Defensive secretion of Eurycantha calcarata - chemical composition and method of collection

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Article

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Abstract

Chemical defense in insects is becoming an increasingly interesting topic and has the potential for providing unexplored compounds with unknown properties for drug and repellent discovery, so the secretions of different species of insects are being studied and new ways of collecting these secretions are being sought. Silica gel and activated carbon are absorbents that were used to collect the gaseous defensive secretion of Eurycantha calcarata. Using gas chromatography coupled with mass spectrometry, 52 compounds were identified, including 19 carboxylic acids, 14 esters, 10 alcohols, 5 hydrocarbons and other organic compounds. The most abundant two compounds from each group are: hexadecenoic acid, octadecanoic acids, 9-hexadecanoic acid octadecyl ester, hexadecanoic acid tetradecyl ester, octacosanol, triacontanol, tridecane and tetradecane. The silica gel turned out to be a better absorbent because it captured more compounds than the activated carbon. The mass of the absorbent did not affect the quality of the analyses. This paper is the first describing the volatile secretions emitted by Phasmid representatives, not originating from the prothoracic glands. The presented results of the analyses and the known properties of found compounds give grounds for the conclusion that these secretions are of importance for defense in this species of phasmid.

Introduction

Insects usually use mimicry, catalepsy or active attack using their legs, stingers and mouth apparatus to defend themselves[1], but some species use chemical defense to scare away or discourage predators. These insects secrete irritating compounds from specialized structures in gaseous[2] or liquid form[3]. Phasmida is one such order of insects whose representatives use chemical defense to deter a predator although their main defense mechanism is to resemble their surroundings. Insects from this order are dorsoventrally flattened or cylindrical stick-like shaped, with an elongated or flattened body often covered with appendages resembling plant parts such as leaves, branches or bark, with the same color and structure[4,5]. If a predator manages to spot them and decides to attack, some species defend themselves by secreting irritating compounds from their prothoracic glands to scare the predator away[6]. One of the Phasmida species that uses such chemical defense is *Eurycantha calcarata*, also known as the giant spiny stick insect, inhabiting New Guinea, the Solomon Islands and the Bismarck Archipelago[7]. This species, where the females are larger than the males, can reach up to 14 centimetres in length, and the adults of both sexes are dark brown covered with spines. Usually *E. calcarata* avoid predators by blending into the tree bark where they spend most of the day, but when the *E. calcarata* males are irritated or feel threatened they raise their third pair of legs armed with a sharp spike about one centimetre long, they bend their abdomen in the shape of the letter S and release a defensive secretion from undescribed abdominal structures[8] (Fig. 1).

The defensive secretion of this insect has not been studied and described so far and it thus gives the potential to find new compounds with unknown properties. Such secretions could be a source of a new drug discovery[6] and of compounds with unknown effects, as confirmed by previous studies such as those that follow. Parectadial (4S)-(3-oxoprop-1-en-2-yl)cyclohex-1-enecarbaldehyde, for example, was a new unexplored compound found by Dossey[9]. It was a main component of the defensive secretion of *Parectatosoma mocquerysi* and from the author's observations it appears to cause a specific reaction on human skin. After contact with the secretion of this insect, the human skin reddens and with greater exposure exfoliates with no pain or itching observed. 4-methyl-1-hepten-3-one was the main compounds found in the defense secretions of *Agathemera elegans*, a species that is recognized and avoided by residents of Chile, because contact with the defensive secretions of this insect can cause temporary blindness due to acute conjunctivitis and corneal damage[10]. This was the first notation of the natural origin of these compounds. Not only is the chemical composition of the defensive secretions of Phasmid representatives studied but also the properties of the compounds contained in them. 3) Another compound, from the defensive secretions of Phasmids with confirmed repellent properties, is actinidine – iridoid, found in the defensive secretion of *Megacrania tsudai*. Authors claim that this

species stores and secretes it to scare away and discourage a predator, because actinidine occurs in the host plant and it has repellent properties both on spiders and birds[11]. Actinidine was also found in *Megacrania alpheus*[12]. 4) Quinoline found in *Oreophoetes peruana* which repels ants, spiders, cockroaches and frogs has similar properties[13].

The various groups of compounds contained in insect secretions can perform many functions as they are involved in chemical communication, chemical signaling, defense and reproduction[14] . For example, hydrocarbons are one of the better known groups of compounds in insects and they can perform all the above mentioned functions.

In insects defensive secretion, various groups of compounds have been found like carboxylic acids, alcohols, esters, carbohydrates, aldehydes and more. For example, in the defensive secretions of the nymphs and the adults of both sexes in *Pyrrhocoris apterus* 40 components were identified from the nymphal posterior dorsoabdominal glands and 35 from the adult metathoracic glands. From these identified compounds 23 were aldehydes, five saturated hydrocarbons, five alcohols, three ketones, three lactones, two terpenes, one phenol and one ester[15].

Because new, unexplored compounds can be found in the defensive secretions of insects it becomes a popular topic and it has potential for drug and repellent discovery[6], and because of it new ways of collecting secreted compounds are being sought. When the secretion is in the liquid form, sampling seems to be easy because to carry out the analysis of the defensive secretion it needs only to be collected at the necessary volume[13]. The problem begins when the secreted irritants are in a volatile form, when the content of the compounds is unknown and it could be not enough to be detected in analytical techniques. Among the available works on volatile defensive secretions from insects, there are works describing samples collected by placing insects in a glass vial with a rubber septum and after a certain amount of time collecting the volatiles secreted by insects sampled from the head space (HS), and analysed with SPME-CGC[16]. Volatile compounds from the defensive spray of *Megacrania tsudai* after irritation by handling were collected with a microsyringe and directly injected GC-MS for analysis) [11]. Another method of colleting volatile compounds is described by Gunbilig (2009)[17] where a whole insect was enclosed in a glass container and the emitted volatiles were trapped onto charcoal traps while air circulated for 24h. The collected volatiles were eluted with dichloromethane containing n-bromodecane as an internal standard and analysed with GC-MS. In order to collect the defensive secretion from males of *Eurycantha calcarata*, however, a new method of sampling had to be created because this species is large and must be manually irritated to release its secretion, so closing it in a container and collecting a sample from the headspace or from charcoal traps would not bring the desired results. The adult male needed to be annoyed, preferably in the terrarium, because then it was the most likely to deposit the secretion. It was also important to collect samples in an open space such as in the terrarium because when a strong characteristic smell was noticeable, it meant that the insect had deposited the secretion. The use of sorbents to collect these secretions seemed to be the best way to collect the compounds in gaseous form.

Silica gel and activated carbon are two absorbents that differ in pore size, thanks to which they capture various volatile chemical compounds[18]. In this work the chemical composition of the defensive secretion of males *E. calcarata* was analysed and the absorbing properties of the silica gel and activated carbon was compared to see if there were going to be differences in the chemical analyses in terms of the number and amount of detected compounds. This, subsequently, is the first description of the chemical composition of the defensive secretion of *E. calcarata* and the method of collection and extraction of the defensive secretion in gaseous form in representatives of Phasmids with the use of silica gel and activated carbon, was used for the first time .

Results and Discussion

From the analysis of the secretions of adult males of *Eurycantha calcarata*, 52 compounds were identified: 19 carboxylic acids, 11 esters, 10 alcohols, 5 hydrocarbons, glycerol, monopalmitate, monooleate, monostearin, cholesterol, manoyl oxide and 7,9,-di-teter-butyl-1-oxaspiro(4, 5)deca-6,9-diene-2,8-dione. Differences in the occurrence of compounds in the analyses differ due to the way the secretions were collected and samples were extracted. Table 1. shows the sample number (used in tables below) along with a description of the collection, extraction method and number of identified compounds. Table 2 shows the number of compounds which were identified from each group in each analysis.

Table 1
Samples of the defensive secretion of the adult male of *E. calcarata* – sample number, used absorbent, method of extraction and number of identified compounds.

Sample number	Absorbent	Amount	Extraction method	Number of identified compounds
1	Silica gel (terrarium)	1g	evaporation with nitrogen + silylating agent	28
2	Silica gel	1g	evaporation in the air	6
3	Silica gel	1g	evaporation with nitrogen	5
4	Silica gel	2g	evaporation in the air	7
5	Silica gel	2g	evaporation with nitrogen + silylating agent	30
6	Activated carbon	1g	evaporation with nitrogen + silylating agent	16
7	Activated carbon	1g	evaporation with nitrogen + silylating agent	12
8	Activated carbon	2g	evaporation with nitrogen + silylating agent	22
9	Activated carbon	2g	evaporation with nitrogen + silylating agent	18

Table 2
Number of identified hydrocarbons, carboxylic acids, alcohols, esters and other groups of compounds (sample numbers from Table 1.).

Sample number	Absorbent	Amount	Extraction method	Hydrocarbons	Carboxylic acids	Alcohols	Esters	Other compounds
1	Silica gel (terrarium)	1g	evaporation with nitrogen + silylating agent	3	13	8	1	3
2	Silica gel	1g	evaporation in the air	2	1	1	2	0
3	Silica gel	1g	evaporation with nitrogen	1	4	0	0	0
4	Silica gel	2g	evaporation in the air	3	1	0	2	1
5	Silica gel	2g	evaporation with nitrogen + silylating agent	5	15	9	0	1
6	Activated carbon	1g	evaporation with nitrogen + silylating agent	0	4	2	6	4
7	Activated carbon	1g	evaporation with nitrogen + silylating agent	0	11	0	0	1
8	Activated carbon	2g	evaporation with nitrogen + silylating agent	0	14	4	2	2
9	Activated carbon	2g	evaporation with nitrogen + silylating agent	0	13	4	0	1

From Table 1 and 2 it can be concluded that the addition of a silylating agent increased the detectability of the compounds contained in the defense secretion of this insect. Analysing the presence of hydrocarbons in the defensive secretion of *E. calcarata*, we observe differences in the number of detected compounds depending on the absorbent that was used. Silica gel turned out to be a better absorbent because it captured more compounds than activated carbon. The amount of the absorbent used did not affect the quality of the analyses. When analysing the compounds detected in the samples, there are not significant differences in the occurrence of carboxylic acids from samples collected with the different absorbents. In samples 2–4 there are less identified acids. There is also no correlation in the amount of detected acids in relation to the amount of absorbent used. This suggests that the amount of absorbents does not affect the quality of the analyses. It is not possible to determine clear dependencies in the presence of alcohols in the defensive

secretion of *E. calcarata* in relation to the used absorbents and their amounts. There are also no clear dependencies in the presence of esters in relation to the amount and type of the absorbent used.

Tables 3–7 shows the identified compounds in each analyses. The most numerous groups of compounds from all the analyses were carboxylic acids (Table 2), 19 different compounds were identified and the most abundant were hexadecanoic acid (Fig. 2) and octadecanoic acid (Table 3). Carboxylic acids have been found in the defensive secretions of insects. In *Carabus lefebvrei* pupa, carboxylic acids occur in the glandular secretion. Authors claim that the compounds including carboxylic acids found in the pygidial gland secretion have been regarded as the deterrent against predators[19]. Hexadecanoic acid is an organic chemical compound from the group of fatty acids, a solid substance. Excess carbohydrates are converted into hexadecanoic acid, which is a precursor to the production of longer-chain fatty acids[20]. It is assumed that the high amount of hexadecanoic acid is related to its function as a precursor for the production of longer chain fatty acids and that it may be involved in metabolic processes leading to the formation of other compounds. Octadecanoic acid, also known as stearic acid, is a white waxy solid with a mild tallow-like odour. Its esters are found in animal fats[20]. It probably performs the function of storing active substances because it is a solid and active compounds can be suspended in it. Among other insects, hexadecanoic and octadecanoic acids were also present in the highest amount in the defensive secretion of the bug *Graphosoma lineatum* (Hemiptera; Pentatomidae)[21].

Table 3
Percentage content of identified carboxylic acids (sample numbers from Table 1.).

Compound	1	2	3	4	5	6	7	8	9
2-hydroxypropanoic acid	-	-	-	-	-	-	2,50	-	-
Hexanoic acid	0,35	-	-	-	-	-	1,91	3,83	3,38
Heptanoic acid	0,10	-	-	-	0,06	-	-	0,76	1,01
Nonanoic acid	-	3,29	0,83	1,91	2,38	-	7,61	8,29	9,40
Octanoic acid	0,68	-	-	-	0,50	-	-	3,06	-
Decanoic acid	-	-	-	-	0,21	-	0,24	0,55	0,53
Adipic acid	-	-	-	-	-	-	1,70	-	-
Dodecanoic acid	0,21	-	-	-	0,04	-	-	0,20	0,31
Azelaic acid	-	-	-	-	0,22	-	2,03	0,29	0,53
Tetradecanoic acid	0,37	-	4,91	-	0,22	-	0,77	0,69	0,94
Pentadecanoic acid	0,33	-	-	-	0,36	-	0,53	0,82	0,79
Hexadecanoic acid	11,82	-	24,92	-	10,47	8,45	35,90	28,86	34,70
Heptadecanoic acid	0,51	-	-	-	0,60	0,34	0,59	1,16	0,71
Octadecanoic acid	8,55	-	10,79	-	5,74	9,83	31,98	19,12	26,67
Eicosanoic acid	0,55	-	-	-	0,62	4,95	-	0,92	0,81
Heneicosanoic acid	0,15	-	-	-	-	-	-	-	-
Docosanoic acid	1,27	-	-	-	1,76	-	-	1,96	2,14
Tetracosanoic acid	-	-	-	-	0,44	-	-	-	-
Hexacosanoic acid	0,42	-	-	-	0,20	-	-	-	-

Esters were the second most abundant group of compounds in the analyzes (Table 2). 9-hexadecanoic acid octadecyl ester (Fig. 3) and hexadecanoic acid tetradecyl ester had the highest percentage content (Table 4). Little is known about the octadecyl ester of 9-hexadecanoic acid and, so far, it has not been found in the defense secretions of insects. The only mention of its occurrence among these organisms is the record of its presence in *Ceratitis capitata* both before and during the breeding period[22]. Hexadecanoic acid tetradecyl ester, or myristyl palmitate, is referred to as a xenobiotic metabolite of bacterial and fungal origin. It comes from tetradecan-1-ol[20]. The presence of this compound in the secretion of *Eurycantha calacarata* may indicate the presence and use of endosymbionts (bacteria or fungi) in this insect. The second possibility is its origin as a xenometabolite from host plants and only its accumulation by phasmids. Determining the origin and possible functions of this compound in *E. calcarata* requires further research. 9-octadecanoic acid 9-octadecyl ester, 9-hexadecanoic acid eicosyl ester, 2,3-hydroxy tetradecanoic acid propyl ester, and hexadecanoic acid octadecyl ester are compounds that are not well understood. There is no information on their occurrence in plants and animals, they have not been identified as compounds found in insects, nor is there information on their occurrence in insect defensive secretions. The presence of these esters was found in only one analysis, but after verification and confirmation of their presence, this would be the first finding of the presence of these substances in insects. The main problem here is to determine whether these are esters that may come from background impurities. Some of the esters identified in the analyses have previously been shown in insects. This includes isopropyl myristate, found in three analyses. It is a polar compound that is used as a moisturizer in topical cosmetics and medical preparations to improve skin absorption. Isopropyl myristate has been extensively researched and used as a skin penetration enhancer[20]. Among insects, it has been detected in analyses of extracts from the head and the body of the ant *Iridomyrmex humilis*[23,24]. Its potential function in the phasmid may be to increase the absorption of irritating substances, thus strengthening their effect on the attacker.

Table 4
Percentage content of identified esters (sample numbers from Table 1.).

Compound	1	2	3	4	5	6	7	8	9
Isopropyl laurate	-	0,15	-	-	-	-	-	-	-
Dodecanoic acid 1-methylethylene ester	-	-	-	0,12	-	-	-	-	-
2,2,4-Trimethyl-3-carboxy isopropyl pentanoic acid isobutyl ester	0,47	-	-	-	-	-	-	1,91	-
Isopropyl myristate	-	0,57	-	0,64	-	-	-	0,21	-
2,3-Hydroxy tetradecanoic acid propyl ester	-	-	-	-	-	2,02	-	-	-
Hexadecanoic acid tetradecyl ester	-	-	-	-	-	5,17	-	-	-
9-hexadecanoic acid octadecyl ester	-	-	-	-	-	7,55	-	-	-
Hexadecanoic acid octadecyl ester	-	-	-	-	-	2,33	-	-	-
9-octadecanoic acid, 9-octadecyl ester	-	-	-	-	-	4,69	-	-	-
9-hexadecanoic acid eicosyl ester	-	-	-	-	-	4,90	-	-	-

From the analyses the two most abundant alcohols are octacosanol (Fig. 4) and triacontanol (Table 5). Researchers describe the alcohols found in insect defense secretions as compounds found in smaller amounts and they do not assign them a greater role in it; however, some insects use alcohols for defense, though not directly. The larvae of many beetles Chrysomelidae sequester phenolglucosides such as salicin from their food plants like Salix and Populus spp. Salicin is hydrolysed in the glandular reservoir of the defense glands. The resulting salicylic alcohol (saligenin) is

oxidized by extracellular oxidase. The product, salicylaldehyde, accumulates as the main defense compound of these beetles[25]. It seems that alcohols may be used in a similar way by *Eurycantha calcarata*, but this requires further research and determining whether they are only accumulated, or whether they are subject to further metabolic transformations. Octacosanol is a straight-chain aliphatic fatty alcohol that is used as a nutritional supplement. This organic compound is the main component of a natural product wax extracted from plants. Octacosanol is reported to possess cholesterol-lowering effects, antiaggregatory properties, cytoprotective use, and ergogenic properties. It has been studied as a potential therapeutic agent for the treatment of Parkinson's disease[20]. Triacontanol is an ultra-long-chain primary fatty alcohol[20] that has shown significant inhibition of feeding activity against the insects, *Spilosoma obliqua* and *Spodoptera litura*[26]. It is possible that insects use it as a deterrent.

Table 5
Percentage content of identified alcohols (sample numbers from Table 1.).

Compound	1	2	3	4	5	6	7	8	9
Dodecanol	0,21	0,88	-	-	0,22	-	-	0,33	0,22
Tetradecanol	0,14	-	-	-	0,21	-	-	-	-
Hexadecanol	0,33	-	-	-	0,37	0,16	-	0,63	0,42
Oktadecanol	-	-	-	-	0,29	2,58	-	-	-
Eicosanol	0,06	-	-	-	-	-	-	-	-
Docosanol	0,47	-	-	-	0,59	-	-	-	-
Hexacosanol	0,78	-	-	-	1,37	-	-	-	-
Octacosanol	8,97	-	-	-	12,77	-	-	10,64	8,21
Triacontanol	5,98	-	-	-	8,38	-	-	5,99	3,62
Dotriacontanol	-	-	-	-	2,24	-	-	-	-

The hydrocarbons occurring in the largest amounts turned out to be tridecane (Fig. 5) and tetradecane (Table 6); although these two compounds were present in the control sample, they were more than twice as high in the defensive secretion samples (Table 6). It is assumed that in a closed terrarium where insects are living, volatile compounds emitted by them will also be present in the atmosphere of the insectarium. Tridecane is a straight-chain alkane with 13 carbon atoms and it is a component of essential oils isolated from plants. In its pure form it is an oily, yellow, transparent liquid with a characteristic smell. Repeated or prolonged contact of tridecane with the skin may irritate it and cause redness, leading to inflammation. Exposure to high vapour concentrations can cause headache and stupor[20]. In insects tridecane occurs in the volatile secretion of the bug *Cosmopepla lintneriana* (Hemiptera; Pentatomidae)[27]. The Pentatomidae family are known for using volatile odorants as a predator deterrent, which they release when threatened[28]. The secretion of this bug, consisting mainly of tridecane, acts as a deterrent to birds and lizards[27]. Tridecane was also found in *Graphosoma lineatum* secretions. It has been identified as a toxic, irritant or repellent compound, that is released by these bugs in response to irritation, and it is believed that the secretion of this compound by the insect also acts as a predator deterrent[21]. This alkane was also found in the secretion from the metatoracal glands of another Pentatomide species – *Piezodorus guildinii*, where it performed the same functions. Tridecane is known as a toxin, irritant or repellent released by Pentatomidae in response to irritation. This suggests that this compound acts as a chemical defense for these species[29]. Tridecane was present in both sexes in the defensive secretion of *Parastizopus transgariepinus*[30]. Chemioreaction experiments shows that tridecan was attractive to females but not males, which indicates that tridecane could also be a sex pheromone. Tetradecane is a straight chain alkane consisting of 14 carbon atoms. It is a natural

compound found in plants in essential oils, known to be s secondary plant metabolite[20]. Interestingly, it has recently been shown[31] that the amount of volatile tetradecane in maize roots increases after the invasion of the larvae of the beetle *Holotrichia parallela* (Melolonthidae). These studies show that tetradecane can act as a signal to induce defenses and prepare plants for the following attacks, including metabolic changes[31]. It was also shown that the growth of *H. parallela* larvae was inhibited when fed with maize roots after exposure to tetradecane. This compound can be extracted from plants and used by *E. calcarata* as a compound to deter potential predators. There is also the possibility that tetradecane is being used as a signal of impending danger to other individuals. Both tridecane and tetradecane were also present in the protective secretion of ground beetles (Carabidae) of the following genera: *Brachinus*, *Stenaptinus*, *Metrius*, *Goniotropis*, *Pachyteles*, *Ozaena* and *Homopterus*[32].

Table 6
Percentage content of identified hydrocarbons (sample numbers from Table 1.).

Compound	1	2	3	4	5	6	7	8	9
Dodecane	0,68	-	-	1,76	0,83	-	-	-	-
Tridecane	0,93	2,62	-	2,95	1,33	-	-	-	-
Tetradecane	21,71	59,86	23,39	66,05	34,14	-	-	-	-
Heptacosane	-	-	-	-	0,47	-	-	-	-
Hentriaceontane	-	-	-	-	0,97	-	-	-	-

Table 7
Percentage content of identified compounds from other groups of compounds (sample numbers from Table 1.)y.

Compound	1	2	3	4	5	6	7	8	9
Glycerol	6,11	-	-	-	3,63	0,48	14,24	3,34	4,41
7,9,-di-teter-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	-	-	-	-	-	-	-	1,12	-
Manoyl oxide	-	-	-	0,54	-	-	-	-	-
Monopalmitate	1,36	-	-	-	-	9,81	-	-	-
Monooleate	-	-	-	-	-	2,82	-	-	-
Monostearin	0,16	-	-	-	-	-	-	-	-
Cholesterol	-	-	-	-	-	21,44	-	-	-

Conclusions

This paper is the first to describe the volatile secretions produced and emitted by Phasmid representatives not originating from the prothoracic glands. For the first time, absorbents such as silica gel and activated carbon were used to collect defensive secretions in a gaseous form from an insect of considerable size, which had to be annoyed for handling. Silica gel turned out to be more effective in detecting organic compounds. The addition of a silylating agent facilitated the chromatographic analysis as the compounds became more volatile and the chromatographic signals were better resolved. Gentler evaporation of the solvent did not significantly affect the quality of the analyses because highly volatile compounds were not detected in the analyses of extracts evaporated in the air.

The presented results of the analyses and the known properties of compounds with proven irritating effects, such as tridecane found in the secretions, give grounds for the conclusion that this secretion is of defensive importance for this species of phasmid. It can be concluded that this insect uses the structures at the end of the abdomen to chemically deter the predator. Research on the chemistry of phasmids may also provide new insights into their phylogenetic relationships. Limonene – a compound with a structure similar to parectadial found in the secretion of *Parectatosoma mocquerysi* – was also found in *Sipyloidea sipylus*– an unrelated stick insect from Southeast Asia[9]. Researchers speculate that similarities in the chemical composition of the secretions of these insects may indicate the relationship of species from different taxonomic groups and shed new light on the evolution of these insects.

Compounds from the defensive secretions of Phasmid representatives have a described deterrent effect on various groups of animals. For example, tridecane, found in the defense secretion of *E. calcarata*, has been described as an irritating repellent compound. In the era of searching for new methods of plant protection that do not have a negative impact on the environment, compounds found in nature that have a deterrent effect on popular crop pests such as insects, birds and snails are sought. Tridecane and tetradecane are compounds found in plants as secondary metabolites that could be used as plant protection products due to their deterrent effect and mobilizing the plant response to invasion[31]. Further research should be conducted on the potential of using compounds from insect defense secretions in plant protection products and on how they impact the insects[33].

Methods

Eurycantha calcarata. Insects used for the analyses were obtained from breeding started in 2019, with the third generation used for the study. Eggs were from sexual reproduction and were kept in a glass incubator at 21–25 °C temperature and 75–85% humidity until they hatched. Young individuals were transferred to a glass terrarium with coconut peat, a glass bowl with fresh water and food plants. From the moment of hatching, the insects were fed with ivy (*Hedera helix* Linnaeus, 1753 it is a common species not under the protection and collecting it did not require permits) and kept in a 21–25°C temperature with a humidity of 70–85% until they reached maturity.

Sample preparation and chemical analysis. Bedford (1975)[8] in his work mentions that a strong-smelling fluid is secreted from unknown structures associated with the males copulating block. This observation was not confirmed by any other researchers. There are no histological and anatomical reports about this structure of *E. calcarata*, so to make sure that the secretion is released from the abdominal structures the vial was put at the end of the abdomen when the male *E. calcarata* was irritated. After characteristic behaviour and a strong smell, the vial was closed and after a week was opened to see if the scent was noticeable. The smell was strong, and no fluid residue was on the vials glass, which indicates the gaseous form of the secretion. For this reason, it was decided to use two absorbents – silica gel and activated carbon – for the sampling.

The defensive secretion from the adult male was collected by placing the end of the insect's abdomen at the irritation time into the vial with the absorbent, when the characteristic smell was perceptible; the insect then deposited the secretion into the vial. Each sample consisted of three deposits of secretion into the vial. In order to determine what chemical compounds there were in the terrarium, a vial containing 1 gram of silica gel was put into the terrarium and left there for exactly the same amount of time that it took for the samples to be taken from the insects - namely 8 minutes.

To obtain a filtrate for further analysis, the absorbents had to be rinsed with dichloromethane. In order for the extract to be as concentrated as possible, it was necessary to determine what would be the smallest amount of dichloromethane needed to rinse the absorbent to obtain the filtrate. This is why various amounts of dichloromethane were added to 1 and 2 grams of the silica gel and activated carbon, and it was found that to 1 gram of silica gel should be added 3 ml of CH_2Cl_2, to 2 grams of this absorbent – 5 ml, for 1 gram of activated carbon – 4.5 ml and to 2 grams – 9 ml of CH_2Cl_2.

After shaking for two minutes, the solution from each vial was filtered through a paper filter and the obtained extracts were poured into smaller vials. In order to check which extraction method would prove to be the most effective and will show the most compounds, four silica gel extracts were prepared for analysis in different ways. To minimize the risk of evaporation of the more volatile compounds, the extract from 1 and 2 grams of silica gel was evaporated in the air and analysed. The other two extracts were evaporated under nitrogen. All the extracts were subjected to the derivatization process. A mixture of 100 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide ($CF_3C[=NSi(CH_3)_3]OSi(CH_3)_3$) was added to each sample weighing approximately 1 mg. The samples were heated for 1 h at 100 ℃.

The analytical technique used to determine the occurrence of hydrocarbons in each sample was gas chromatography coupled with mass spectrometry. The Shimadzu GCMS-QP-2010-SE gas chromatograph coupled with the QP-2010 SE (Shimadzu Corporation, Kyoto, Japan) quadrupole mass detector was used to analyse the compounds obtained from the insects. The samples were introduced through the gas chromatograph equipped with a ZB-5 capillary column, 30 m x 0,25 mm x 0,25 µm (Zebron, Phenomenex, USA). Helium was the carrier gas and the process occurred in the temperature range of 60 (held for 3 min.) to 310 ℃ at a rate of 4 ℃/min. The ion source was 200 ℃. The transfer line and injector were heated to 310 ℃. Identification of the compounds was made based on the retention time, the obtained masses spectra and comparing them with the data in the spectrum library[34,35].

Declarations

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Author Contribution Statement

W.K., J.S. and M.G. conceived and designed the experiments.

W.K. and M.G. performed the experiments, analyzed the data and wrote the article.

Competing interests

The authors declare no competing interests.

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Statement

this study complies with relevant Polish guidelines and legislation. *Hedera helix* was taken from a private breeding, it is a common species not under the species protection and collecting it did not require permits. The identification of the plants was made by one of the authors - Weronika Koczur. Specimen copy of this material has not been deposited in a public herbarium.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article.

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Figures

Figure 1

Adult male of *Eurycantha calcarata* releasing defense secretion.

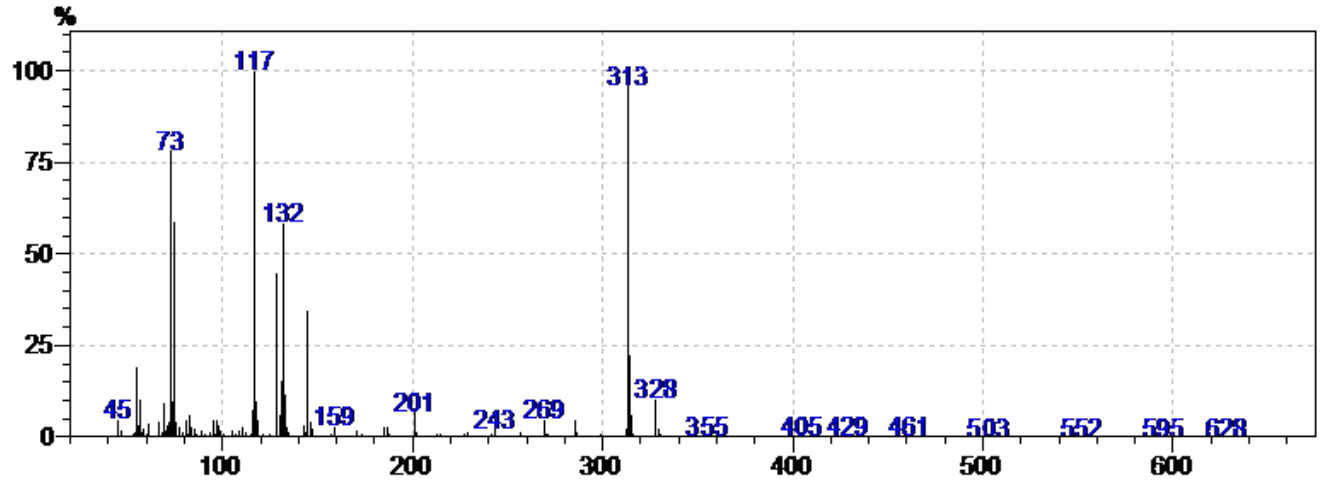

Figure 2

Mass spectrum of hexadecanoic acid

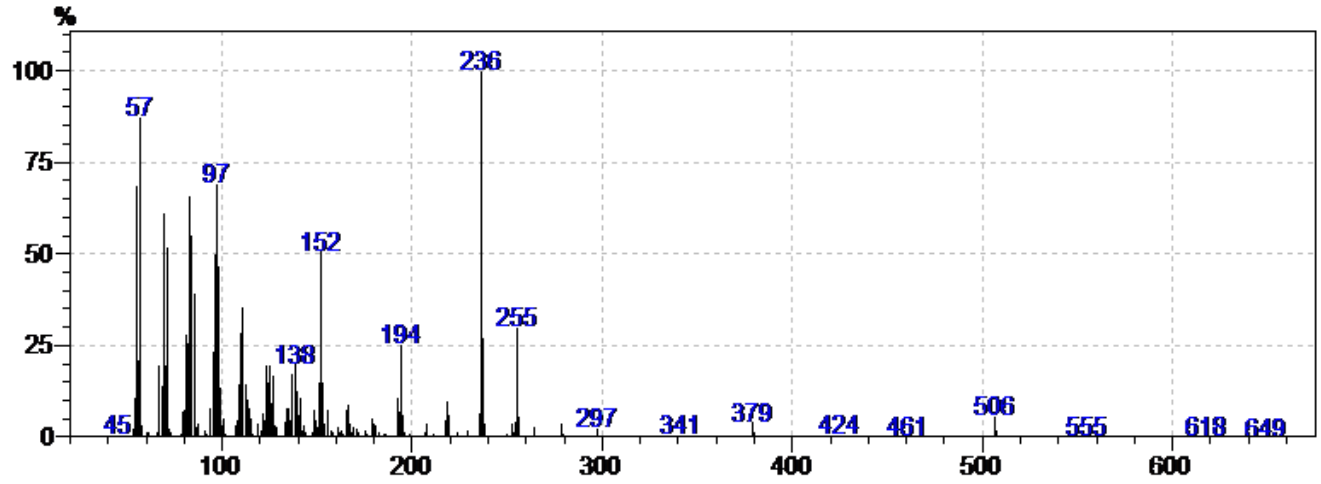

Figure 3

Mass spectrum of 9-hexadecanoic acid octadecyl ester

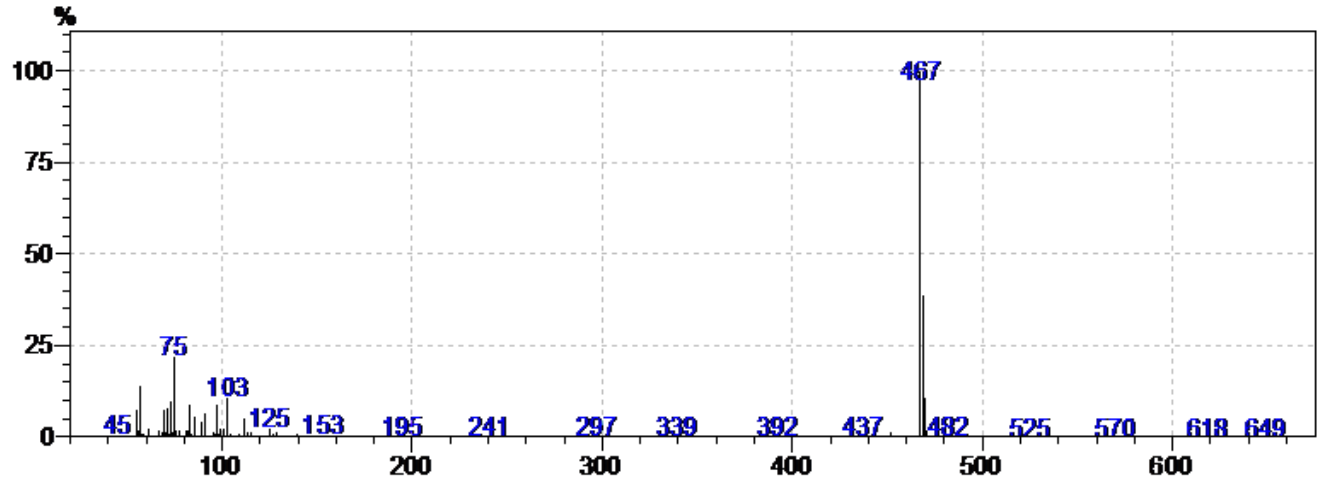

Figure 4

Mass spectrum of octacosanol

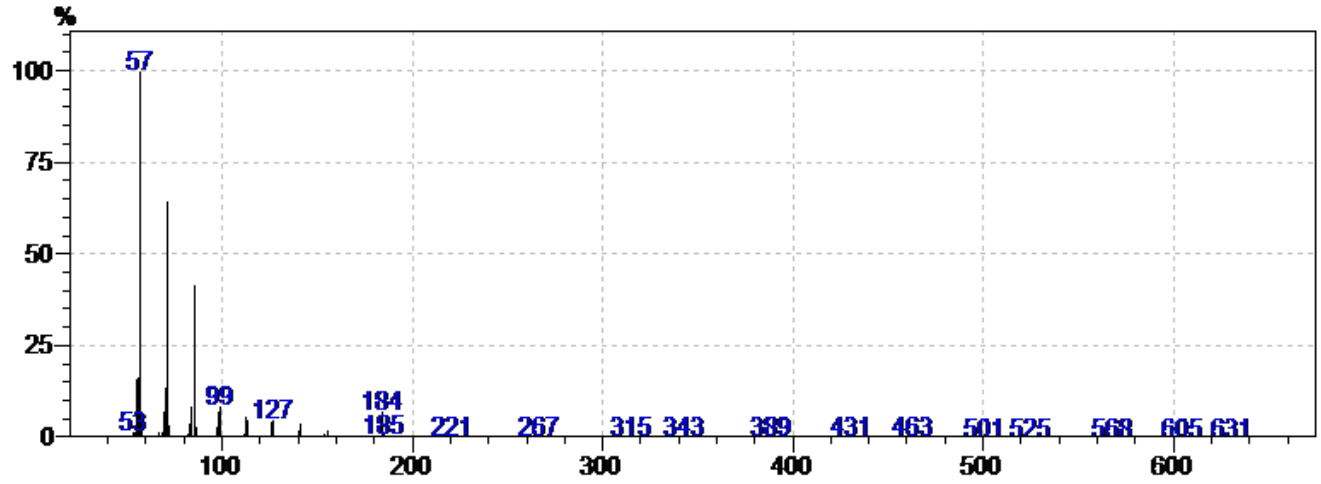

Figure 5

Mass spectrum of tridecane