WHY DO POLLINATORS BECOME "SLUGGISH"? NECTAR CHEMICAL CONSTITUENTS FROM *EPIPACTIS HELLEBORINE* (L.) CRANTZ (ORCHIDACEAE)

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Abstract. Eight populations of *Epipactis helleborine* (L.) Crantz originating from the area of Lower Silesia in Poland (Central Europe) were examined in respect to composition of their nectar and its influence on the insect attraction in field conditions. The chemical composition of *Epipactis helleborine* (L.) Crantz nectar was studied by means of GC/MS SIM. A number of compounds with potential narcotic properties were identified in the nectar, namely 3-{2-{3-{3-(benzyloxy)propyl}-3-indol, 7,8-didehydro-4,5-epoxy-3,6-d-morphinan and oxycodone. Pollinator and visitor insects were identified. The key role of ethanol in the process of alluring and stunning of the insects was discussed. A scheme of the influence of the identified compounds on the pollinators was proposed.
Keywords. *Epipactis helleborine, toxic nectar, morphinan derivatives, indol derivatives, ethanol*

Introduction

Various species of plants attract their pollinators in a variety of ways, namely releasing specific attractants or offering the plant rewards as e.g. oils, stigmatic exudates, pollen [17] and nectar secretion [44]. Nectar does not perform any other role in the flowers of angiospermous plants apart from alluring the pollinators [20]. The main ingredient of the nectar in the majority of plants is sugar: glucose, fructose and saccharose [3, 47], which amount ranges from 50% to 75% [16]. Apart from it, it contains amino acids, lipids, organic acids, as well as various vitamins, enzymes, antioxidants, mineral ions and secondary metabolites [2, 17, 32].

Therefore, the aim of our research was to determine, which chemical compounds are responsible for alluring insects and testing the hypothesis, if the key role in the process of alluring the insects might be ascribed to ethyl alcohol or perhaps to other chemical compounds, that can have "narcotic" properties, as it was found for other plant-insects interactions for example in *Datura* species [8, 37, 42] and orchids such as *Cypripedium* sp. [5], *Cryptostylis* sp., *Dendrobium* sp., *Polyradicion* sp. that can have "narcotic" properties [1, 9].

Review of literature

So far, the research on the composition of sugars and amino acids in the nectar of orchids including *Epipactis* orchids was conducted by Pais & Chaves das Neves [39] and Pais *et al.* [40]. According to the literature, nectar produced by the *Epipactis helleborine* (L.) Crantz contains also ethanol [13, 31, 34]. Kevan *et al.* [27] and Ehlers & Olsen [13] found that ethanol is most likely of microbial origin, produced by microorganisms that expanded in the nutrient medium rich in sugars and amino acids, which is nectar. Probably these microorganisms reach the lip together with the pollinators which are visiting the flowers [13].

Literature data on the composition of secondary metabolites of the nectar of *Epipactis helleborine* (L.) Crantz are scarce and insufficient.

Materials and methods

Eight populations of *Epipactis helleborine* (L.) Crantz growing under various habitat conditions in the area of Lower Silesia (South-Western Poland – Middle Europe) were chosen. Studies were performed at the sites of: Srebrna Góra, Karpacz, Kletno, Kotowice-Siechnice, Gozdnik, Krowiarki Mts., Orlickie Mts. and The Stolowe Mts. National Park. The size of the studied populations averaged from 69–428 individuals. The research was conducted during the years 2001–2004. Nectar was collected during the maximal secretion by the plants, namely between 11:00 a.m. and 15:00 p.m. Composition of the nectar was fairly stable amongst the eight populations.

Nectar was collected from flowers by using capillary and methylene chloride as eluent. Then the methylene chloride extract was dried over anhydrous Na_2SO_4 and evaporated up to volume 0.2 ml followed by analysis GC-FID and GC-MS. Thus, sample of extract was injected into ELWRO N504 gas chromatograph equipped with flame ionization detector (FID). The instrumental parameters and operational conditions were as follows: fused silica capillary column (60 m × 0,25 mm i.d.), a film thickness of 0.25 µm with a temperature program: 50 °C to 300 °C at rate of 3 °C/min, with nitrogen as carrier gas (about 1.2 cm^3/min).

Each analysis was performed in triplicate to assess the reproducibility of results.

For the purpose of definition of compounds present in the sample, extract was analysed by means of Hewlett Packard 5973 GC-MS system.

Nectar’s influence on the behaviour and activity of the insects were examined under natural conditions. Microcapillary tubes containing collected nectar were placed in field conditions at a significant distance from *Epipactis helleborine* (L.) Crantz plants in order to observe insects interest in these capillary tubes. Observations were made from 11:00 a.m. to 15:00 p.m. from 15 July to 28 August 2001, 2002, 2003 and 2004. All possible pollinators and vectors were caught, identified by specialists and deposited in Department of Zoology, Wrocław University. The plant vouchers were kept in Herbarium, Department of Plant Systematics and Phytosociology, University of Wrocław.

Results

Chromatographic analysis of methylene chloride extracts of nectar revealed the presence a set of well separated analytical signals (*Fig. 1*). Chemical formulas of compounds found in the nectar of *Epipactis helleborine* (L.) Crantz and discussed in this work are shown in *Fig. 3*.

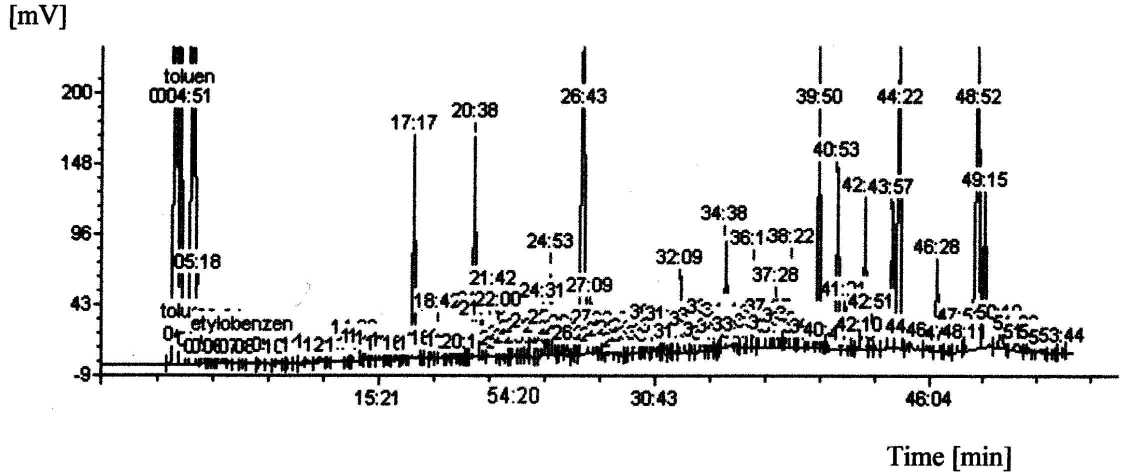


__Figure 1.__ GC-FID chromatogram of the nectar components contained in Epipactis helleborine (L.) Crantz

In order to identify individual substances the extract was examined by means of GC-MS. As seen from *Fig. 2*, the applied conditions resulting from the differences of the equipment used, gave different chromatograms. Methylene chloride extract of the nectar contains over 100 chemical species, which belong to the class of polar chemical substances. The identification of extract components was possible by searching for ions 72, 77, 91, 93, 95, 96, 103, 107, 108, 110, 121, 122, 126, 131, 134, 135, 136, 138, 150, 152, 153, 154, 168, 170, 180, 194 characteristic for indoles, alcohols, phenols, alkaloids, terpenes and individual substances were identified basing on NBS75K and NIST MS libraries.

Both chromatograms indicate a wide variety of compounds present in the nectar. Among them, set of derivatives of phenol, eugenol, indole and as well as derivatives of morphine were identified. Also occurrence of numerous of carboxylic acids was determined. Substances that occur in the nectar in higher amounts were identified and are shown in *Table 1*.

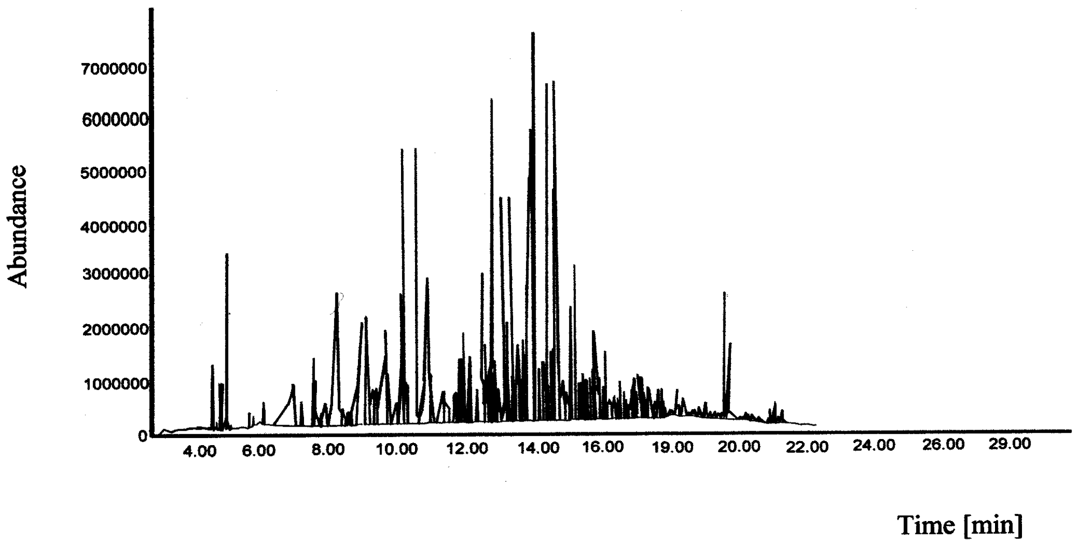
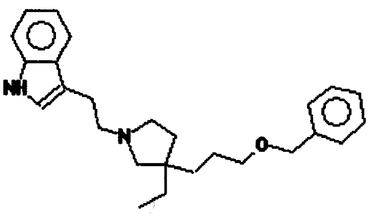


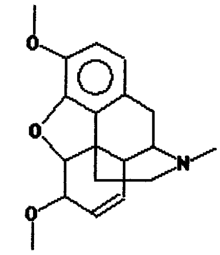
__Figure 2.__ GC-MS chromatogram of the nectar components contained in Epipactis helleborine (L.) Crantz

Table 1. *Nectar chemical composition of Epipactis helleborine (L.) Crantz*

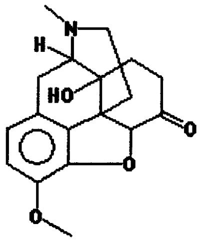
chemical	molecular formula	properties
3-{2-{3-{3-(benzyloxy)propyl}-3-indole	$C_{26}H_{34}N_{2O}$	antimicrobial [12], overpowering substance
xanthatin	$C_{15}H_{18}O_3$	antimicrobial [18]
7,8-didehydro-4,5-epoxy-3,6-D-morphinan	$C_{19}H_{23}NO_3$	soporific, sedative, narcotic [4, 46]
4,5*a*-epoxy-14-hydroxy-3-methoxy-17-methyl-morphinan-6-one (oxycodone)	$C_{18}H_{21}NO_4$	narcotic [41, 51]
androstane-3,17-dione	$C_{19}H_{28}O_2$	hormone
2-furancarboxyaldehyde (furfural)	$C_5H_4O_2$	toxic [22, 23], insect attractant, characteristic "almond/benz-aldehyde" odour
2(5*H*)-furanone	$C_4H_4O_2$	antibacterial [30]
3-methyl-1,2-cyclopentanedione	$C_6H_8O_2$	—
2,6-dimethoxy-phenol (syringol)	$C_8H_{10}O_3$	antimicrobial [24, 33]
2-metoxy-4-(2-propenyl)-phenol (eugenol)	$C_{10}H_{12}O_2$	antifungal [36, 48], antimicrobial [38], insect attractant [26, 43, 50]
2,6-dimethoxy-4-(2-propenyl)-phenol (methoxyeugenol)	$C_{11}H_{14}O_3$	antifungal, antimicrobial [19], insect attractant [26]
benzylalcohol	C_7H_8O	—
4-hydroxy-3-methoxy-benzylalcohol	$C_8H_{10}O_3$	—
4-hydroxy-3-methoxy-benzaldehyde (vanillin)	$C_8H_8O_3$	characteristic odour
ethanol	C_2H_6O	insect attractant [13, 31, 34]
2,2-diethoxyethanol	$C_6H_{14}O_3$	—
2-hydroxy-benzene-methanol	$C_7H_8O_2$	—
4-hydroxy-benzene-methanol	$C_7H_8O_2$	—
pentadecanol	$C_{15}H_{32}O$	—
heptadecanol	$C_{17}H_{36}O$	—
eicosanol	$C_{20}H_{42}O$	—
benzoic acid	$C_7H_6O_2$	—
tetraeicosanoic acid	$C_{20}H_{32}O_2$	—
octadecenoic acid	$C_{18}H_{36}O_2$	—
pentadecenoic acid	$C_{15}H_{30}O_2$	—
heptadecenoic acid	$C_{17}H_{34}O_2$	—
9-hexadecenoic acid	$C_{16}H_{30}O_2$	—
oleic acid	$C_{18}H_{34}O_2$	—
eicosane	$C_{20}H_{42}$	—
heneicosane	$C_{21}H_{44}$	—
tricosane	$C_{23}H_{48}$	—
pentacosane	$C_{25}H_{52}$	—
hexacosane	$C_{26}H_{54}$	—
heptacosane	$C_{27}H_{56}$	—
octacosane	$C_{28}H_{58}$	—
eicosanoic acid methyl ester	$C_{21}H_{42}O_2$	—
tetracosanoic acid methyl ester	$C_{25}H_{50}O_2$	—
pentadecenoic acid methyl ester	$C_{26}H_{52}O_2$	—
hexadecenoic acid methyl ester	$C_{27}H_{54}O_2$	—
heptanal	$C_7H_{14}O$	characteristic odour
nonanal	$C_9H_{18}O$	—
hexadecanal	$C_{16}H_{32}O$	—
octadecanal	$C_{18}H_{36}O$	—
nonadecanal	$C_{19}H_{38}O$	—



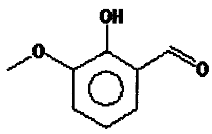
3-{2-{3-{3-(Benzyloxy) propyl}-3-indole



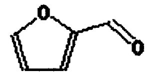
7,8-Didehydro-4,5-epoxy-3,6-d-morphinan



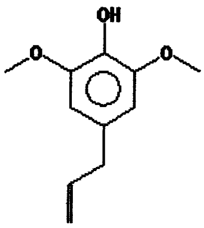
Oxycodone



Vanillin



2-Furancarboxaldehyde



2,6-Dimethoxy-4-(2-propenyl)-phenol
(Methoxyeugenol)

Figure 3. *Molecular formulas of some compounds identified in nectar of Epipactis helleborine (L.) Crantz*

Attraction of insects

We have observed attracting properties of the capillary tubes containing nectar extract towards some insects in field conditions. It is possible, that insects react mostly to eugenol derivatives and vanillin acting as attractants. It is well documented that these compounds have the greatest importance in the first stage of pollination, because of their well established role to have an ability to allure insects (*Fig. 4*). The process of pollinating in vivo was also a subject of our research. Nectar of Epipactis orchids is attractive to both group of insects: pollinators and visitors. We have been observing strong attraction of a big group of insects belonging to families such as: Syrphidae, Mycetophilidae (Diptera), Ichneumonidae, Formicidae, Vespidae, Apidae (Hymenoptera) and Anaspididae, Cantharidae, Coccinellidae, Nitidulidae, Lagriidae, Malachiidae (Coleoptera) as well Arctiidae (Lepidoptera).

Discussion

The characteristic feature of the large number of species from Orchidaceae family is, that their nectar contains a group of alkaloids of various chemical origin. So far over 1500 different compounds with overpowering properties, coming from the orchids, have been described [1]. The majority of them belongs to two classes of compounds:

pyrrolizidine alkaloids and dendrobine alkaloids [28, 29, 45]. Bell [7] suggests that some of the plants may produce hallucinogenic or narcotic substances, which addict the pollinators and cause visible disorientation of flight described by term „drunken insects”. Such behaviour of insects may also be caused by the action of ethanol, which is formed by fermentation process, as it was suggested by Løjtnant [31], Müller [34], Ehlers & Olsen [13] for *Epipactis* orchids. According to Kevan *et al.* [27], bumblebees became „drunken“ after visiting *Asclepias* flowers, sized by lot of yeast species.

In our opinion, the „drunken insects” effect might be also the result of the presence of other substances, such as: derivatives of indole, morphinans and derivatives of phenol, which we identified basing on GC-MS analysis (*Table 1).* The characteristic features of these compounds may have an impact on the behaviour of the insects. Furthermore co-occurrence of these compounds in the nectar may point at synergic effect. Such effect is not usual as seen from studies on eugenol, which is a substance that allures the insects, while methyl eugenol acts as a sex attractant of fruit fly *Daucus dorsalis* [50]. Methyl-eugenol is produced by many plant species, i.e. *Cassia fistulosa*, *Fagraea berteriana*, *Valeriana tuberosa*, and probably its role in the process of alluring pollinators is also important [15, 20, 43].

Identification of the presence of vanillin (4-hydroxy-3-methoxy-benzaldehyde), a compound which was for the first time isolated from *Vanilla planifolia* (Orchidaceae) confirms that plants which belong to one family are similar to each other not only in respect to genetics, but also in respect to presence of similar secondary metabolites. Interestingly, *Epipactis atrorubens* has an intensive vanilla scent [11].

Data presented in this paper show that *Epipactis helleborine* nectar is definitely not a scentless substance. It has not been known so far, which compounds or a group of compounds are responsible for its odour. The presence of compounds with strong scent properties such as vanillin, methyl eugenol, or carboxylic acid esters suggest that *Epipactis helleborine* scent comes from a mixture of several compounds. It also depends on the climate conditions in which the plants grow.

The results of GC/MS analysis of the nectar indicate, that probably its overpowering properties come not only from ethanol, but also from the other group of compounds, present in nectar, namely alcohols, phenols, organic acids and aldehydes. The effect of ethanol cannot be quantitatively estimated, because of its volatility and because that it is a product of metabolism of fungal species such as: *Cladosporium*, *Candida* and *Aspergillus* sp. There is no data on the correlation of the size and intesity of growth of fungal colonies with factual amount of ethanol in the nectar. It is also very probable, that the chemical decomposition of some of the compounds present in nectar might be also a source of ethanol and its derivatives. In Poland, the florescence period of *Epipactis* is the end of July and the beginning of August, so during the time when air temperatures during the day often exceed 28 °C. The quantity of ethanol that is produced by the microorganisms would have to be enormous, to counterbalance its evaporation speed and to maintain its overpowering properties.

The growth of microorganism colonies, which produce ethanol in natural conditions might be effectively restrained by the complex action of chemical compounds which have bactericidal and fungicidal properties. For example antifungal and antibacterial activity of furfural [22, 23], syringol [24, 33], indole derivatives, eugenol and methyl-eugenol which constituted major plant metabolites, is well documented [6, 21, 35]. Moreover, numerous literature data prove, that orchids belong to plants, which protect themselves very strongly from the attack of microorganisms, by producing various

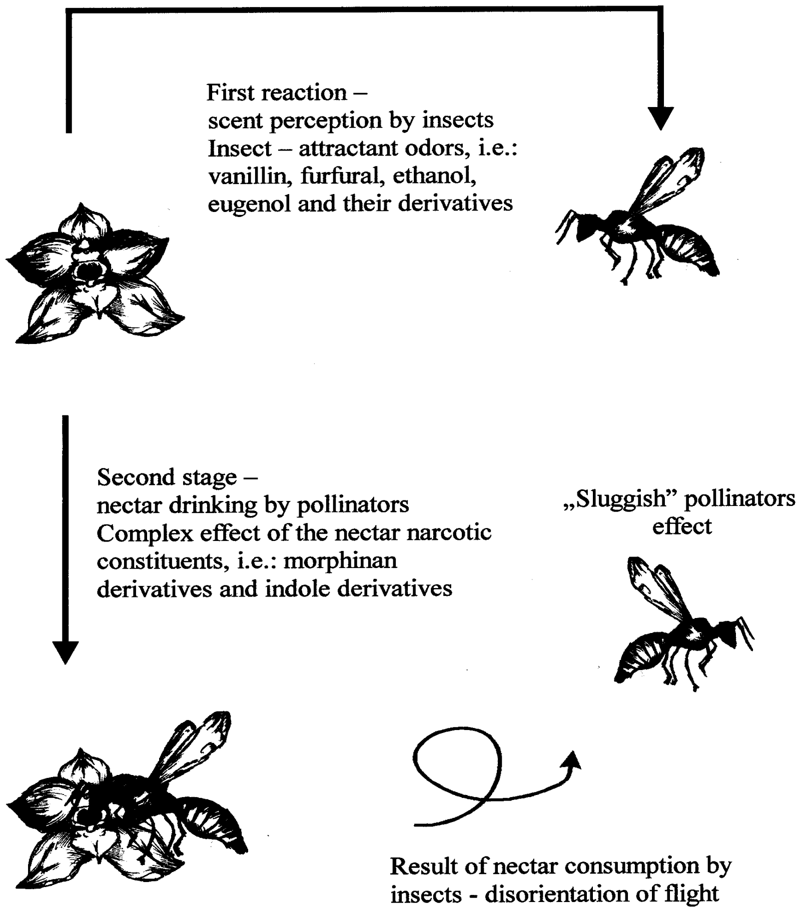


***Figure 4.** Hypothetical scheme of the chemical compounds' influence on the pollinators and visitors insects of Epipactis helleborine (L.) Crantz*

chemicals including phytoalexins [14]. The species of *Cladosporium* and *Candida* observed by Ehlers & Olsen [13] belong to these, which occur commonly in the nature, and therefore their role in the process of insect overpowering might not be as significant as the authors suggest. Finding these microorganisms in the laboratory conditions and therefore in artificial conditions, does not necessarily mean that they can reproduce in the nectar of *Epipactis helleborine* (L.) Crantz upon environmental conditions.

Why do orchids produce „drunken” insects?

Helleborine grow in various phytocoenoses. The majority of species grow in deciduous and mixed forests, but in Poland they may be seen on dunes, peat bogs and meadows, as well upon highly anthropogenic conditions, such as roadsides, cemeteries and municipal parks [10, 25, 49]. Such a diversity of habitats results in the diversity and size of the insect species visiting the plants.

Production of narcotic compounds such as 3-{2-{3-{3-(benzyloxy)propyl}-3-indol, 7,8-didehydro-4,5-epoxy-3,6-D-morphinan and oxycodone is one of the attempts to attract potential pollinators. The presence of oxycodone, which was reported to be a semisynthetic morphinan [41, 51], is of special interest here. These substances make the insects, which drink the nectar to become „sluggish”, what prolongs the time, which they spent on the inflorescence and therefore increases chance of pollinating larger

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number of flowers. Such a strategy is very effective if taking into consideration the fact, that *Epipactis helleborine* flowers are not morphologically attractive to insects and that attendance of potential pollinator is dependent on habitat.

Summing up, we propose two-stage reaction of insect to attractive components of orchids as shown in *Fig. 4*.

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