *T. collina* | *T. terminata* (MAS) | *A. florea* | *A. andreniformis* |
| 1 st Quick column chromatography | | | | | | | |
| Hexane | 0.5072±0.0407 | 0.5037±0.0302 | 0.4857±0.0178 | 0.5294±0.0384 | 0.4857±0.0178 | - | - |
| 5% Hexane in Ethyl acetate | 0.5156±0.0253 | 0.8639±0.0277 | 0.5057±0.0187 | 0.4877±0.0204 | 0.6547±0.0239 | - | - |
| 15% Hexane in Ethyl acetate | 0.5651±0.0289 | 0.5281±0.0268 | 0.5004±0.0425 | 0.5162±0.0246 | 0.4901±0.0235 | - | - |
| 20% Hexane in Ethyl acetate | 0.7645±0.0366 | 0.6988±0.0529 | 0.7259±0.0659 | 0.5874±0.0446 | 0.4951±0.0138 | - | - |
| 40% Hexane in Ethyl acetate | 0.9523±0.0327 | 0.8837±0.0269 | 0.8442±0.0937 | 0.7663±0.0741 | 0.6973±0.0771 | - | - |
| 80% Hexane in Ethyl acetate | 0.4723±0.0512 | 0.6548±0.0164 | 0.5812±0.0890 | 0.5170±0.0176 | 0.4874±0.0166 | - | - |
| Ethyl acetate | 0.4795±0.0236 | 0.5009±0.0211 | 0.5059±0.0219 | 0.5452±0.0264 | 0.5059±0.0219 | - | - |
| 10% Methanol in Ethyl acetate | 0.4825±0.0229 | 0.5106±0.0084 | 0.5224±0.0367 | 0.5209±0.0312 | 0.5224±0.0368 | - | - |
| 2 nd Silica column chromatography | | | | | | | |
| Fraction 1 | 0.8205±0.0143 | 0.8753±0.0771 | 0.6648±0.0183 | 0.6801±0.0225 | - | - | - |
| Fraction 2 | 0.7663±0.0333 | 0.8302±0.0415 | 0.7432±0.0189 | 0.6441±0.0732 | - | - | - |
| Fraction 3 | 0.9366±0.0086 | 0.5329±0.0260 | 0.7699±0.1463 | 0.6419±0.0608 | - | - | - |
| Fraction 4 | 0.8546±0.0087 | 0.8871±0.0465 | 0.4854±0.0207 | 0.6527±0.0641 | - | - | - |
| Fraction 5 | 0.5895±0.0107 | 0.7309±0.0531 | 0.5019±0.0232 | - | - | - | - |
| Fraction 6 | 0.7470±0.0251 | - | 0.3947±0.0734 | - | - | - | - |
| Fraction 7 | 0.6428±0.0178 | - | - | - | - | - | - |
| Fraction 8 | 0.5602±0.0120 | - | - | - | - | - | - |
| Fraction 9 | 0.9080±0.0173 | - | - | - | - | - | - |
| Fraction 10 | 0.6595±0.0146 | - | - | - | - | - | - |
| Fraction 11 | 0.4779±0.0204 | - | - | - | - | - | - |
| Fraction 12 | 0.5354±0.0441 | - | - | - | - | - | - |
| Fraction 13 | 0.5226±0.0418 | - | - | - | - | - | - |

### 4.4.2 Effect of material used by bees at the nest on *F. polyctena*

**The biological activity of raw material**

***A. florea***

Samples of 7 *A. florea* colonies from Thailand were tested. The mean repellent index was 0.53 ± 0.01. The repellent indices range from 0.51 in colony no. 4 to 0.55 in colony no. 1.(fig. 4.49).

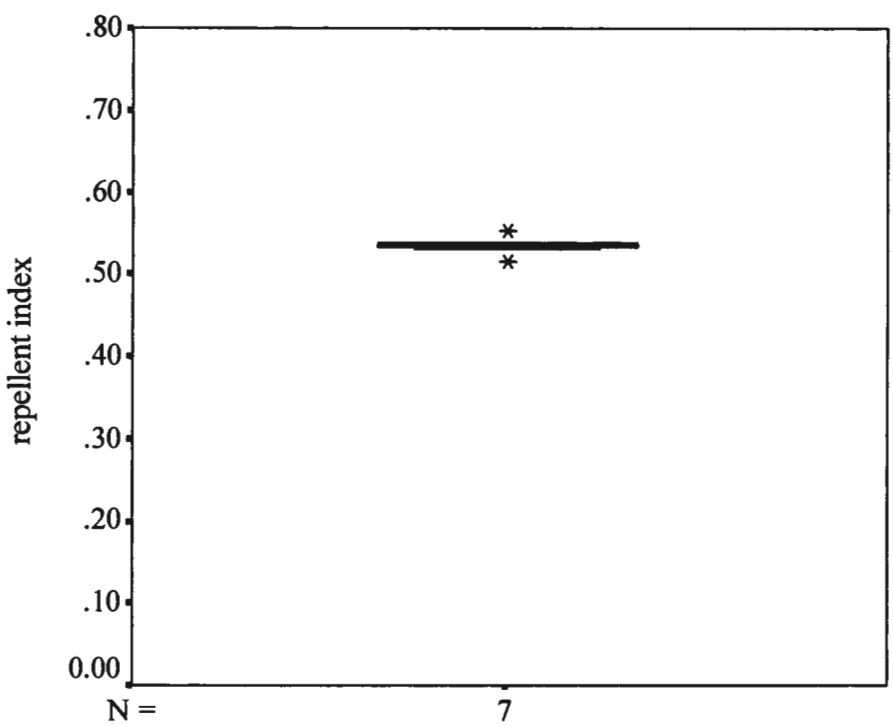


**Figure 4.49**: The repellent indices of sticky band raw material from 7 colonies of *A. florea*. Samples collected in Thailand. N=number of tests. See Figure 4.22 for explanation of boxplots.

### *A. mellifera* (THA)

Samples of 7 *A. mellifera* colonies from Thailand were tested. The mean repellent index was 0.64 ± 0.07. The repellent indices range from 0.59 ± 0.06 in colony no. 7 to 0.69 ± 0.07 in colony no. 2. (fig. 4.50). Within the samples collected in Thailand, the repellent indices were not significantly different ($p < 0.147$, Kruskal Wallis-tested, table: 4.10 )

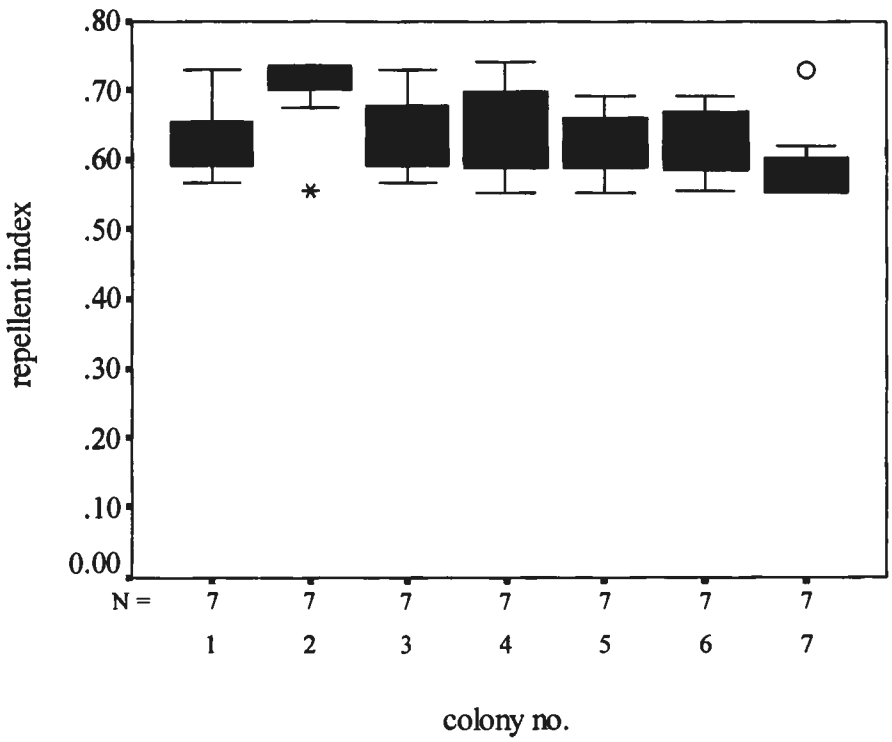


**Figure 4.50**: The repellent indices of raw material from 7 colonies of *A. mellifera.* Samples collected in Thailand. N=number of tests. See Figure 4.22 for explanation of boxplots.

### *A. mellifera* (GER)

Samples of 7 *A. mellifera* colonies from Germany were tested. The mean repellent index was 0.57 ± 0.04. The repellent indices range from 0.55 ± 0.04 in colony no. 1 to 0.59 ± 0.08 in colony no. 7. (figure 4.51). Within the samples collected in Germany, the repellent indices were not significantly different ($p< 0.915$, Kruskal Wallis-tested, table 4.10 )

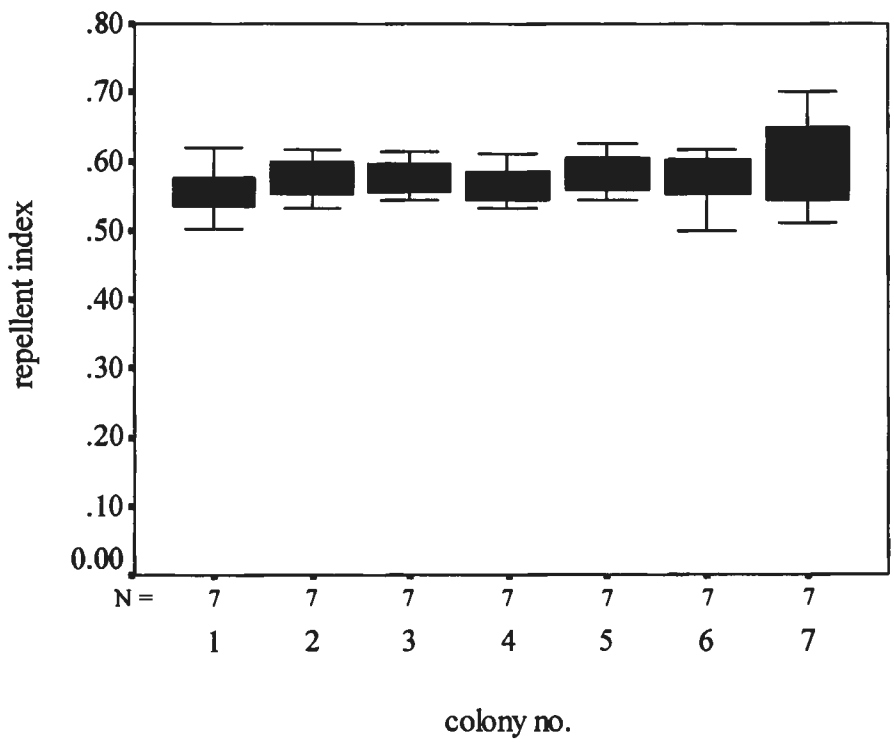


**Figure 4.51** : The repellent indices of raw material from 7 colonies of *A. mellifera*. Samples collected in Germany. N=number of tests. See Figure 4.22 for explanation of boxplots.

### *T. apicalis*

Samples of 9 *T. apicalis* colonies from Thailand were tested. The samples show relatively no repellent activity. The mean repellent index RI was was 0.49 ± 0.09. The repellent indices range from 0.42 ± 0.14 in colony no. 7 to 0.54 ± 0.03 in colony no. 3. (fig. 4.52). Within the samples collected from same geographic region, the repellent indices were significantly different ($p < 0.052$, Kruskal Wallis-tested, table 4.10)

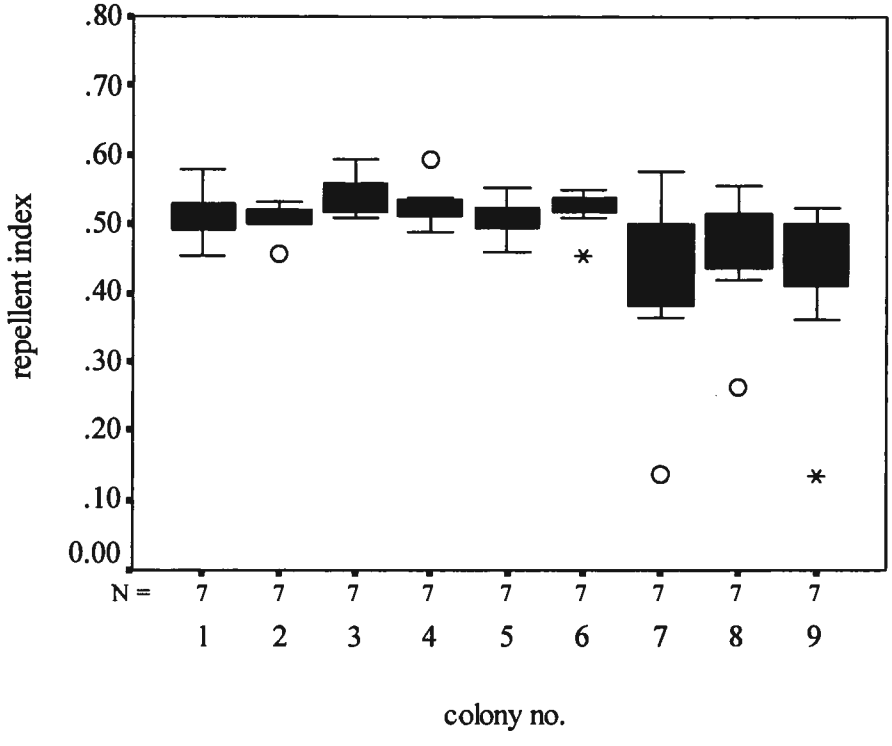


**Figure 4.52**: The repellent indices of raw material from 9 colonies of *T. apicalis*. N=number of tests. See Figure 4.22 for explanation of boxplots.

***T. collina***

Samples of 7 *T. collina* colonies from Thailand were tested. The mean repellent index was 0.49 ± 0.04. The repellent indices range from 0.49 ± 0.01 in colony no. 7 to 0.54 ± 0.03 in colony no. 3. (fig. 4.53). Within the samples collected from same geographic region, the repellent indices were not significantly different ($p<$ 0.172, Kruskal Wallis-tested, table: 4.10 ).

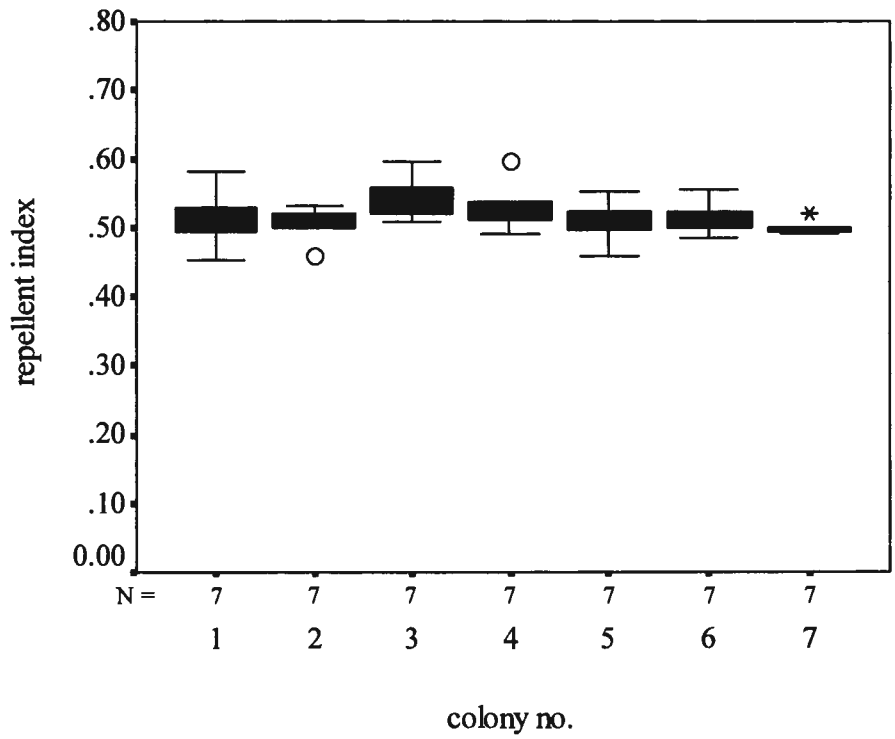


**Figure 4.53** The repellent indices of raw material from 7 colonies of *T. collina.* N=number of tests. See Figure 4.22 for explanation of boxplots.

### *T. terminata* (THA)

Samples of 7 *T. terminata* colonies from Thailand were tested. The mean repellent index was 0.49 ± 0.03. The repellent indices range from 0.49 ± 0.03 in colony no. 7 to 0.51 ± 0.02 in colony no. 2. (fig. 4.54). Within the samples collected from the same geographic region, the repellent indices were not significantly different ( $p< 0.911$, Kruskal Wallis-tested, table 4.10 ).

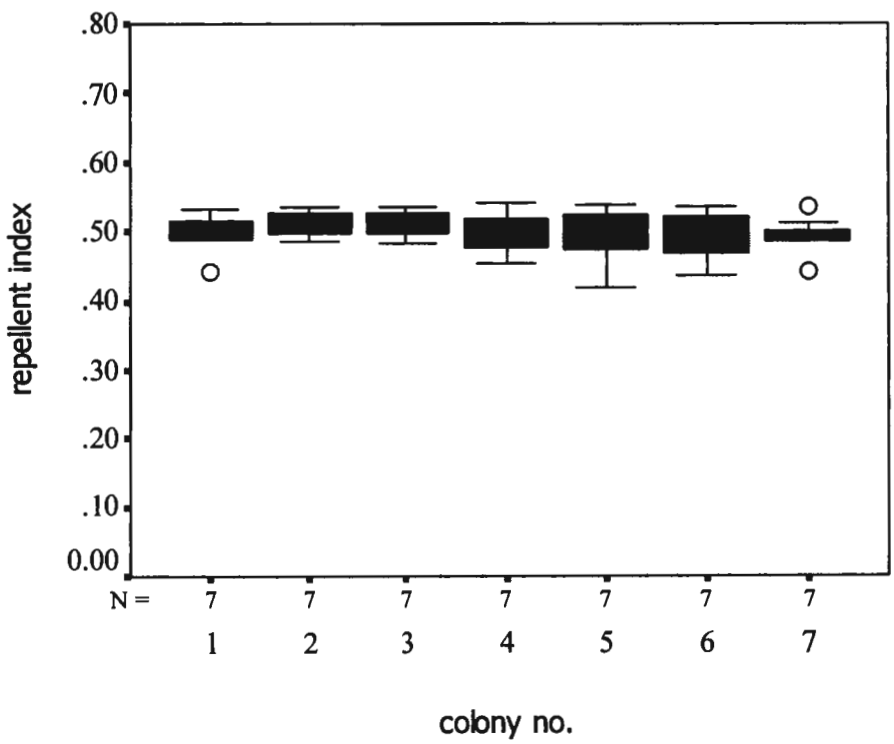


**Figure 4. 54** The repellent indices of raw material from 7 colonies of *T. terminata.* N=number of tests. See Figure 4.22 for explanation of boxplots.

*T. terminata* (MAS)

Samples of 6 *T. terminata* colonies from Tenom, Malaysia were tested. The mean repellent index was 0.52 ± 0.05. The repellent indices range from 0.49 ± 0.09 in colony no. 6 to 0.56 ± 0.05 in colony no. 7. (fig. 4.55). Within the samples collected from the same geographic region, the repellent indices were not significantly different ($p$<0.154, Kruskal Wallis-tested, table 4.10 ).

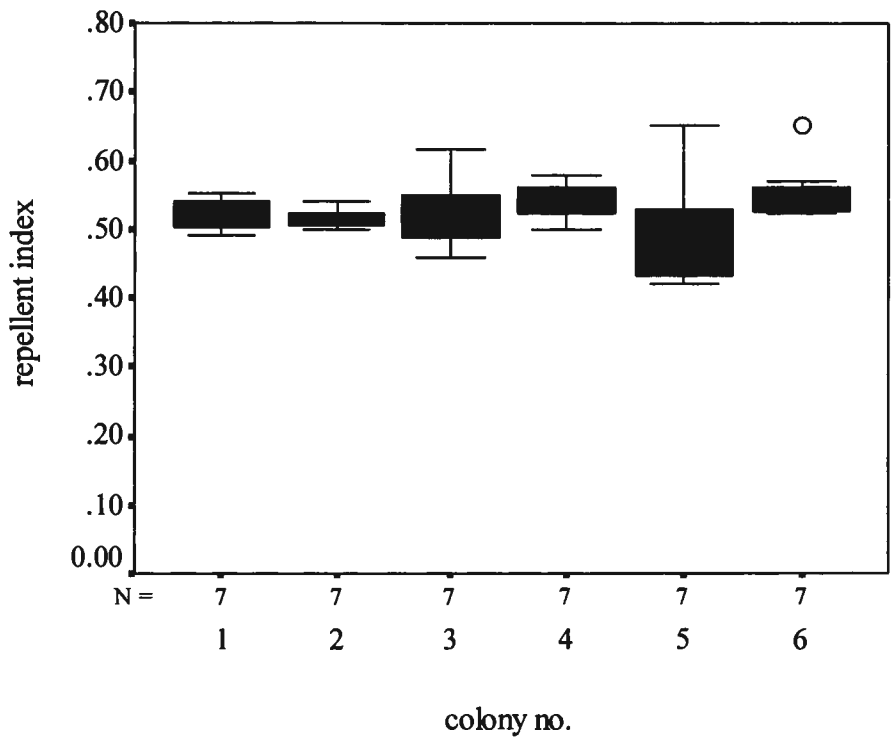


**Figure 4.55** The repellent indices of raw material from 6 colonies of *T. terminata*, samples collected from Tenom, Malaysia. N=number of tests. See Figure 4.22 for explanation of boxplots.

***T. minor***

Samples of 2 *T. minor* colonies from Thailand were tested. The mean repellent index was 0.49 ± 0.03. The repellent indices range from 0.49 ± 0.03 in colony no. 6 to 0.49 ± 0.02 in colony no. 7 (fig. 4.56). Within the samples collected from same geographic region, the repellent indices were not significantly different ($p< 0.620$, Kruskal Wallis-tested, table 4.10).

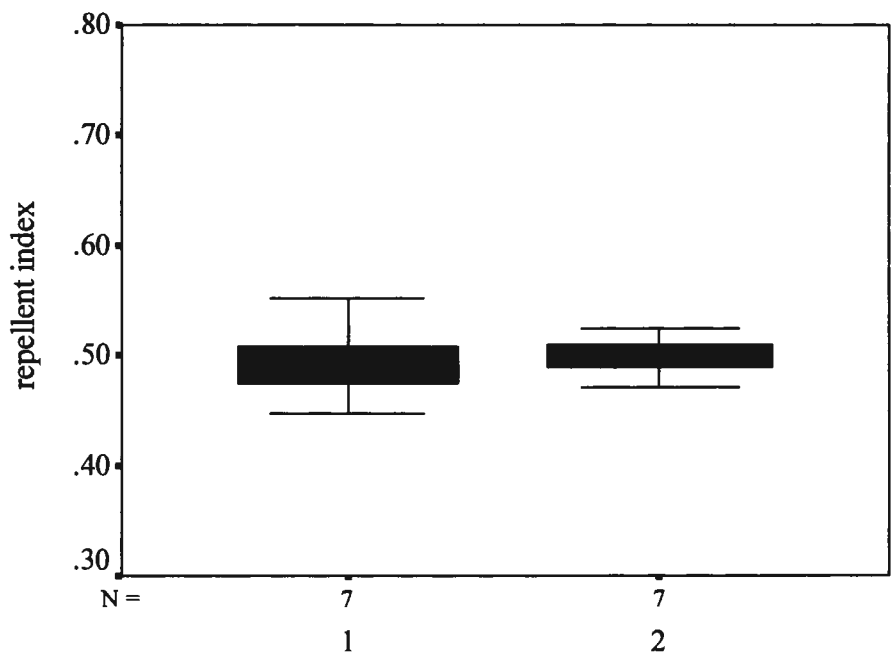


**Figure 4.56** The repellent indices of raw materials from 2 colonies of *T. minor*. N=number of tests. See Figure 4.22 for explanation of boxplots.

**Table 4.10:** The comparison of repellent indices within species (Kruskal Wallis-tested).

| Species | R (mean± SD) | *p* (Kruskal Wallis-tested) |
|---|---|---|
| *A. florea* | 0.5335±0.0112 | - |
| *A. mellifera* (THA) | 0.6367±0.0652 | >0.147 |
| *A. mellifera* (GER) | 0.5747±0.0411 | >0.915 |
| *T. apicalis* | 0.4907±0.0863 | >0.052 |
| *T. collina* | 0.4983±0.0446 | >0.172 |
| *T. terminata* (THA) | 0.4996±0.0308 | >0.911 |
| *T. terminata* (MAS) | 0.5250±0.0494 | >0.154 |
| *T. minor* | 0.4959±0.0269 | >0.620 |

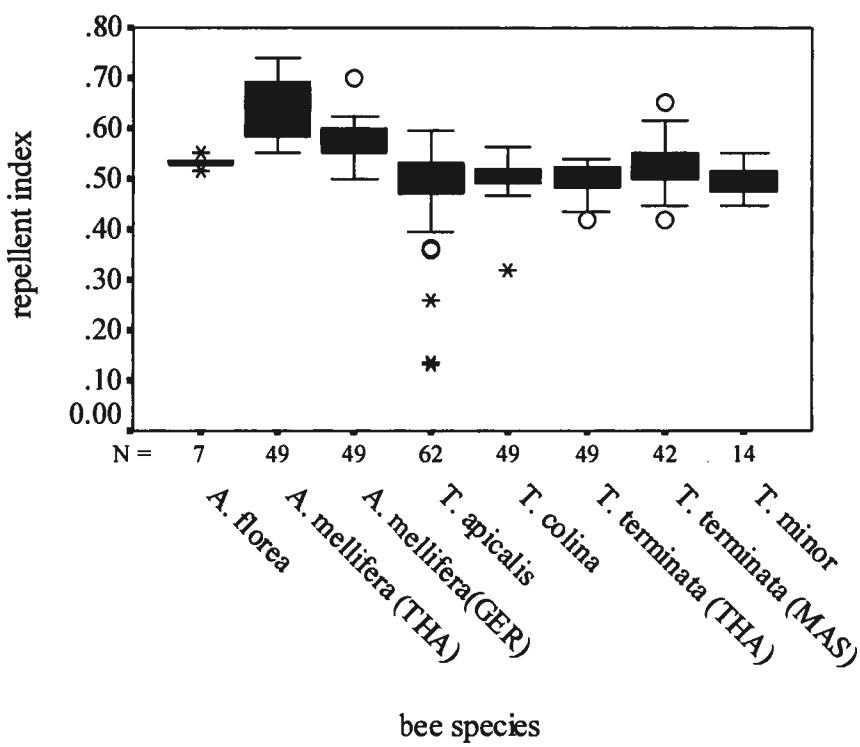


**Figure 4.57** The repellent indices of raw material. Samples were collected from 8 species of social bees. N=number of tests. See Figure 4.22 for explanation of boxplots.

**Tests of fractions on repellent activity**

**A. Extract from Soxhlet**

The repellent indices were approximately about 0.5 (table 4.11, fig. 4.58-59.) which means no repellent. Only, the material of *T. minor* and the propolis samples of *A. mellifera* collected from Thailand, showed a small repellent efficacy, with similar results for the raw material and the pentane extract (Table 4.11).

**Table 4.11** The repellent indices of bee material.

| Species/bee material | Fraction (R, mean ± SD) | | |
|---|---|---|---|
| | Raw material | Pentane | Acetone |
| *A. florea* | 0.5335±0.0112 | 0.5604±0.0260 | 0.4881±0.0167 |
| *A. mellifera* (THA) | 0.6367±0.0652 | 0.6391± 0.0381 | - |
| *A. mellifera* (GER) | 0.5747±0.0411 | 0.6568±0.0459 | 0.5629± 0.0231 |
| *T. apicalis* | 0.4907±0.0863 | 0.5156± 0.0738 | - |
| *T. collina* | 0.4983±0.0446 | 0.5493±0.0128 | 0.4967±0.0116 |
| *T. terminata* (THA) | 0.4996±0.0308 | 0.4996± 0.0118 | 0.5038± 0.0152 |
| *T. terminata* (MAS) | 0.5250±0.0494 | 0.5322±0.0125 | 0.4933±0.0179 |
| *T. minor* | 0.4959±0.0269 | 0.6548± 0.0219 | 0.5159± 0.0129 |

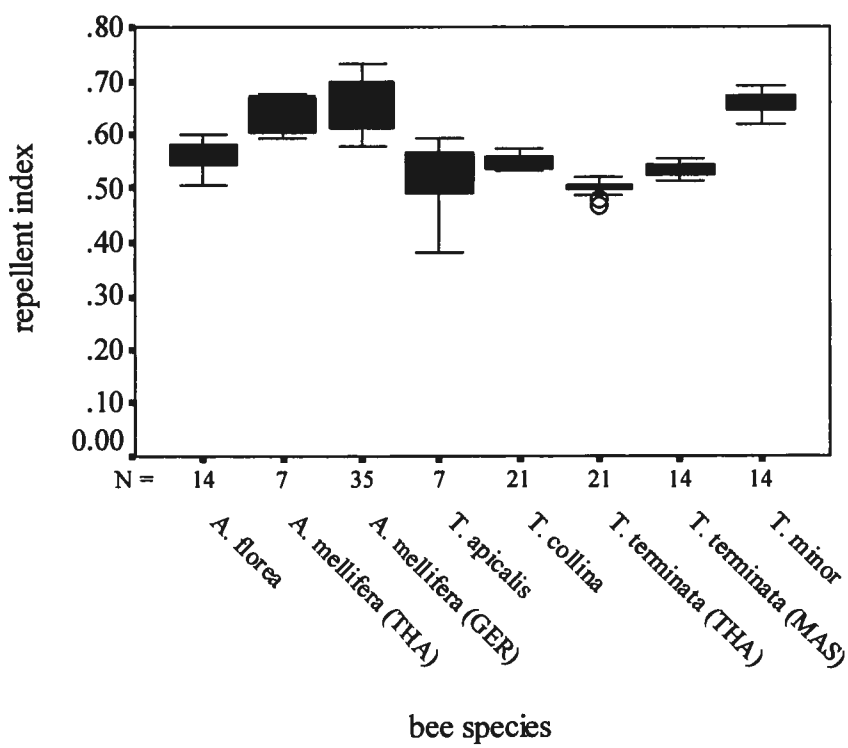


**Figure 4.58** The repellent indices of pentane extracts. Samples were collected from 7 species of social bees. N=number of tests. See Figure 4.22 for explanation of boxplots.

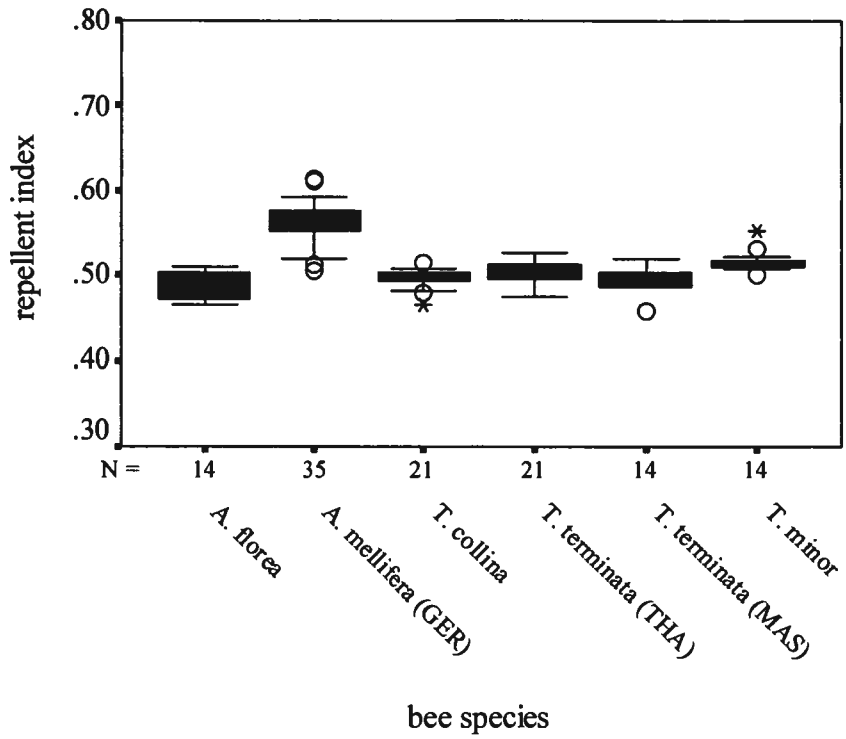


**Figure 4.59** The repellent indices of acetone extracts. Samples were collected from 5 species of social bees. N = number of tests. See Figure 4.22 for explanation of boxplots.

**B. Chromatographed fractions**

For each species, we chose the fractions which had shown the highest repellent acitivity against weaver ant, *O. smaragdina*. For comparison, the fractions which did not show the repellent activity was also tested.

***A. mellifera* (THA)**

The 4 fractions from quick column chromatograph were tested, hexane (1), 40% ethyl acetate in hexane (2), 60% ethyl acetate in hexane(3) and ethyl acetate (4). The repellent indices of fractions were R(1) = 0.49 ± 0.03, R (2) = 0.53 ± 0.08, R(3) = 0.49 ± 0.04 and R(4) = 0.49 ± 0.06, respectively (fig. 4.60); indicating no repellent effect.

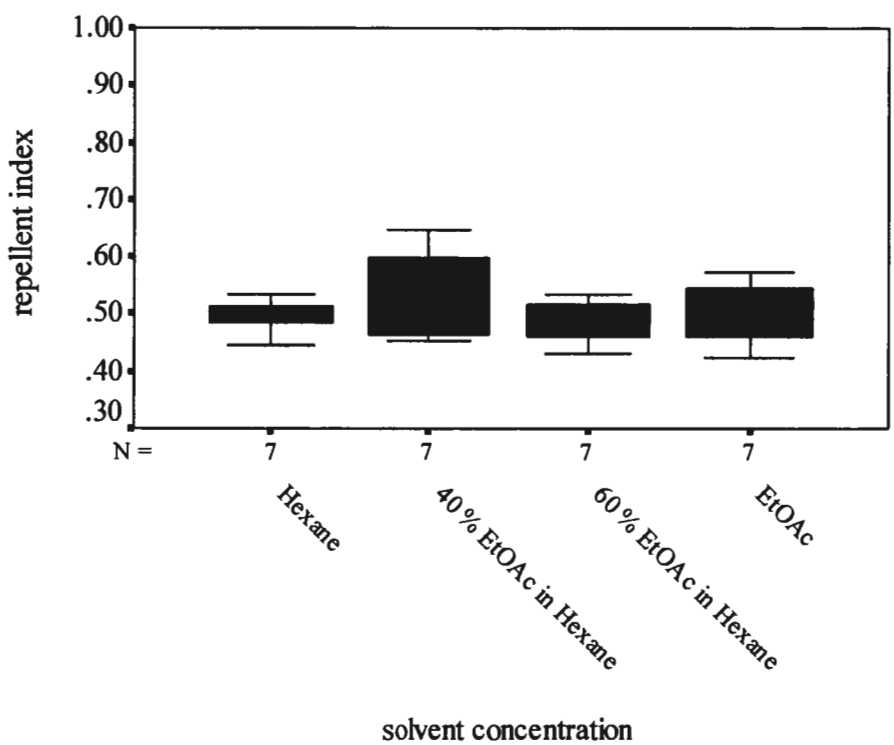


**Figure 4.60** The repellent indices of pentane extracts of *A. mellifera* collected from Thailand after quick column chromatography (solvent system: hexane/ethyl acetate). The last fraction was eluted with 10% methanol in ethyl acetate. N=number of tests. . See Figure 4.22 for explanation of boxplots.

*A. mellifera* (GER)

The two fractions from quick column chromatograph were tested, 15% ethyl acetate in hexane (R= 0.62 ± 0.04) and 40% ethly acetate in hexane (R= 0.72 ± 0.02 (fig 4.61). Accordingly the at 40% ethyl acetate in hexane showed a light repellent activity.

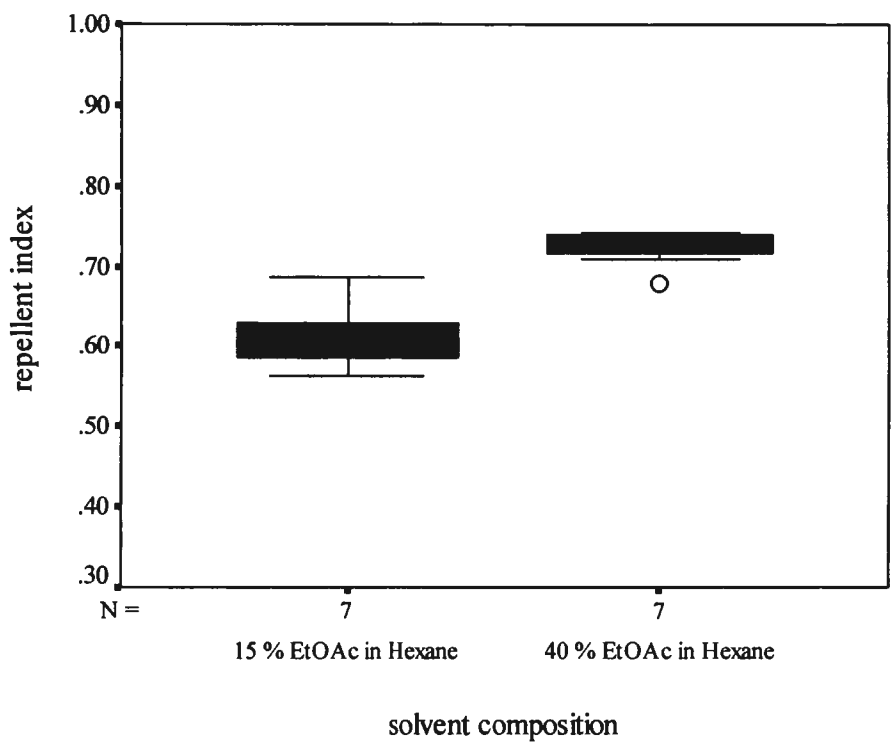


**Figure 4.61** The repellent indices of pentane extracts of *A. mellifera* collected from Germany after quick column chromatography (solvent system: hexane/ethyl acetate). The last fraction was eluted by 10% methanol in ethyl acetate. N = number of tests. See Figure 4.22 for explanation of boxplots.

*T. apicalis*

The two fractions from quick column chromatograph were chosen for testing, hexane and 40% ethyl acetate in hexane. The repellent activity in fractions were 0.4786 ± 0.0527, and 0.4796 ± 0.0789, respectively (fig. 4.62). According to the repellent indices, the fractions showed relatively no repellent activity.

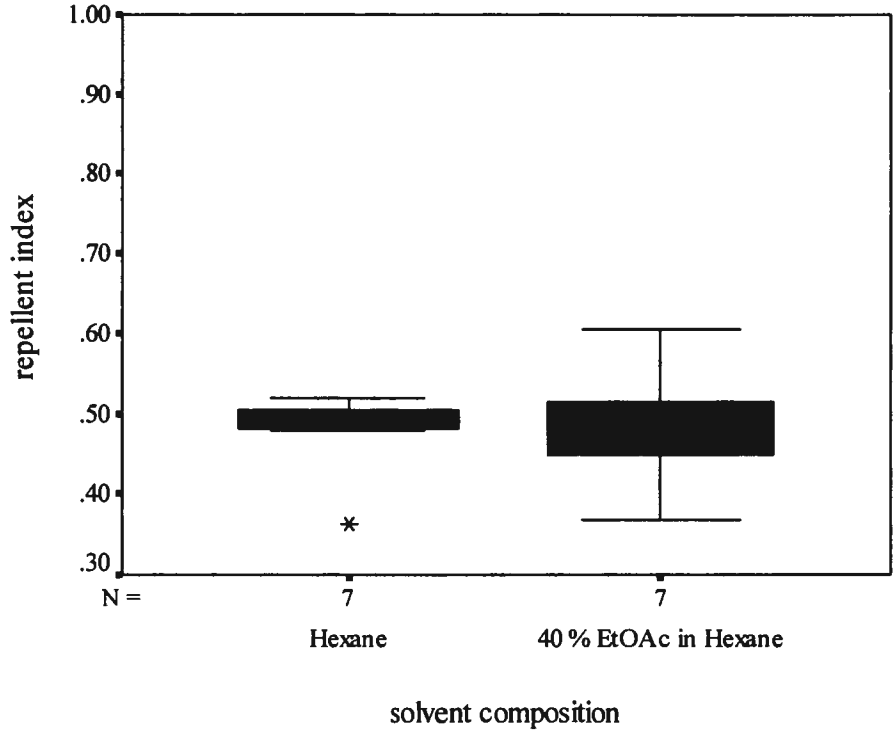


**Figure 4.62** The repellent indices of the pentane extract of *T. apicalis* after quick column chromatography (solvent system: hexane/ethyl acetate). The last fraction was eluted with 10% methanol in ethyl acetate. N = number of tests. See Figure 4.22 for explanation of boxplots.

*T. terminata* (MAS)

The four fractions were tested. The repellent activity from three fractions,15% ethyl acetate in hexane (R= 0.53 ± 0.04), 40% ethyl acetate in hexane (R=0.55 ± 0.03) and 80% ethyl acetate in hexane (R= 0.49 ± 0.03) did not show repellent activity. Only the fraction from 5% ethyl acetate in hexane had a high repellent activity (R=0.79 ± 0.06) (fig. 4.63).

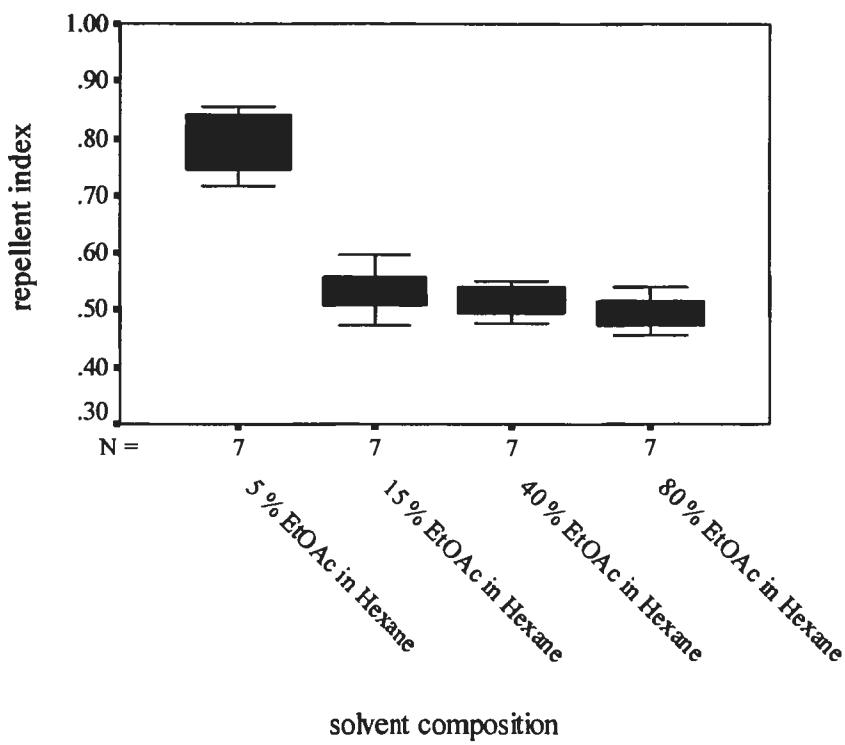


**Figure 4.63** The repellent indices of the pentane extract of *T. terminata*, samples collected from Tenom, Malaysia, after quick column chromatography (solvent system: hexane/ethyl acetate). The last fraction was eluted with 10% methanol in ethyl acetate. N = number of tests. See Figure 4.22 for explanation of boxplots.

### 4.4.3 The constituents in active fractions

*A. florea*

The pentane extract was identified (fig 4.64). The chromatograms are presented in fig 4.65. The most abundant groups of compounds are triterpene (45.82 %) and steriods (30.32%).

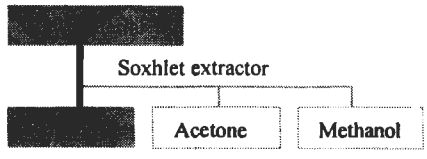


**Figure 4.64** The separation chart of the repellent fraction from samples of *A. florea.*

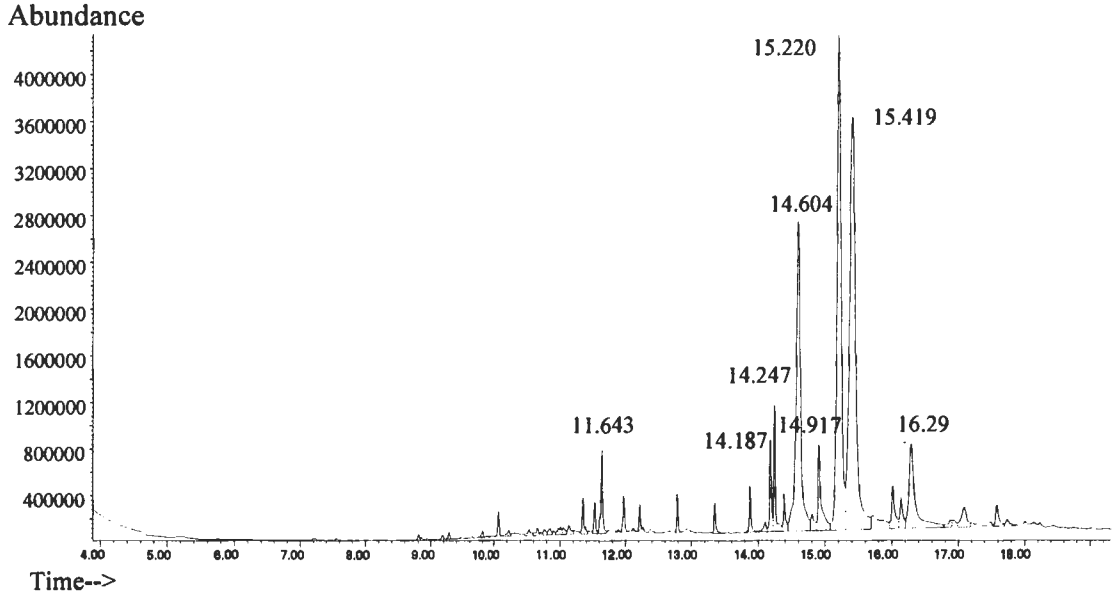


**Figure 4.65** Chromatograms of the pentane raw extracts of the sticky band material collected at *A. florea* nests, from Nakorn Sawan province. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 11.643 = no satisfying library match, $R_t$ 14.187 = phenol derivative, $R_t$ 14.247 = phenol derivative, $R_t$ 14.6 = triterpene, $R_t$ 14.917 = heptacosane, $R_t$ 15.220 = steroid, $R_t$ 15.419 = triterpene, $R_t$ 16.294 = steroid ; $R_t$=retention time.

*A. mellifera* (THA)

The pentane extract was separated by column chromatography (fig 4.66). Four fractions displayed the repellent activity. Thus the chemical identification of these fractions was proceeded. The chromatograms of fraction 1, 2, 4 and 9 are presented in fig. 4.67-70, respectively. The mixture is dominated by triterpene found in fraction 4 (73.71%) and fraction 9 (59.85%). Another abundant group is long chain hydrocarbons found mostly in fraction 1 (100%). Steriods are also found moderately, 26.29% in fraction 4 and 35.97 % in fraction 9.

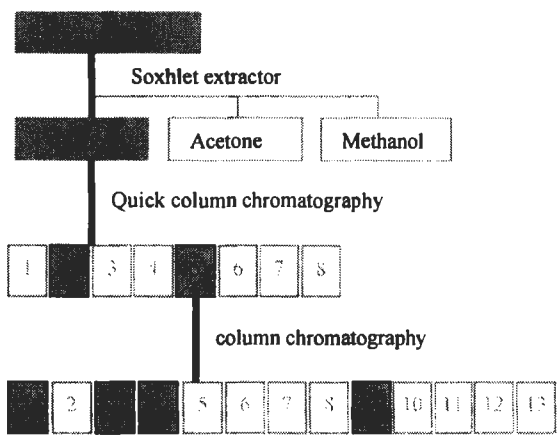


**Figure 4.66** The separation chart of the repellent fraction from samples of *A. mellifera* in Thailand.

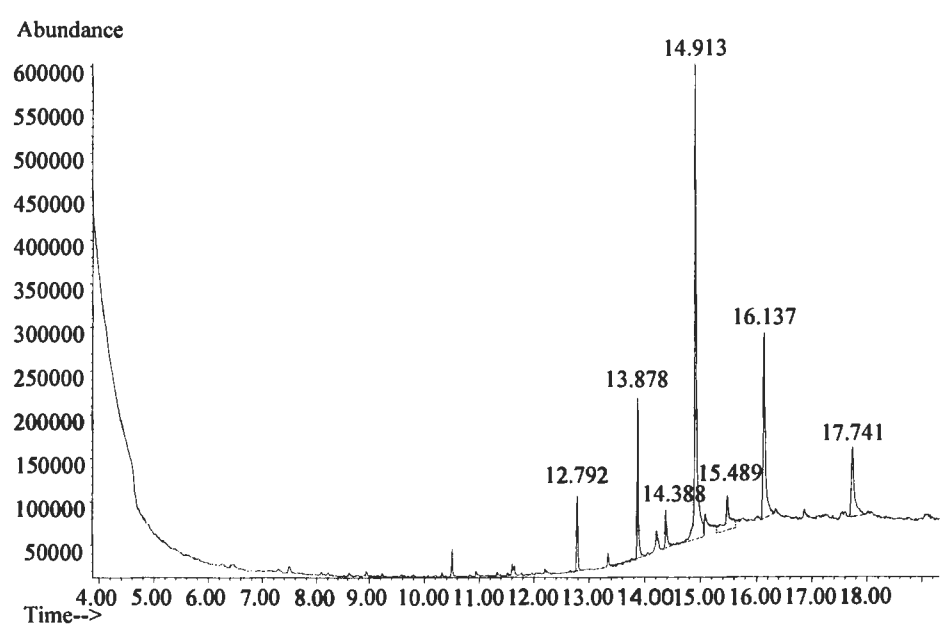


**Figure 4.67** Chromatograms of fraction 1, which was chromatographed from propolis collected from nest of *A. mellifera* in Thailand. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 12.792 = tricosane, $R_t$ 13.878= pentacosane, $R_t$ 14.388 = hexacosane, $R_t$ 14.913 = heptacosane, $R_t$ 15.489 = octadecane, $R_t$ 16.137= tetracosane, $R_t$ 17.741 = octadecane, $R_t$=retention time.

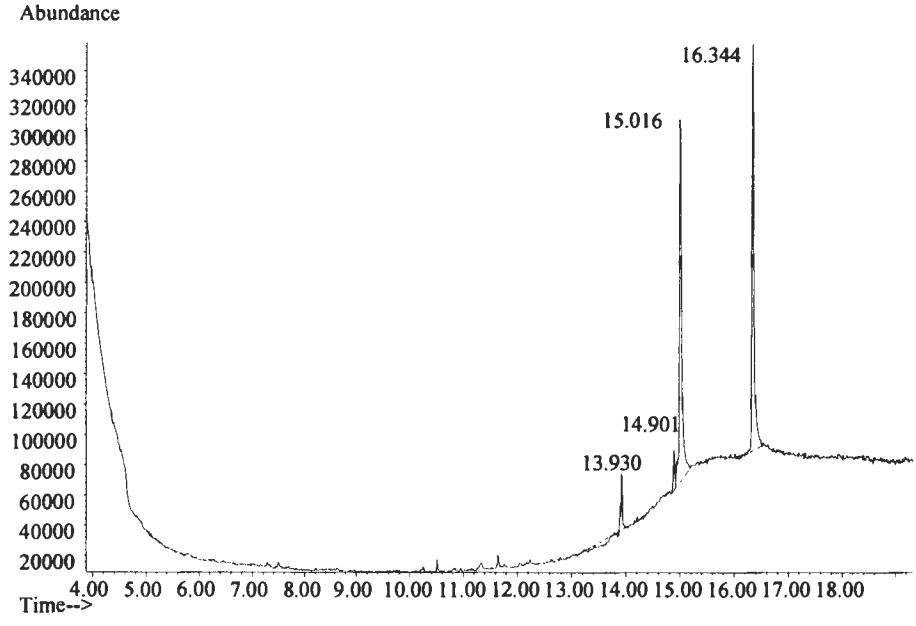


**Figure 4.68** Chromatograms of fraction 3, which was chromatographed from propolis collected from nest of *A. mellifera* in Thailand. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 13.930 = phenol derivative, $R_t$ 14.901= no satisfying library match, $R_t$ 15.016 = phenol derivative, $R_t$ 16.344 = phenol derivative, $R_t$=retention time.

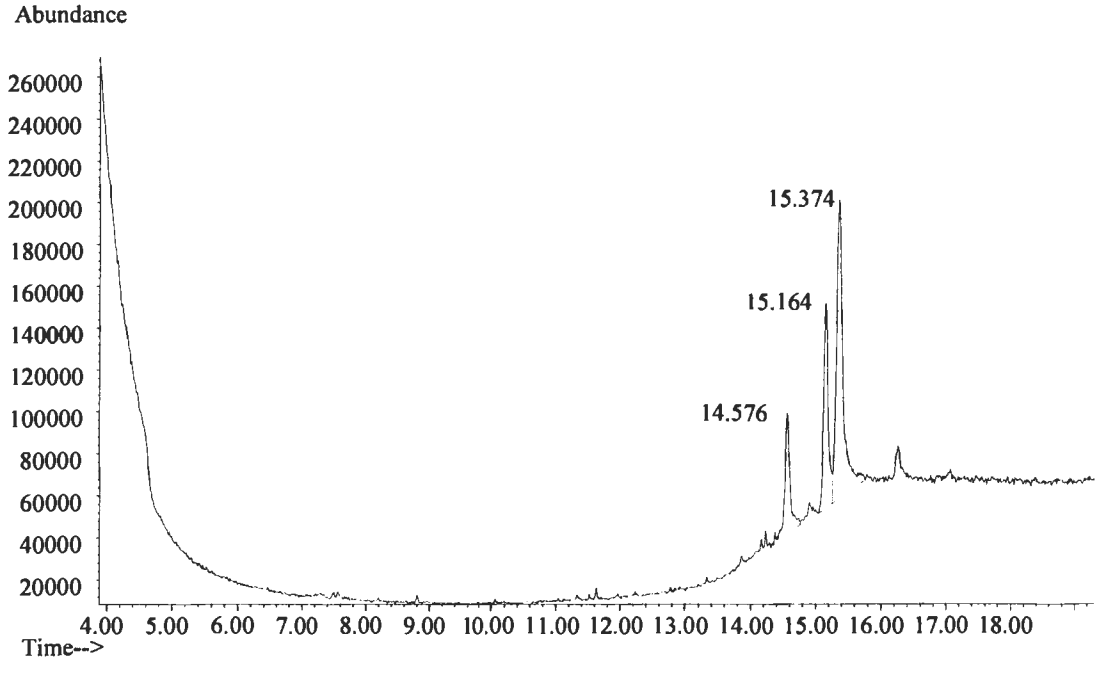


**Figure 4.69** Chromatograms of fraction 4, which was chromatographed from propolis collected from nest of *A. mellifera* in Thailand. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 14.576 = triterpene, $R_t$ 15.164 = steroid, $R_t$ 15.374 = triterpene, $R_t$ = retention time.

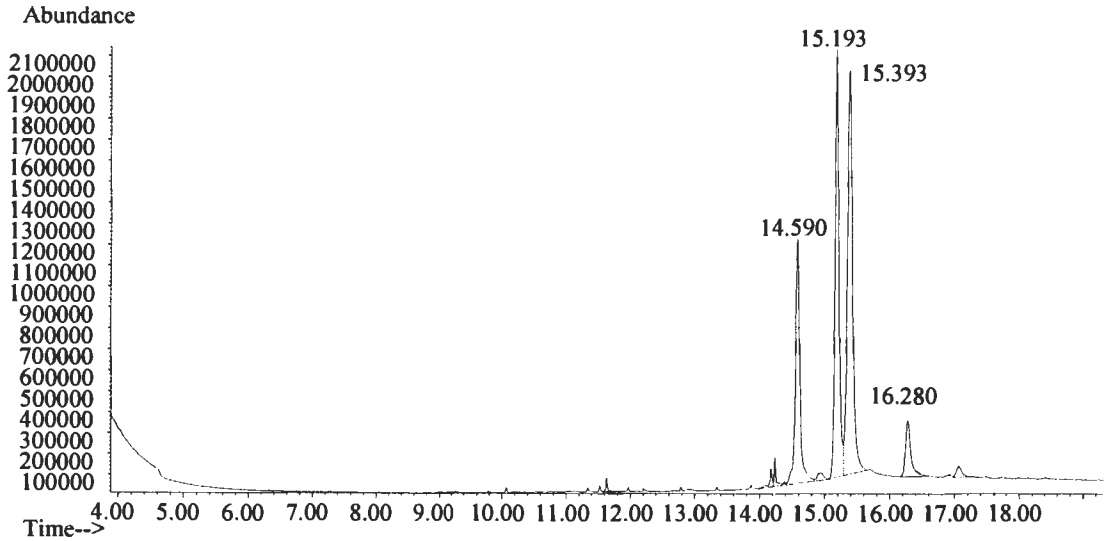


**Figure 4.70** Chromatograms of fraction 9, which was chromatographed from propolis collected from nest of *A. mellifera* in Thailand . Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 14.590 = triterpene, $R_t$ 15.193 = steroid, $R_t$ 15.393 = triterpene, $R_t$ 16.280 = steroid, $R_t$ = retention time.

### *A. mellifera* (GER)

The pentane extract was chromatographed by quick column chromatography (Fig 6.71). Two fractions, fraction 2 (eluent = 5% ethyl acetate in hexane) and fraction 5 (eluent = 40% ethyl acetate in hexane) exhibited repellent activity and were later identified. The chromatograms are presented in fig: 4.72-73, respectively. The fraction 2 contains very high proportions of long chain hydrocarbon (75.53%). Another abundant group is triterpene found in fraction 5 (54.29%). Steroids were also found in significant abundance, 35.98% in fraction 5.

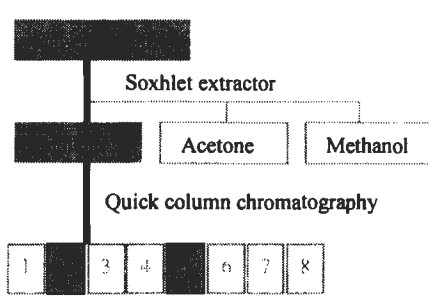


**Figure 4.71** The separation chart of the repellent fractions from samples of *A. mellifera* in Germany.

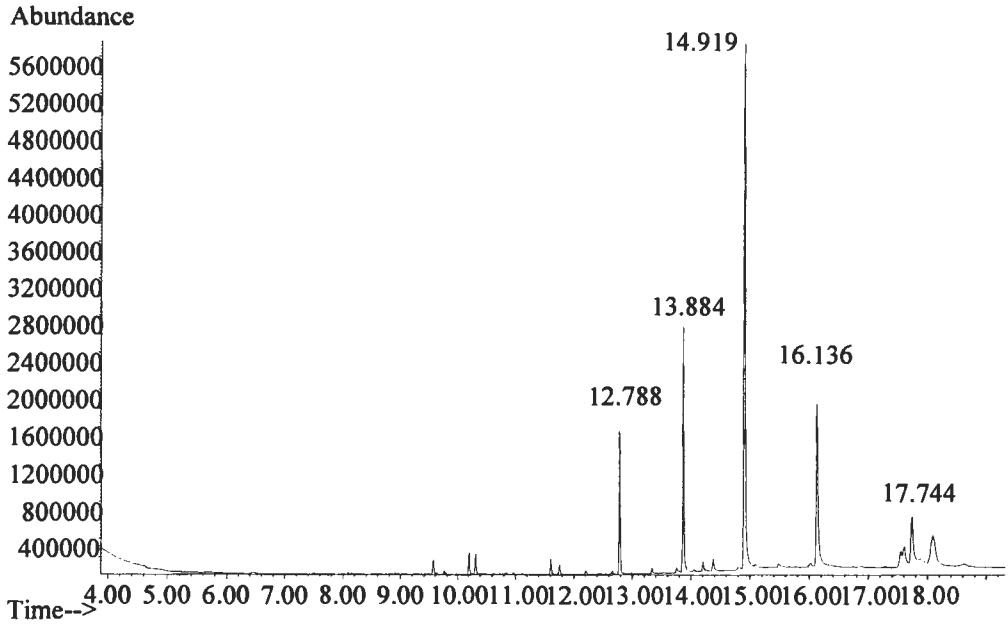


**Figure 4.72** Chromatograms of fraction 2 (eluent =5% ethyl acetate in hexane), which was chromatographed from propolis collected from nest of *A. mellifera* in Germany. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 12.788 = tricosane, $R_t$ 13.884 = pentacosane, $R_t$ 14.919 = heptacosane, $R_t$ 16.136 = octacosane, $R_t$ 17.744 = hentriacontane, $R_t$ = retention time.

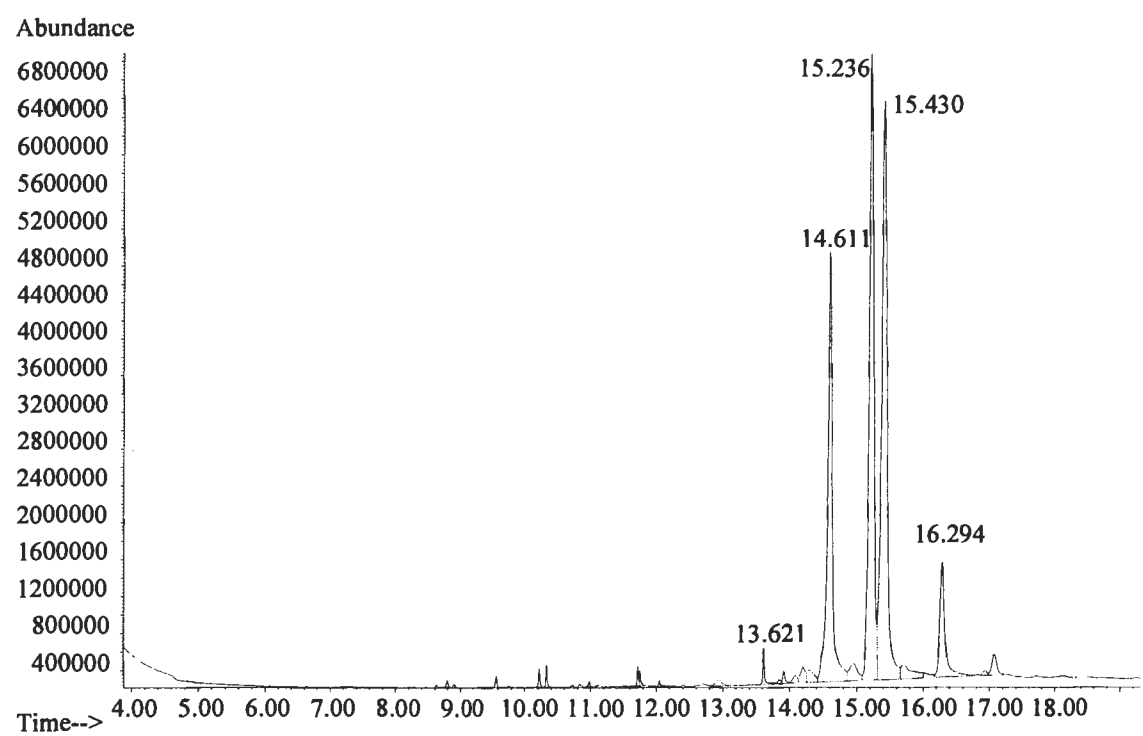


**Figure 4.73** Chromatograms of fraction 5 (eluent =40% ethyl acetate in hexane), which was chromatographed from propolis collected from nest of *A. mellifera* in Germany. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 13.621 = aromatic ketone, $R_t$ 14.611 = triterpene, $R_t$ 15.236 = steriod, $R_t$ 15.430 = amyrin, $R_t$ 16.294 = triterpene, $R_t$ = retention time.

***T. apicalis***

The pentane extract was chromatographed by quick column chromatography and later by column chromatography (fig 4.74). Fraction 3 exhibited repellent activity and was later identified. The constituents were presented in fig 4.75. The dominant group is unknown (52.83%). Naphthalene derivative is also variable in minor components (4.90%).

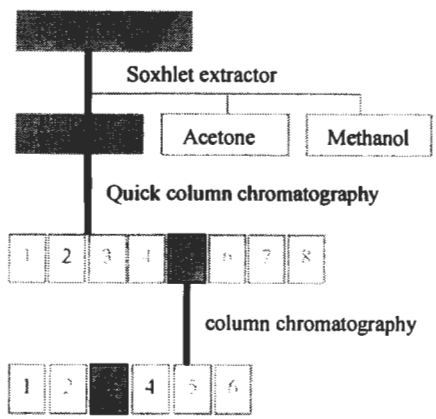


**Figure 4.74** The separation chart of the repellent fraction from samples of *T. apicalis*.

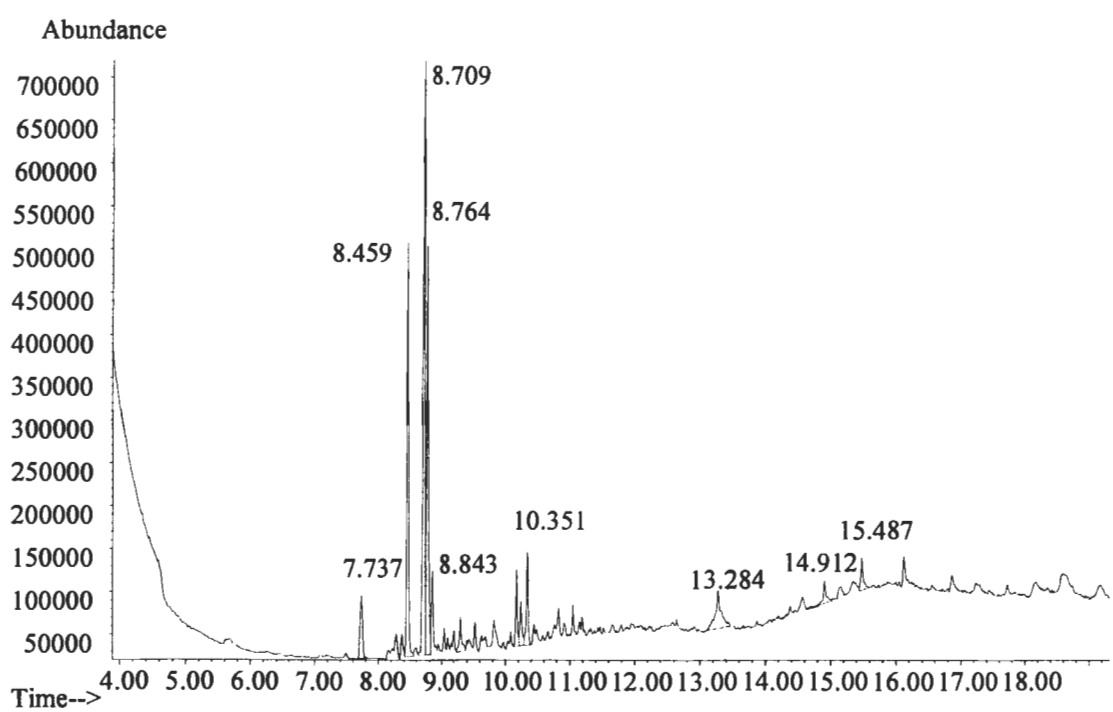


**Figure 4.75** Chromatograms of fraction 3, which was chromatographed from entrance tubes from nest of *T. apicalis* in Thailand. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 7.737 = aromatic heterocyclic compound, $R_t$ 8.459 = no satisfying library match, $R_t$ 8.709 = no satisfying library match, $R_t$ 8.764 = heteroaromatic, $R_t$ 8.843 = no satisfying library match, $R_t$ 10.351 = naphthalene derivative, $R_t$ 13.284 = dehydroabietic acid, $R_t$ 14.912 = eicosane, $R_t$ 15.487 = octacosane, $R_t$ = retention time.

*T. collina*

The pentane extract was chromatographed by quick column chromatography and later by column chromatography (Fig 4.76). Fraction 5 exhibited repellent. Thus the identification was proceeded. The chromatograms are presented in fig 4.77. The major abundance are caryphyllene oxide like compound and sesquiterpenes. The library database also suggested some compounds that occured in the naphthalene group.

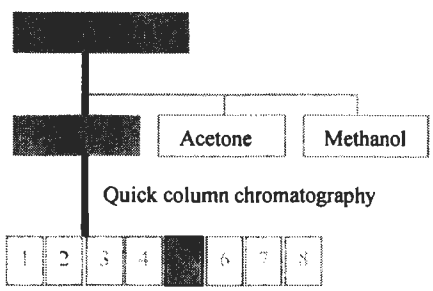


**Figure 4.76** The separation chart of the repellent fraction of samples from *T. collina.*

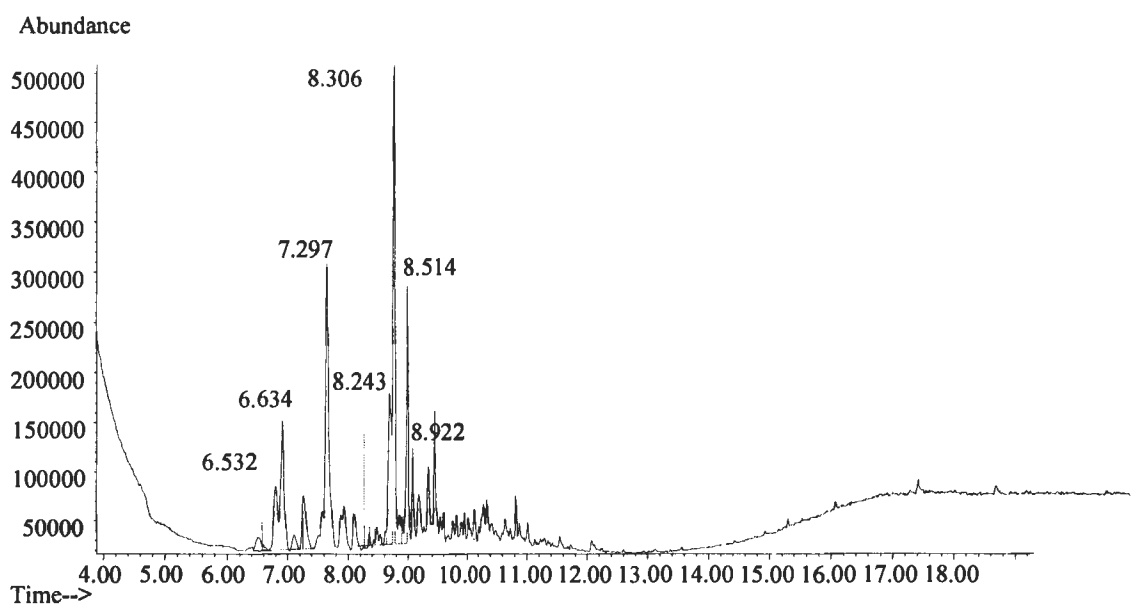


**Figure 4.77** Chromatograms of fraction 5 (eluent = 40% ethyl acetate in hexane), which was chromatographed from entrance tube from nest of *T. collina* in Thailand. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 6.532 = sesquiterpene, $R_t$ 6.634 = no satisfying library match, $R_t$ 7.297 = sesquiterpene, $R_t$ 8.243 = cyclopropazulenol, $R_t$=8.306 = caryphyllene oxide, $R_t$=8.514 = naphthalene derivative, $R_t$ 8.922 = no satisfying library match, $R_t$ = retention time.

*T. terminata* (MAS)

Two fractions of pentane extracts (separated by chromatgraphy) gave distinctive bioassay results (fig.4.78). Therefore, fraction 2 (eluent = 5% ethyl acetate in hexane) and fraction 5 (eluent = 40% ethyl acetate in hexane) were further analyzed. The constituents are presented in fig 4.79-80. Fraction 2 carried 97.18% long chain hydrocarbons. Like incidentally found in the other species, triterpenes and steroids were found at 80.51% and 12.24% respectively.

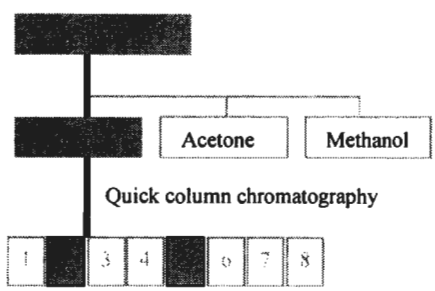


**Figure 4.78** The separation chart of the repellent fractions of samples from *T. terminata* in Tenom. Malaysia.

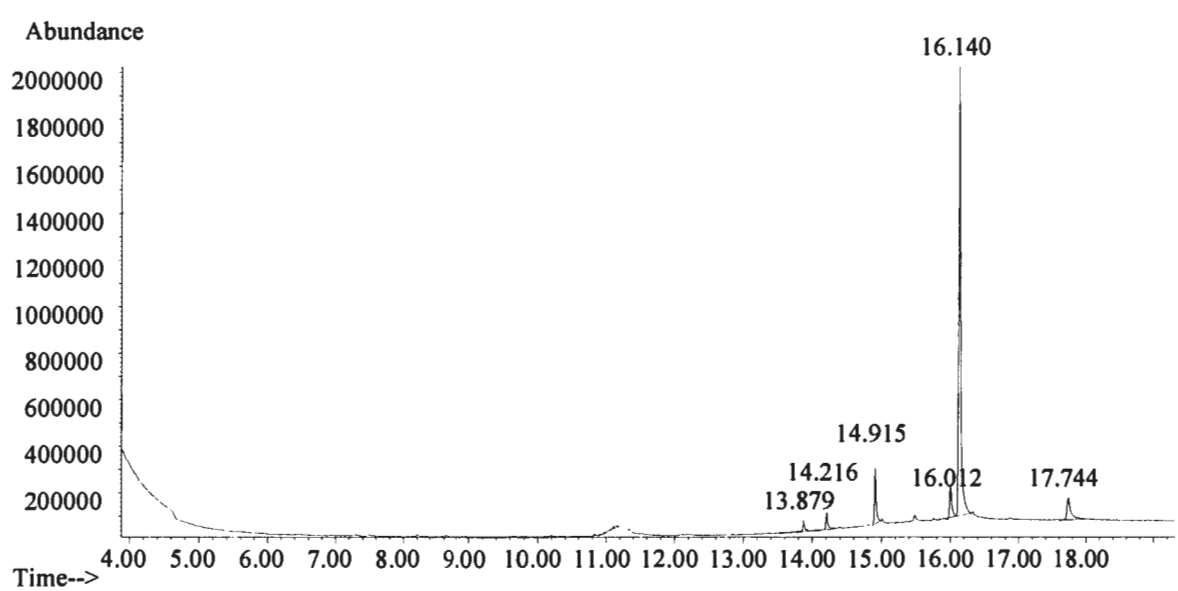


**Figure 4.79** Chromatograms of fraction 2 (eluent= 5% ethyl acetate in hexane) which was chromatographed from entrance tube collected from nest of *T. terminata* in Tenom, Malaysia. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 13.879 = pentacosane, $R_t$ 14.216 = aromatic dicarboxylic acid, $R_t$ 14.915 = heptacosane, $R_t$ 16.012 = nonadecene, $R_t$ 16.140 = hexatricontane, $R_t$ 17.744 = octacosane, $R_t$ = retention time.

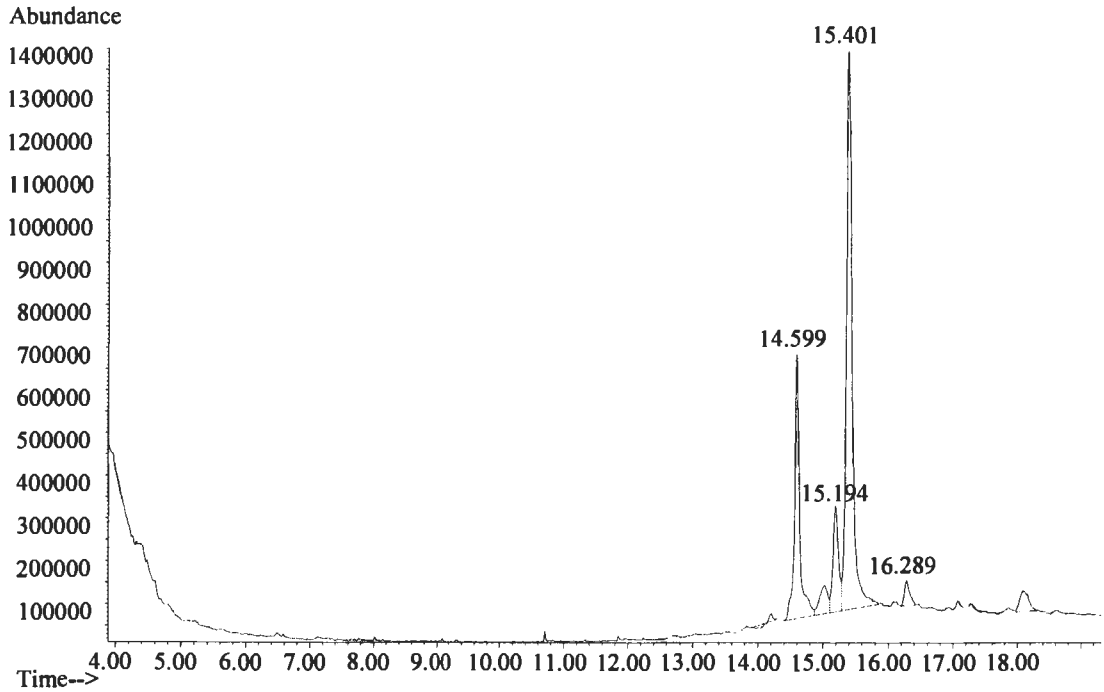


**Figure 4.80** Chromatograms of fraction 5 (eluent= 40% ethyl acetate in hexane) which was chromatographed from entrance tube collected from nest of *T. terminata* in Tenom, Malaysia. Preliminary identification based on WILEY 7N library database. Only main constituents mentioned: $R_t$ 14.599 = triterpene, $R_t$ 15.194 = steroid, $R_t$ 15.401= triterpene, $R_t$ 16.289 = steroid, $R_t$ = retention time.

In summary, the GC-MS data were compared to an identification library and the components, which we frequently found in active fractions were categorized into four groups. Table 4.12 list the substances identified in repellent active fractions.

**1. Terpenoids**

This chemical group is found abundantly in various fractions. The amyrin-like terpenoids frequently found in active fraction of entrance tube of *T. terminata* from Malaysia (80.51% in fraction 5), propolis of *A. mellifera* from Thailand (59.85% in fraction 9 and 73.71% in fraction 4) and from Germany (60.59% in fraction 5) and sticky band of *A. florea* (45.82 %). Sesquiterpene was also found significantly proportion in active fraction from material from *T. collina* nest (24.80%). Steroids were also repeatedly found in many fractions. It was a main component of the active fraction of propolis of *A. mellifera* from Thailand (35.97% in fraction 9 and 26.29% in fraction 4) and from Germany (29.68 %), sticky band of *A. florea* (30.32%) and entrance tube of *T. terminata* collected from Tenom, Malaysia (12.24%).

**2. Long chain hydrocarbon**

Long chain hydrocarbons presented significantly constituent. We found docosane, eicosane, tricosane, tetracosane, pentacosane, hexacosane, heptacosane, nonadecene, octacosane, and heneicosane. Fraction 1 from propolis of *A. mellifera* collected from Germany, contained nearly 100% long chain hydrocarbons. Moreover, fraction 2 of entrance tubes of *T. terminata* from Malaysia, and fraction 2 of propolis from Germany had a very high proportion of hydrocarbons, 97.18% and 76.68%, respectively. The pentane extract of sticky bands of *A. florea* contained about 10% hydrocarbons (10.98%).

**3. Phenol derivatives**

Phenol derivatives were found in fraction 3 of material from *A. mellifera* (96.88 %). It was also found in *A. florea* (4.60%).

**4. Naphthalene derivatives**

These compounds contributed 0.05% in fractions of entrance tubes of *T. collina* and 4.90% in fractions from *T. apicalis*. In addition, the compound which revealed naphthalene groups was found as minute (0.16%) component in fraction 5 of propolis of *A. mellifera* from Germany.

**Table 4.12** Characterized compounds from fraction exhibited repellent activity separated from samples collected from bee nests. The calculations were based on the value of the area under the chromatographic peak.

| Constituents | *Area (%)* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A. florea* | *A. mellifera (THA)* | | | | *A. mellifera (GER)* | | *T. apicalis* | *T.collina* | *T. terminata (MAS)* | |
| | **Pentane** | **F1** | **F3** | **F4** | **F9** | **F2** | **F5** | **F3** | **F5** | **F2** | **F5** |
| **Terpenoids** | | | | | | | | | | | |
| Amyrin-like | 45.82 | | | 73.71 | 59.85 | | 60.59 | | | | 80.51 |
| Sesquiterpene | | | | | | | | | 24.8 | | |
| Steroids (Cyclolanostenol-like) | 30.32 | | | 26.29 | 35.97 | | 29.68 | | 9.42 | | 12.24 |
| **Long chained Hydrocarbon** | | | | | | | | | | | |
| Docosane | 3.95 | 4.00 | | | | 0.50 | | | | | |
| Eicosane | 0.66 | | | | | 0.33 | | 1.65 | | | |
| Hentriacontane | | | | | | 7.61 | | | | | |
| Heptacosane | 3.16 | 43.35 | | | | 30.75 | | | | 8.16 | |
| Hexacosane | | 2.82 | | | | | | | | | |
| Nonadecene | 0.69 | | | | | | | | | 80.46 | |
| Pentacosane | | 8.85 | | | | 13.23 | | | | 1.26 | |
| Octacosane | | 19.68 | | | | 14.94 | | 2.20 | | 7.30 | |
| Tetracosane | 0.88 | 21.30 | | | | | | | | | |
| Tricosane | 1.64 | | | | | 7.32 | | | | | |
| **Naphthalene derivatives** | | | | | | | 0.16 | 4.90 | 10.05 | | |
| **Phenol derivative** | 4.6 | | 96.88 | | | | | | | | |
| **total** | **91.72** | **100** | **96.88** | **100** | **95.82** | **74.68** | **90.43** | **8.75** | **44.27** | **97.18** | **92.75** |

## 4.5 Discussion

### 4.5.1. Repellent activity of bee material against ants

Propolis of *A. mellifera* and some *Trigona* spp. have been mentioned to inhibit bacteria, virus and fungi (Chapter II, page 22-26). But the use as repellent of natural enemies has not been studied. The material which we collected from colonies of social bees was not tested before for its properties to repel ants. So we present the first experimental evidence on its effectiveness. *A. florea* showed a specific response to intruded ants by an increase number of bees on sticky area and increased material deposition on sticky area. These bees responded to the invaded ants by a rise in the activities related to constructing sticky band (Duangphakdee, *et al.*, 2005). This barrier seems to be rather effective as is demonstrated by dead ants which were observered on sticky band (fig. 3.1, personal observation). The experiment compared to other crawling insects, *Tenebrio* larvae showed no significantly reaction. The results also showed that *A. florea* was able to distinguish between two arboreal ant species, *C. rogenhoferi* and *O. smaragdina* (Duangphakdee *et al.*, 2005). Consequently, we were able to demonstrate that *A. florea* reacted specially to *O. smaragdina* and strengthen the sticky band in relation to presentation of *O. smaragdina*. The results from repellent experiments and reaction on weaver ant worker (unpublished data) in *A. andreniformis* are corresponding to those from *A. florea*. Moreover, the *A. andreniformis* is the sister taxon to the dwarf honeybees. Even with lack of data on the specific experiments, we may imply that the small dwarf honeybees categorized in the same group together with *A. florea*. We conclude that the bees perceive a semiochemical (kairomone) of *O. smaragdina*. The advantage of recognition is to save the cost of energy. Bees do not invest on the sticky band where is no *O. smaragdina*.

Our results suggest that some substances of the resin collected and processed by bee colonies hindered the approach of *Oecophylla*. Generally, the resin was more effective to repel ants when it derived from the same geographical region. Accordingly, the samples collected from the Asian bees, *A. florea*, *A. andreniformis* and *Trigona* spp. showed higher repellent activity to Asian weaver ants, *O. smaragdina*. Similarly, samples collected from the nest of European bees, *A.*

*mellifera*, showed higher repellent effect to the ant species from the same region, European red wood ants, *F. polyctena*. This indicated that the repellent index was higher against sympatric predatory ant species. The repellent effect seems to be more conspicuous in the weaver ant, *O. smaragdina*, while the resin processed by the bees seemed to have little or no effect on red wood ants, *F. polyctena*. We found no general repellent principle to repel ants suggested that the repellent activity is a result of a specific adaptation.

*T. apicalis*, *T. terminata*, *T. melanocephala*, *T. laeviceps* and *T. minor* are highly defending themselves against weaver ants (no specific experiments were performed). Their entrance tubes show highly repellent activity against weaver ants. *T. collina* is only one species of *Trigona* in our study whose entrance tube shows significantly lower repellent activity. Their entrance tubes exhibit only a slight repellent effect against weaver ants. Like the honeybees, stingless bees are highly eusocial living in permanent colonies in cavities (Michener, 1974) and have also a common feature to encounter with weaver ants because of overlapping habitat. The differences between these species probably represent recent adaptive divergence forced by ant predations.

The comparative study (table 4.13) suggested that each species had strategy of defense against weaver ants. Although in a heterogenous environment like a tropical forest, each species evolved phenotypic plasticity (Bradshaw, 1965; Stearns, 1992; DeWitt *et al.*, 1998; Van Buskirk and Relyea, 1998; Dahl and Peckarsky, 2002). A comparison of nest structure of social bees related to defense (Table 4.13) revealed differences between the groups with resinous material present (*A. florea, A. andreniformis*, *A. mellifera*, *T. apicalis*, *T. collina*, *T. terminata*, *T. melanocephala*, *T. laeviceps* and *T. minor*) and those without resinous material present (*A. dorsata, A. nuluensis, A. cerana*, *A. nigrocincta*, *A. koschevnikovi* and *A. nuluensis*). Two possible factors may influence the "construction of a barrier" by using resinous material are size of the worker bees and nest architecture.

**Table 4.13** Comparative study of social bee's defensive strategies.

| Bee species | Traits | | | | |
|---|---|---|---|---|---|
| | **Worker body length (mm)** | **Nest site** | **Resinous material barrier** | **Nest Height (m)** | **Nest Visibility** |
| ***A. laboriosa*** | 16.5-17.5 [a] | Tree lib or cliff | absent | <15 | Conspicuous |
| ***A. dorsata*** | 16.5-17.5 [a] | Tree lib or cliff [b] | absent | <15 [b] | Conspicuous [b] |
| ***A. mellifera*** | 10-14 [a] | Cavity | Propolis ? | <2 [b] | Conspicuous [b] |
| ***A. cerana*** | 10-14 [a] | Cavity [b] | absent | <2 [b] | Conspicuous [b] |
| ***A. koschevnikovi*** | 10-14 [a] | Cavity [a] | absent | <5 [b] | Conspicuous [b] |
| ***A. nuluensis*** | 10-14 [a] | Cavity [a] | absent | <2 [b] | Conspicuous [b] |
| ***A. florea*** | 7-10 [a] | Branch of shrub [b] | Sticky band | <5 [b] | Hidden [b] |
| ***A. andreniformis*** | 8-9 [a] | Branch of shrub [b] | Sticky band | <5 [b] | Hidden [b] |
| ***T. thoracica*** | 8 [d] | Cavity | Entrance tube | < 2 | Conspicuous |
| ***T. apicalis*** | <5 | Cavity | Entrance tube | ~6 [c] | Conspicuous |
| ***T. terminata*** | <5 | Cavity | Entrance tube | ~3 [c] | Conspicuous |
| ***T. laeviceps*** | 3-3.5 [e] | Cavity | Entrance tube | ~3 [c] | Conspicuous |

**a** Oldroyd and Wongsiri, 2006, **b** Seeley, 1985, **c** Eltz *et al.*, 2003, **d** Sakagami, 1989, **e** Sakagami, 1978.

Morse and Flottum (1997) reported that colonies of *A. dorsata* are rarely bothered by ants and are successful in repelling them. The *A. dorsata* guard bees protect the nest from arboreal ants by grabbing them or causing them to scurry away (Seeley, 1983). The preliminary observations on the reaction of the *A. dorsata* against worker of weaver ants showed that the workers of weaver ants were bitten to death (unpublished data). We imply that bees which have size as large as *A. dorsata* workers or bigger seem not need the barrier against weaver ants because they win the direct combat. The size of a guard bee probably relates to success winning a direct combat with weaver ants. Workers of *A. cerana* are ~25% larger than those of *A. florea*, and workers of *A. dorsata* are ~50% larger than those *of A. cerana* (Seeley, 1983) (fig. 4.81). Seeley (1983) reported that the workers of these species differ strikingly whereas the drones do not. Possibly, the size of worker bees is a result of natural selection (Seeley, 1983). However, the tropical cavity dwelling species, *A. cerana*, *A. nigrocincta*, *A. koschevnikovi*, and *A. nuluensis*, apparently do not use resinous material as a barrier to protect their nests against ants. These bees have worker size ~10-14 mm (Oldroyd and Wongsiri, 2006) which are much less than *A. dorsata* – about 16.5-17.5 mm (Oldroyd and Wongsiri, 2006). Occasional observations revealed that *A. cerana* may lose a direct combat with *O. smaragdina* and sometimes whole colony of *A. koschevnikovi* fells prey to weaver ants (fig 4.83). Nevertheless, overall Asian cavity dwelling honey bee species survive in the habitat of *O. smaragdina*. Their mode of defense against weaver ants is still unclear. This argument indicated that small size is not the only factor which forces the bees to use resinous material as a barrier.

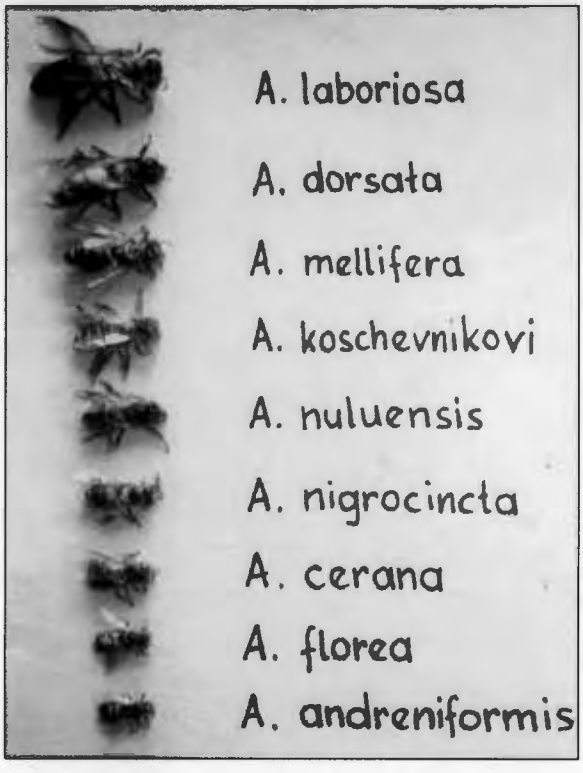


**Figure 4.81** Comparison the worker size of honey bees. Photo by N. Koeniger.

**Figure 4.82** *A. dorsata* nest has no sticky band on the nesting branches. Photo by M. Phiancharoen.

**Figure 4.83** The weaver ants attacked the *A. koschevnikovi* colony by waiting near the nest entrances and captured the bees flying in and out. Photo by O. Duangphakdee.

Other two characteristics that may affect the strength of selection to "barrier construction" phenomenon are the position of nests and the architecture of entrance tube. This related to the variable of predation risk by weaver ants. The position of the nest seems to influence the possibility of encounter with the weaver ants. Bee species which nest in the foraging area of weaver ants would face higher risk of predation. As a result, bee species with arboreal nests were exposed to higher selection pressure caused by weaver ants than ground nesting bees. Dwarf honey bees have open nest on the small trees or shrub (Wongsiri, *et al.*, 2000). They have to face a high probability of encounters with ants and they rely on the specific defense against weaver ants (Duangphakdee *et al.*, 2005). Consequently, *A. florea* perceive weaver ants as a very important predator. They invest high costs on the construction of sticky bands. Because *A. florea* is small bees, therefore, its defense against visually hunting predators relies primary on avoiding detection (Seeley, 1983). Accordingly, it appears that the material of the sticky band favour to repels the scout ants than the massive attack of *O. smaragdina*. *T. collina* nests on the lower part of the tree trunk downed to the ground (Sakagami, 1983; Eltz *et al.*, 2003). This trait probably resulted the species faces less selective pressure than the arboreal nesting bees like *A. florea*, *A. andreniformis*, *T. apicalis*, *T. terminata*, *T. larviceps*, *T. melanocephala* and *T. minor*. Another phenomenon which may influence the occasion of encounter to weaver ants is the architecture of the entrance tubes. *T. collina* builds narrow and long (15-30 cm.) tubular entrances tubes directed upwards (Sakagami, 1983) whereas the other stingless bee species construct funnel like or short tubular shaped nest entrances (see more in details 4.3.2). So the elaborated architecture of the entrance tube of *T. collina* may balance the lower repellent activity against weaver ants. On the other hand, the architecture of the *T. collina* may be also related to another predatory insect, the Assassin bug (Silva and Gil-Santana, 2004) (fig. 4.84).

**Figure 4.84** The Assassin bug (Hemiptera: Reduviidae) at the nest entrance of *T. collina* await for the arrival bees close to the entrance in order to capture them. Photo by O. Duangphakdee.

*T. melanocephala* entrance tube is often found on the base of the crack on the rock or the base on the partition of the house. This position is similar to entrance tube of *T. collina* but much shorter (3-5 cm.). Most likely, that the length and architecture of tube may reflect the strength of predatory selective pressure as mentioned before.

The samples collected from Asian bees show none or a very low repellent effect on European red wood ant, *F. polyctena*. This can be explained by the zoogeographic situation. Natural interactions occur between sympatric species and never between allopatric species. Unanimously, propolis from European bee show more effect on European red wood ants even through the effectiveness is much lower compared to the Asian bee material versus Asian ants. This significant lower effect may be explained with the difference in strength, mode, uniformity of selection, and specificity of the relationship (Thomson, 1982, 1994). Even though, the red wood ants are reported to attack and destroy Apiary in Romania (Prosie, 1959; Morse and Flottum, 1997) and in West Germany (Muthel, 1955; Morse and Flottum, 1997). The red wood ant is still not the serious enemy compare to weaver ants which are the major ant predators of Asian bees (Seeley, 1983). Furthermore, the propolis from *A.*

*mellifera* collected from Germany show also the repellent activity against Asian weaver ant. This probably points out that the non-specific repellent property which found the propolis of *A. mellifera* may be the indirect result from the ant-plants interaction. In summary of predator prey arms race, the intense biotic interactions have produced a greater selection pressure on both predator and prey, thus it results in higher degree of specific adaptation.

**Figure 4.85** The nest entrance of *A. cerana*. No obvious resinous material barrier between the nest and outside. Photo by M. Phiancharoen.

### 4.5.2 Predator-prey arms race

The results indicated that resinous material from colonies of Asian bees, *A. florea*, *A. andreniformis* and *Trigona* spp., strongly repelled weaver ants. The relationship was similar in Thailand and Malaysia. This indicated that the Asian bees perceive ants as a potential threat. The *Oecophylla* is a relatively old genus that prospered especially during the Oligocene (23-34 ma) and Miocene (5 - 23 ma) (Azuma et *al.*, 2006). On the other hand, subfamily Apinae is known from 80 million year ago on late Cretaceous (stingless bees) - the oldest know specimen (Michener and Grimaldi, 1988; Crane, 1999) - and 54.8 to 33.7 million years ago on Eocene epoch (honeybees) (Crane, 1999). Therefore, weaver ants and bees might have

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Encounters with predators, with their concomitant risks of injury and possible death, can be a source of selection pressure (Brodie and Brodie, 1999). The predator / prey arms race is actually an elaborate defensive adaptation of prey (Cott, 1940, Edmunds 1974, Endler, 1986). The defensive trait of preys can be a result from the interaction with predators that drives evolutionary system and occurs at a phenotypic interface that comprises the characters determining the outcome behavior of the prey. These reactions or the arms race resulted in phenotypes which became more elaborate after generations of interaction. Furthermore, the interactions must adapt to favor changes in the opponent and also must likewise generate selection and evolution response (Abrams and Matsuda, 1997). On the other hand, the natural enemies seem to behave in a similar way to improve abilities to be successful (Abram and Matsuda, 1997). Therefore, the prey must improve by escaping from being captured. The encounter between ants and bees were likely to have considerable influences in the

evolutionary shape of each other. In the ant's perspective, attacking the bee colony could be a rewarding goal because the bee colony contains 5000-100,000 individual workers, stored food (honey and pollen) and brood (larvae and pupae) (Michener, 1974, 2000). Vice versa, if ants are predators with high possibility of encounter, the social bees must try as much as possible to protect themselves. Losing against ants would mean a disaster of its species.

### 4.5.3 Results of Arms-Races in Predator Perspective

Predator-prey arms-races produce the selection pressures on both predator and prey (Abrams and Matsuda, 1997). Thus with a strong interaction in tropical area, the results of the reaction should also exhibit in the predator (weaver ants) traits. Nevertheless, the empirical and theoretical evidences suggest that predators may not be forced to evolve in response to prey adaptation, especially the interaction which characterized by asymmetrical selection that prey included arms races (the selection on prey is thought to be stronger than on predators) (Brodie and Brodie, 1999). In contrary, prey almost certainly evolves in response to predation. This is asymmetrical selection which by prey on predators is expected to be much weaker than selection by predators on prey (Brodie and Brodie, 1999). In this case, the adaptive change of social bee traits affected the rate at which they are caught by predator is more substantiation.

### 4.5.4 Bees defense against ants using the resinous material from plants: An analogy to general defense of plants against herbivorous insects?

Plants have evolved the array of mechanical and chemical defense against arthropods which feed on them to minimize damage incurred from feeding (Rhoades, 1979). The chemical defense of plant come from a wide variety of chemicals known as secondary metabolic products (Rhoades, 1979). These chemicals are not an essential part in plant metabolism. So it can be hypothetically explained that some of these chemicals serve as repellents to herbivorous insects (Fraenkel, 1959; Ehrlich

and Raven, 1964). In the comparable cause of events, bee nests have been invaded by the ant predators. The bees evolved also to avoid fitness loss which may include mechanical and chemical defense. According to optimal defense hypothesis, many kinds of chemical defense have been secondarily lost in many termitids, perhaps because it was so "costly" (Pasteels and Gregoire, 1983). Like in social bees, the chemical defense caused a high cost in terms of energy and the material, thus it has been secondary lost. Social bees, however, have developed "corbiculate" (Michener, 2000), which permit an effective mode of food collection. Perhaps by chances they collected some resinous material from plants and then transferred it to intricate nest structures. Nest material of social bee species may be viewed as another analogy to the quantitative defenses of trees which have been selected through evolutionary mechanism for long periods to be a ant defensive material.

### 4.5.5 The Bioassay

Our bioassay is meaningful because it used the natural material and was performed in weaver ant's natural habitat. Therefore, the screened bioactivity is an imitated results from natural event. Noticeably, the bioassay was carried out close to the ant colony in natural habitat (about 5 meters). It is likely that the material of those bees is more effective against ants foraging in area more distant from the ant nest.

The foraging of weaver ants is based on feedback mechanisms in the form of pheromone trails (Hölldobler and Wilson, 1990). Therefore, the consequence designed of bioassay based on the pheromone- foraging model in Pharaoh's ants which study by Beekman *et al.* (2001).

$$\frac{dx}{dt} = (\alpha + \beta x)(n + x) - \frac{sx}{s + x}$$

**Figure 4.86** The model of recruitment pattern (Beekman *et al.*, 2001 ).

$\chi$= total increase, n = no of ants walking to the single food source (colony size), $\alpha$ = probability of ants begin foraging at the food source through searching, $\beta$ = probability of ants begin foraging at the food source leading by pheromone trail, *s*= maximum rate which ants can leave the trial.

The model assumes that the probability that ants begin foraging at the food source depends on both probability per min per individual, that she finds it through independent searching, and the probability per min per individual, $\beta x$, = the rate that x ants following the pheromone and lose it. $\alpha$, $\beta$ and S are constants that depend on the topology of foraging environment and the ability of individual ant to follow the pheromone trail (fig. 4.86). During the foraging period, the constant number of ant walking to the feeder was performed even though the number of ant marking

pheromone is increased (fig. 4.87). In the preliminary study of *O. smaragdina*, our data was found similar stable equilibrium. We found at the stable foraging stage number of ants on ant road is approximately 15-25. As that reason, we picked up this stage (number of ants on the stick is at least 15 ants) to test the bioassay (4.2.1) to minimize the variable on number of ants between tests.

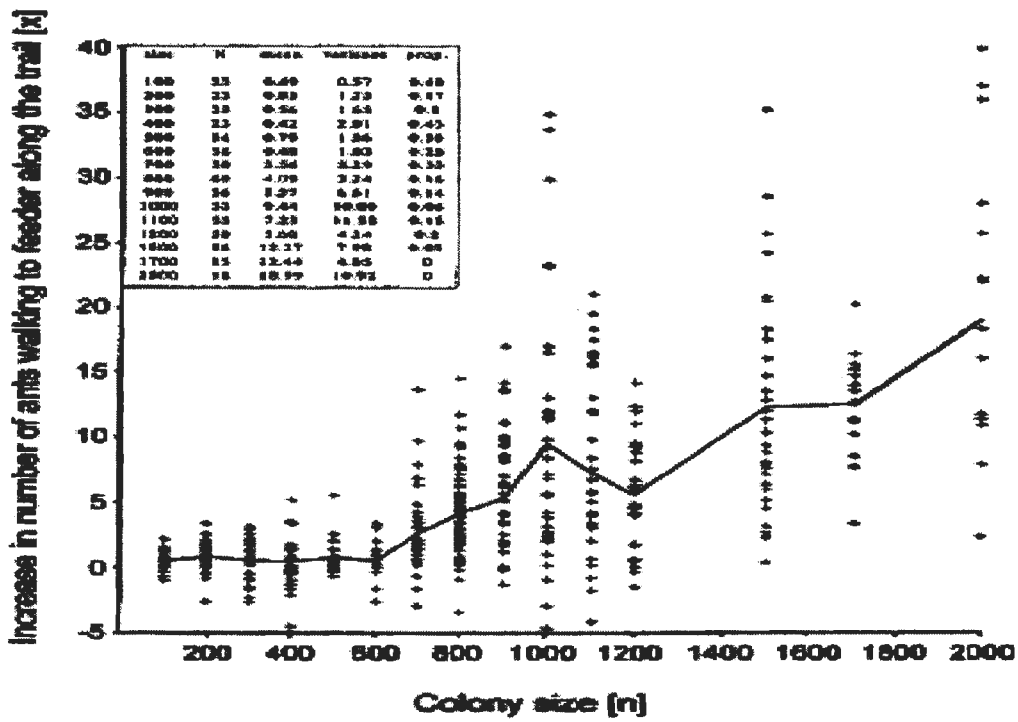


**Figure 4.87** The increase in the number of ants walking to the feeder along the trail in relation to colony size n. The colony size (*n*) vs. the increase in the number of ants walking to the feeder along the trail (x). The line connects the means of all trials at a given colony size. Crosses represent single trial. Also shown (in box) are the numbers of trials per colony size, mean, variance, and the proportion of trials with a mean increase of less than 1 ant per min. (Beekman *et al.*, 2001).

The results from all trials from bee material in same species were correspondent to each other. These indicated that our bioassay is efficient and practical. The entire process takes about 8 hours per experiment to sample of one colony. The test requires a simple system consisted of a platform, a Petri dish, food and ant species. The assay is also available for screening other compounds in natural sources which have an effect on ant activity.

### 4.5.6 Compositions in ant- repellent fractions

We found no activity in polar solvents (acetone, methanol) and all active compounds were dissolved in pentane which is nonpolar. This result is significant because all insects are covered by the epicuticular which is built by hydrocarbons mainly (Bagnères and Morgan, 1991, Elzinga, 1997, ). For penetration of the epicuticular of predatory ants, bees used nonpolar substances. From the GC-MS identification of active fractions, the terpenoids play a major role in the active fraction of two species of *Apis*, *A. florea* and *A. mellifera*, whereas, in *Trigona*, *T. apicalis*, *T. collina* and *T. terminata*, the active fractions showed high diversity in the proportions of terpenoids, hydrocarbons, phenol and naphthalene derivatives. Our results indicated that the material in *Apis* are more uniform in the chemical composition pattern but the material from *Trigona* possess a higher diversity. Probably because the *Trigona* are older than *Apis*, they adapted through longer time. The parallel adaptation may also appeare evidently in high diversity of entrance tubes.

In this study, chemical data accumulated from various species of bees and locations, therefore a conviction that the complex and unpredictable substances were assumed. Under that circumstance, attempts to establish patterns of ant repellent substances may be frustrated. Although, we did not directly test the single compound for repellent experiments, we cannot rule out the possibility that these compounds identified can be ant repellent. According to previously reported, terpenoids are commonly found in plant secretions (Pare and Tumlinson, 1999) and resinous material from bee nest (see table 2.1). Several volatile terpenes were reported that plant released in response to insect herbivory; ocimene (monoterpene) is very active repellent of leafcutter ants *Atta cephalotes* (Hymenoptera, Formicidae, Attini) (Chen *et al.*, 1984). Farnesol, the sesquiterpene alcohol presented in many essential oils in plant, had a high degree of ant repellant activity (Shorey *et al.*, 1992). Neo-clerodane diterpenoids are also significant repellents of the leafcutter ant *Acromyrmex octospinosus* (Chen *et al.*, 1992). Phenol derivatives which carry long side chains

were also one group which widely distributed in plants and commonly found in propolis (table 2.1). Phenolic compounds are known to act as repellents in insects such as salicylaldehyde, volatilic defense, has repellent effect on ants (Pasteels *et al.*, 1986). Naphthalene and derivatives have also known for its insecticidal and repellent properties. Generally, in commerce, naphthalene is used as an antimicrobial, an insecticide, an insect repellent, an anthelmintic and vermicide (Bolton and Eaton, 1968 cited in Daisy *et al.*, 2002). It has been reported as possible chemical in the nest material serve as protection from insects of *Formosan subterranean* termites (Azuma *et al.*, 1996; Chen *et al.*, 1998). Our results suggested that the repellent fraction containing high molecular weight substances (C10-C30) which are presented in significant quantity. Although, low molecular weight compounds with more volatile property have been shown to have stronger effects, but less-volatile or heavier analogues, may retain their activity for longer time. Moreover, with more volatile substances could caused disadvantage to bees because some predators may emit it as a specific allomones (Pasteels and Gregoire, 1983) to locate their prey.

Long chained hydrocarbons typically play a part in species recognition in insects (Hadley *et al.*, 1981; Bagneres and Morgan, 1990). It has also semiochemical fuctions and may be the principal constituent of surface pheromones in insects (Ruther *et al.*, 2002; Abdalla *et al.*, 2003). Until recently, none of literatures mentioned that the long chained hydrocarbons were known to have ant repellent activity. But the intriguing point is that they were found as major components (almost 100%) in two active fractions (Fraction 1 in *A. mellifera* and Fraction 2 in *T. terminata* from Tenom, Malaysia). More investigation on that point would be needed to answer if the long chained hydrocarbons encompass the ant repellent property.

From the chemical literatures, it is possible that the identified compounds are mostly naturally occurring and containing some secondary metabolites of known repellents. More than seven compounds in ant-repellent fractions were not matched with the data from the library. It is possible that these compounds are new substances

and should be marked that they may have the ant repellent property. As several compounds were identified, their importance is mostly unknown, and most are still unrecognized. Consequently, the number of possible mechanisms of their activity is obviously high. Moreover, the synergistic interactions which may occur within the repellent compounds make it more complex. Further exploration of the existence and role of chemicals in ant repellent would help to resolve the questions.