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# THE CHEMICAL COMPOSITION OF THE FLORAL EXTRACT OF *EPIPOGIUM APHYLLUM* SW. (ORCHIDACEAE): A CLUE FOR THEIR POLLINATION BIOLOGY

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*Abstract* – *Epipogium aphyllum* is a rare European obligate mycoheterotrophic orchid lacking chlorophyll. It has not been studied previously with respect to pollination biology. We studied the association between the composition of floral scent emission and its pollination systems. Field observation indicates that the main pollinators of *Epipogium aphyllum* are representatives of the genus *Bombus* (Hymenoptera), *B. lucorum*, *B. hortorum*, *B. terrestris*, *B. pascuorum* and *B. proteus*, and the genus *Apis* (Hymenoptera) namely *A. mellifera*. The main potential vector (observed to accidentally carry pollen), is most likely *Episyrphus balteatus* (Diptera, Syrphidae). The chemical composition of the floral extracts of 4 populations of *Epipogium aphyllum* Sw. growing naturally in Poland and the Czech Republic was examined by gas chromatography coupled with mass spectrometry (GC-MS) and high-performance liquid chromatography coupled with diode-array detection and electrospray ionization mass spectrometry (LC-ESI-MS) techniques. According to GC-MS analysis, 9-tricosene, nonadecane, 1-nonadecene and nonacosane predominated in the floral extracts. The studied samples were also characterized by relatively high amounts of benzenoids, e.g. methyl cinnamate, which is known as an attractant to the males of various orchid bees. LC-ESI-MS revealed the presence of flavor compounds such as vanillin (4-hydroxy-3-methoxybenzaldehyde) and its derivative acetovanillone, together with higher amounts of aliphatic and phenolic acids. Additionally, we detected the presence of indole and morphine derivatives.

*Key words: Epipogium aphyllum;* floral extract; pollination strategy

## INTRODUCTION

Many plants, including orchids, emit floral scents in order to attract a variety of animal pollinators, mostly insects (Dudareva and Pichersky, 2000). Therefore, floral scents are well known as a basic medium of chemical communication between plant species and pollinators, who use them for orientation, approach, landing, feeding and associative learning (Dobson, 1987). Rare achlorophyllous orchids like *Epipogium* provoke many controversies about pollination ecology. It still needs to be explained whether the fact that they are scarce is due to the lack of pollinators, ineffective pollination or other factors, among which a lack or unsuitability of chemical attractants might play an important role.

*Epipogium aphyllum* Sw. is a very rare and enigmatic European orchid species, an obligate mycoheterotrophic orchid lacking chlorophyll. It is famous for its unpredictable appearance; in many localities, it has been seen just once. It is found in beech, oak, pine and spruce forests on base-rich soils. The plants grow from an underground, burrowing stem that lacks chlorophyll and possesses ephemeral leaves that have the appearance of small, translucent scales. They only emerge above ground to flower and produce seeds. Large plants of this species can produce a rather stunning woodland display with up to a dozen flower stalks, at once bearing from 1 to 6 nonresupinate flowers each, growing out of leaf litter. Sometimes *Epipogium aphyllum* blossoms underground. Its shoots never survive until the next growing season. After blossoming, they disappear and they hardly ever blossom again in the same place (Delforge, 2006). The Ghost Orchids have an extremely wide range of distribution, but are exceptionally rare in habitat and critically endangered, as well as protected in habitat in many countries (Roy et al., 2009).

*Epipogium aphyllum* is known from Europe and Asia, where bumblebees are recorded as the main pollinators, attracted by their nectar, which has the scent of vanilla or fermented banana (Delforge, 2006; Taylor et al., 2011). Seed set, however, is very low and this is probably due to its growing in dark places in woods, where insects rarely appear (Van der Cingel, 2001). Little is known about the biology of this genus and its ecological relationships with insects. Literature review and our own observation confirmed that Ghost Orchids are rarely visited by insects, although they produce magnificent and medium-large flowers.

The data concerning the pollination biology of this species is scarce in literature, and it is not even known whether it secretes any chemical attractants or why it is so rarely pollinated by insects. Learning about the biology and pollination ecology of *Epipogium aphyllum* may provide the answer to the problem of its extinction in natural habitats. The aim of the present study was to analyze the chemical composition of the flower extract, including compounds that are ingredients of *Epipogium aphyllum* nectar, in a search for potential insect attractants and their potential influence on insect activity.

## MATERIALS AND METHODS

### *Study site*

Populations of *Epipogium aphyllum* growing under various habitat conditions in Poland and the Czech Republic were chosen. Studies were performed at the sites of: 1. Wejherowo in Kashubia region of Pomeranian Voivodiship (northwestern Poland); 2. "Kozi Rynek" Nature Reserve in Augustów Forest in Podlaskie Voivodiship (northeaster Poland); 3. Ojców National Park in Lesser Poland Voivodeiship (southern Poland), and 4. Habrůvka village at the south Moravian Region of the Czech Republic (see Fig. 1.).

The size of studied populations ranged from 30 to 600 individuals. The samples of flowers were collected during the maximal secretion by the plants, i.e. between 9.00 a.m. and 5.00 p.m. The presented studies were done with the consent of the Minister of the Environment, No DOPpn – 4102-179/13447/12/RS, the Director of Ojców National Park, No NB-0620/120/2/2012 and the Regional Directors for Environmental Protection, No. RDOŚ-Gd-PNII.6400.20.2012. MaK.1.,WPN.6400.27.2012.MW, OP-I.6400.18.2012. PWi and No.WPN.6402.2.23.2012.IW.1.

### *Extraction procedure*

Fresh flowers (10-15) were extracted with 10 ml of three organic solvents differing in hydrophobicity – methanol, methylene chloride and *n*-hexane. The obtained extracts were concentrated to dryness under reduced pressure in a rotary evaporator (40°C) and solid residue was reconstructed in 0.5 ml of appropriate organic solvent. In the case of LS-MS analysis, the samples in methanol were analyzed. All used solvents had HPLC grade and were from Sigma-Aldrich, Poland.

### *GC-MS Analysis*

Analysis of the volatile compounds of floral extracts was performed using an HP 5890 GC equipped with

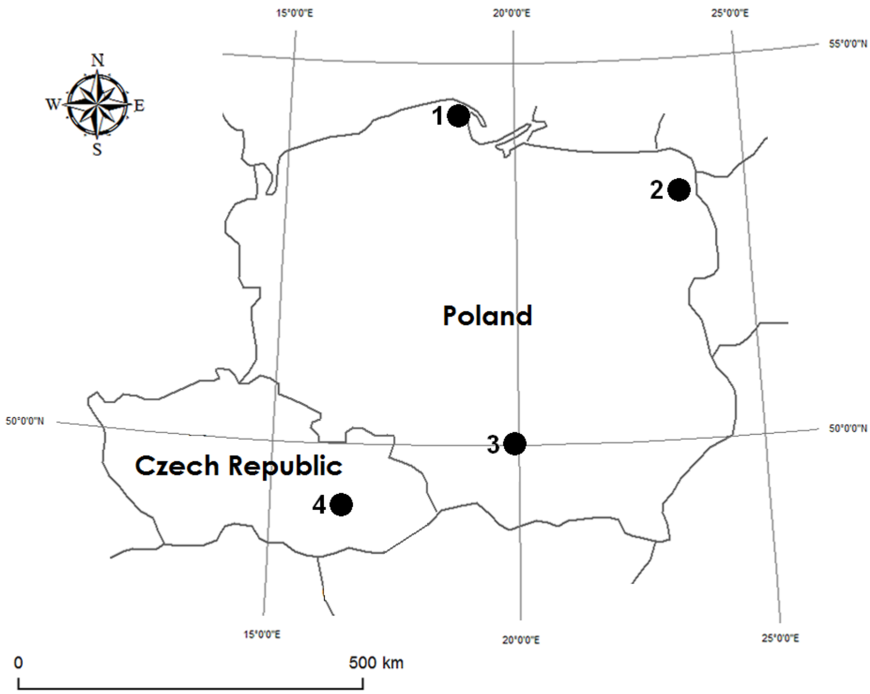


**Fig. 1.** Localities of studied populations of *Epipogium aphyllum*. Numbers from 1 to 4 refer to the populations studied.

an HP 5972 MS detector. The column used was an HP-5MS (Cross-linked 5% phenylmethylsiloxane) capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness) and the gas carrier was helium, at 1 mL/min rate. The injector and MS-transfer line temperatures were maintained at 220 and 290°C, respectively. Oven temperature was held at 50°C for 3 min, raised to 160°C at 3°C/min and then to 280°C at 5°C/min. Mass spectra were recorded in the electron ionization mode at 70 eV, scanning the 40-500 m/z range. Each analysis was performed in triplicate to assess the reproducibility of results. Compounds were identified using the HP Workstation software with the NIST98 mass spectral library.

### *LC-ESI-MS analysis*

The HPLC analysis was performed on a Dionex Ultimate 3000 series instrument coupled to a binary pump, a diode-array detector (DAD), an autosampler and a column compartment. Flower methanolic extracts were separated on a Phenomenex Gemini C18 column (3μm, 150 × 3.0 mm i.d.; Phenomenex) with a sample injection volume of 10 μl. A gradient elution with a mobile phase consisting of acetonitrile (A) and 0.2% formic acid (B) was used as follows: 95%B (0-1.5 min), 5%B (12-15 min), 95%B (20-25 min). The flow rate was 0.4 ml/min. For LC-ESI/MS$^n$ analysis, the Dionex HPLC system was coupled with a Bruker microOTOF-Q II system (Bruker Daltonics, Bremen, Germany) fitted with an ESI source. Both ionization modes (negative and positive) were recorded in the range 50- 1000 m/z. High purity nitrogen was used both as a drying and as a nebulizing gas. The capillary temperature was set at 280°C and spray voltage ±4500 V.

### *Insect identification and behavior*

Observations of the behavior of pollinators and insect visitors were performed from 9:00 a.m. to 5:00 p.m. Consecutive visits to flowers by pollinators were observed at 5 min-3 h intervals. In compliance with the methodology adopted from earlier studies of the pollination biology of the *Epipactis* genus (Jakubska et al., 2005a, 2005b; Jakubska-Busse and Kadej, 2011), we divided the Arthropods observed into 4 groups: 1) pollinators = typical vectors; 2) visitors = potential vector; 3) invertebrates visiting orchids; 4) insects connected with vegetative sprouts of orchids that feed on their juices. Insects were captured in natural conditions, and then identified by specialists. Voucher specimens are deposited in the Department of Biodiversity and Plant Cover Protection, University of Wrocław, Poland.

## RESULTS AND DISCUSSION

### *Pollination biology*

An interesting and as yet unexplained issue is why *Epipogium aphyllum* are mainly visited by bumblebees. The main pollinators of *Epipogium aphyllum* were representatives of the genus *Bombus* (Hymenoptera), namely *Bombus lucorum* (Linnaeus, 1758), *Bombus hortorum* (Linnaeus, 1761), *Bombus terrestris* (Linnaeus, 1758), *Bombus pascuorum* (Scopoli, 1763) and *Bombus proteus* (Gerstaecker, 1869) and by honeybees *Apis mellifera* (Linnaeus, 1758). Their interest in flowers was low and their visits were single and rarely ended in pollen transfer. Most bumblebees live in underground nests; they also perform flights close to the ground surface, which increases their chance of detecting attractants emitted by *Epipogium aphyllum*. Bumblebees are one of the most important and effective insect groups. In one minute, a worker bumblebee visits 20-25 flowers while in the same time a honeybee worker visits only 13 flowers (Krzysztofiak et al., 2004). Bumblebees also have a bigger surface for collecting pollen than the honeybee (they are bigger and more hairy), and they are relatively little sensitive to unfavorable weather conditions, which from the orchid's point of view seems to be very advantageous, particularly when the orchid is rarely visited by insects. Most *Epipogium aphyllum* habitats are poor in terms of other blooming plants that could be an alternative source of food for insects; therefore, the specialization in pollination by bumblebees seems to be valuable from the evolutionary point of view.

The potential vector, which was observed to accidentally carry pollen, was Diptera, Syrphidae, *Episyrphus balteatus* (De Geer, 1776). The most diverse and interesting group were invertebrates visiting orchids: Hymenoptera (Formicidae) and Coleoptera (Nitidulidae and Oedemeridae). We observed workers of *Myrmica rubra* (Linnaeus, 1758), workers of *Myrmica ruginodis* (Nylander, 1846) and *Meligethes aeneus* (Fabricius, 1775) and *Chrysanthia geniculata* (W. Schmidt, 1846). The presence of Coleoptera representatives on flowers is related to the fact that imagines feed on pollen or nectar and thereby they become pollinators. Moreover we observed the representatives of Homoptera (Scraptiidae): *Anaspis frontalis* (Linnaeus, 1758), Diptera (Syrphidae): *Melanostoma scalare* (Fabricius, 1794), *Meliscaeva cinctella* (Zetterstedt, 1843), *Syrphus* sp. (Fabricius, 1775), and flies from the Sciaridae family. These insects did not participate directly in pollination but feed on orchid juices, in particular from the calli on the lip.

No representatives of Vespidae were observed, which seems to be understandable, as *Epipogium aphyllum* nectar does not contain visible amounts of alcohols, which is the main attractant for this group of insects.

Interestingly, another species, *Epipogium roseum*, usually open, successfully attracts insect visitors, with the Asian honeybee (*Apis cerana cerana*) being the only visitor observed as regularly visiting its flowers for nectar (Zhou et al., 2012). We observed *Apis mellifera* visiting or pollinating *E. aphyllum*.

### *Flower extract composition*

### *GC-MS analysis*

The role of floral fragrances for floral visitors has been well-established (Dodson et al., 1969; Jakubska et al., 2005a, 2005b; Jakubska-Busse and Kadej, 2011) as well as the influence of their composition on their attractiveness for insects. Gas chromatography-mass spectrometry analysis of methylene chloride and *n*-hexane extracts from inflorescences of *Epipogium aphyllum* revealed the presence of 48 compounds.

**Table 1.** GC-MS profile of methylene chloride and *n*-hexane extracts from *Epipogium aphyllum* Sw. ("-": not identified).

| Compound | Molecular formula | Average relative amounts (%) of floral scent compounds of *Epipogium aphyllum* Sw. | |
|---|---|---|---|
| | | n-Hexane extract | $CH_2Cl_2$ extract |
| Benzenoids | | | |
| 4-(methoxymethyl)phenol/α-methoxy-*p*-cresol | $C_8H_{10}O_2$ | - | 3.53 |
| 4-hydroxybenzeneethanol/ p-Thyrosol | $C_8H_{10}O_2$ | - | 4.67 |
| 3-phenyl-2-propenoic acid methyl ester/methyl cinnamate | $C_{10}H_{10}O_2$ | - | 1.17 |
| 3-hydroxybenzyl alcohol/3-hydroxy-benzenemethanol | $C_7H_8O_2$ | - | 0.16 |
| 2-methylresorcinol | $C_7H_8O_2$ | - | 0.58 |
| 4-hydroxybenzaldehyde | $C_7H_6O_2$ | - | 3.51 |
| 4-hydroxy-3-methoxybenzaldehyde/ vanillin | $C_8H_8O_3$ | - | 1.27 |
| butylated hydroxytoluene/BHT | $C_{15}H_{22}O$ | - | 1.10 |
| Summary: | | - | **16.09** |
| Saturated hydrocarbons (alkanes) | | | |
| cyclododecane | $C_{12}H_{24}$ | 2.47 | 1.19 |
| cyclotetradecane | $C_{14}H_{28}$ | 2.34 | 1.17 |
| pentadecane | $C_{15}H_{32}$ | 1.84 | 1.02 |
| hexadecane | $C_{16}H_{34}$ | 1.97 | 0.23 |
| heptadecane | $C_{17}H_{36}$ | 0.33 | 0.18 |
| nonadecane | $C_{19}H_{40}$ | 10.81 | 10.71 |
| 2-methylnonadecane | $C_{20}H_{42}$ | 0.15 | 0.13 |
| eicosane | $C_{20}H_{42}$ | 1.16 | 1.75 |
| heneicosane | $C_{21}H_{44}$ | 2.23 | 1.11 |
| docosane | $C_{22}H_{46}$ | 3.45 | 1.12 |
| tricosane | $C_{23}H_{48}$ | 3.56 | 1.66 |
| cyclotetracosane | $C_{24}H_{48}$ | 1.78 | 0.81 |
| tetracosane | $C_{24}H_{50}$ | 1.57 | 1.03 |
| pentacosane | $C_{25}H_{52}$ | 2.29 | 0.22 |
| heptacosane | $C_{27}H_{56}$ | 4.72 | 2.47 |
| nonacosane | $C_{29}H_{60}$ | 12.78 | 11.78 |
| 11-decylheneicosane | $C_{31}H_{64}$ | 5.39 | 0.44 |
| Summary: | | 58.84 | 37.02 |
| Unsaturated hydrocarbons (alkenes) | | | |
| 1-heptadecene | $C_{17}H_{34}$ | 0.33 | 0.12 |
| 1-octadecene | $C_{18}H_{36}$ | 0.21 | 0.23 |
| 3-octadecene | $C_{18}H_{36}$ | 0.49 | 0.32 |
| 1-nonadecene | $C_{19}H_{38}$ | 11.02 | 23.63 |
| 1-docosene | $C_{22}H_{44}$ | 0.48 | 5.10 |
| 9-tricosene | $C_{23}H_{46}$ | 3.60 | 7.40 |
| 9-hexacosene | $C_{26}H_{52}$ | 1.38 | 0.54 |
| 17-pentatriacontene | $C_{35}H_{70}$ | 0.97 | 0.60 |
| hexatriacontane | $C_{36}H_{74}$ | 3.85 | 2.36 |
| Summary: | | 22.33 | 40.30 |

**Table 1.** *Continued*

| Compound | Molecular formula | Average relative amounts (%) of floral scent compounds of *Epipogium aphyllum* Sw. | |
|---|---|---|---|
| 3Ketones, aldehydes, acids & eters | | | |
| 1,1'-oxybis hexane/hexyl ether | $C_{12}H_{26}O$ | - | 0.25 |
| cyclodecanone | $C_{12}H_{22}O$ | - | 0.45 |
| tetradecanal | $C_{14}H_{28}O$ | - | 0.32 |
| hexadecanal | $C_{16}H_{32}O$ | 1.54 | 1.16 |
| octadecanal | $C_{18}H_{36}O$ | 0.63 | 0.28 |
| 9-octadecenal | $C_{18}H_{34}O$ | 1.30 | 0.31 |
| 17-octadecenal | $C_{18}H_{34}O$ | 2.18 | 1.04 |
| hexadecanoic acid/palmitic acid | $C_{16}H_{32}O_2$ | 2.53 | 1.11 |
| octadecanoic acid/stearic acid | $C_{18}H_{36}O_2$ | 1.97 | 0.33 |
| 9,12-octadecadienoic acid/linoleic acid | $C_{18}H_{32}O_2$ | 3.80 | 0.16 |
| 9-octadecenoic acid/oleic acid | $C_{18}H_{34}O_2$ | 2.13 | 0.44 |
| eicosanoic acid/arachidic acid | $C_{20}H_{40}O_2$ | 1.15 | 0.37 |
| Sterols | | | |
| stigmasterol | $C_{29}H_{48}O$ | 0.43 | 0.16 |
| β-sitosterol | $C_{29}H_{50}O$ | 0.78 | 0.18 |
| Total: | | 99.61 | 99.97 |

Their chemical composition is given in Table 1. Of course, the composition of extracted compounds is highly dependent on the used solvent, but it usually gives quite an exact insight into the presence of certain components in flower nectar.

Hydrocarbons (both saturated and unsaturated) identified in the floral extracts comprise more than 75% of all identified compounds. Major compounds found in methylene chloride extract were 1-nonadecene (23.63%), nonacosane (11.78%) and nonadecane (10.71%), constituting around 45% of extracted floral fragrances (Fig.2).

The predominance of alkanes and alkenes in the species *Epipogium aphyllum* has been also reported in other orchids species, e.g. in the genera *Serapias* and *Ophrys* (Schiestl and Marion-Poll, 2002; Pellegrino et al., 2012). Our findings confirm the occurrence of saturated hydrocarbons with chain lengths of 21-29. These chemicals were previously identified as a group of substances mimicking female bees' mating signals (Schiestl et al., 1999).

In both extracts, pentacosane and tricosane were also identified. These compounds were found previously in the genus *Serapias* (Pellegrino et al., 2012) as strong attractants for many groups of insects, including Diptera, Homoptera, Hymenoptera and Lepidoptera. These results taken together confirm the assumption that *Epipogium* flowers may be attractive to many insect groups (http://www.pherobase.com). Among the alkanes detected in the *n*-hexane extract, the highest percentages of nonadecane and nonacosane were observed. In particular, nonacosane (C29) represented 12.78 % of total alkanes in this extracts. According to the literature, *Epipogium* is visited by the ant *Formica rufa* L. (Claessens and Kleynen, 2011). We observed that only *Myrmica rubra* and *Myrmica ruginodis* inspected the flowers at various times, but the ants were far too small to reach the viscidium. The observed ant species feed on nectar and honeydew, which confirms their interest in *Epipogium* flowers. The fact that insects are interested in the plant can also be explained by the occurrence of the emission of eicosane. This compound is a known attractant for the whole Hy-

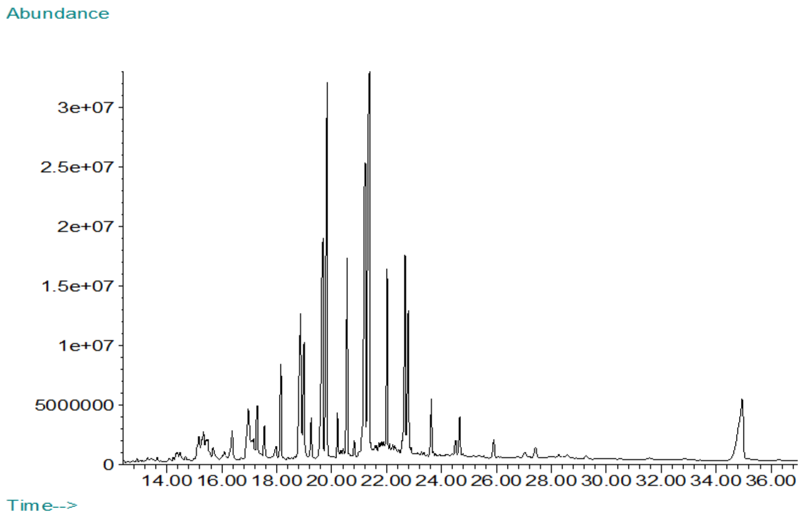


**Fig. 2.** Gas chromatogram of *E. aphyllum* methylene chloride extract with dominated 9-tricosene (Rt 19.70 min), nonadecane (Rt 19.83 min) and 1-nonadecene (Rt 21.25 min), nonacosane (Rt 21.36 min).

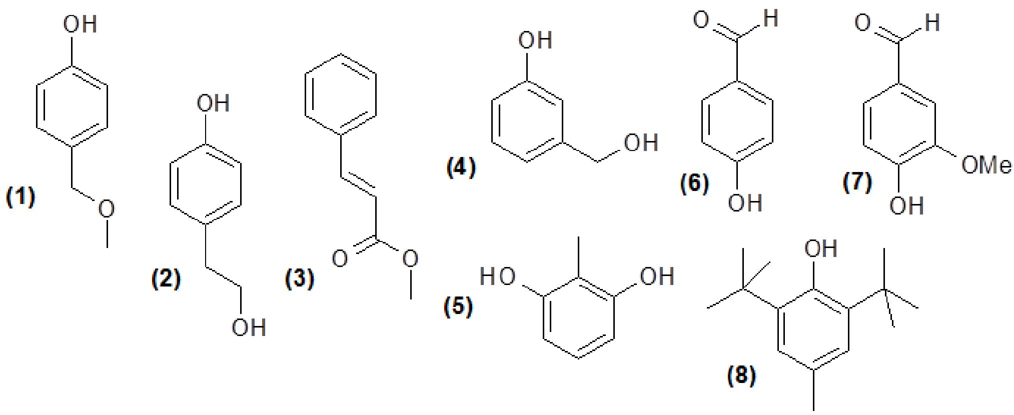


**Fig. 3.** Structure of identified benzenoids: (1) 4-(methoxymethyl)phenol, (2) 4-hydroxybenzeneethanol, (3) methyl cinnamate, (4) 3-hydroxybenzyl alcohol, (5) 2-methylresorcinol, (6) 4-hydroxybenzaldehyde, (7) 4-hydroxy-3-methoxybenzaldehyde, (8) butylated hydroxytoluene.

menoptera group, which includes the observed ants, bumblebees and honeybees (http://www.pherobase.com). Moreover, the ants eagerly feed on the rich-in-disaccharide juice secreted by *Epipogium aphyllum*. Another aliphatic hydrocarbons, 1-heptadecene, is a compound produced by other Orchidaceae; cyclododecane was also identified in Passifloraceae: *Passiflora galbana* and *Passiflora mucronata* (Varassin et al., 2001), while both taxa, i.e. *Epipogium* and *Passiflora* sp. also produce palmitic acid, which was identified in many orchids, such as the *Bulbophyllum* genus (Da Silva et al., 1999).

Additionally, several other identified compounds are widely acknowledged to be attractive to Hymenoptera, including Apidae (represented here by *Bombus* sp. and *Apis* sp.). The most important were octadecanoic and hexadecanoic acids. Therefore, it is quite surprising that we did not observe *Meliscaeva cinctella* reported by Claessens and Kleynen (2011)

**Table 2.** Compounds identified in *Epipogium aphyllum* by HPLC-ESI-MS.

| NO. | Identification | Composition | $[M-H]^-$ (m/z) | $[M+H]^+$ (m/z) |
|---|---|---|---|---|
| 1. | Disaccharide | $C_{12}H_{22}O_{11}$ | 341.11 | ----- |
| 2. | 3-{2-{3-{3-(benzyl)propyl}-3-indole | $C_{26}H_{34}N_2O$ | | 391.26 |
| 3. | 7,8-didehydro-4,5-epoxy-3,6-D-morphinian | $C_{19}H_{23}NO_3$ | | 314.17 |
| 4. | N-methylfindersine | $C_{15}H_{15}NO_2$ | 240.10 | |
| 5. | Hydroxyoctadecanoic acid | $C_{18}H_{34}O_3$ | 297.24 | |
| 6. | oxononadecanoic acid | $C_{19}H_{36}O_3$ | 311.25 | |
| 7. | Hydroxy-eicosanoic acid | $C_{20}H_{38}O_3$ | 325.27 | |
| 8. | Oxoheneicosanoic acid | $C_{21}H_{40}O_3$ | 339.29 | |
| 9. | 2-oxoooctadecanoic acid | $C_8H_{12}O_5$ | 187.06 | |
| 10. | Acetovanillone | $C_9H_{10}O_3$ | 165.06 | |
| 11. | Galactitol | $C_6H_{14}O_6$ | 181.07 | |
| 12. | Unidentified dihydromacrocyclic diester | | | 340.11 |
| 13. | Azafrin | $C_{27}H_{38}O_4$ | 425.27 | |
| 14. | Quercitrin | $C_{21}H_{20}O_{11}$ | 447.09 | |
| 15. | Isosteviol | $C_{20}H_{30}O_3$ | 317.21 | |
| 16. | Chlorogenic acid | $C_{16}H_{18}O_9$ | 353.08 | |
| 17. | Citric acid | $C_6H_8O_7$ | 191.01 | |
| 18. | Shikimic acid | $C_7H_{10}O_5$ | 173.04 | |
| 19. | Genistin | $C_{21}H_{20}O_{10}$ | 431.09 | |
| 20. | 5,6,7-trihydroxyflavone | $C_{15}H_{10}O_5$ | 269.04 | |
| 21. | Isoquercitin malonate | $C_{24}H_{22}O_{15}$ | 549.08 | |
| 22 | Vanillin | $C_8H_8O_3$ | 15.04 | |
| 23 | Unknown | $C_9H_{19}NO_4$ | 204.13 | 206.14 |

(Zetterstedt, 1843) [= *Scaeva cinctella* Zetterstedt, 1843]. Amongst insects attracted by these acids, only *Episyrphus balteatus* was found. It was previously found to be a pollinator of *Epipactis* species (e.g. Van der Cingel, 2001; Jakubska et al., 2005a, 2005b; Jakubska-Busse and Kadej, 2011). Furthermore, it seems that *Episyrphus* is a universal pollinator of all flower plants. The presence of *Meligethes aeneus* (Coleoptera, Nitidulidae) should not, however, be directly related to the pollination of *Epipogium aphyllum* due to small body size of these beetles. Literature data also confirm the significance of several other organic acids produced by *Epipogium* for pollination biology. For example, stearic acid is an attractant for numerous insect groups, including Hymenoptera, Lepidoptera and Coleoptera (Dafni et al., 1995; Fortunato et al., 2001), whereas linoleic and oleic acids are in turn vital ingredients of the scent of another insect-pollinated orchid, *Ophrys exaltata* (Mant et al., 2005).

In addition, another interesting group of compounds – benzenoids, were identified in the studied extracts. They were found to be present in relatively high amounts. Among these compounds, methyl cinnamate is well known as an attractant of males of various orchid bees, such as *Aglae caerulea* (Norris et al., 1983). Chosen structures of identified benzenoids are presented in Fig. 3.

Interestingly, the presence the popular fragrance vanillin (4-hydroxy-3-methoxybenzaldehyde) and vanillin precursors such as 4-hydroxybenzaldehyde, was also observed. These compounds are widley acknowledged as being of great importance in attracting potential pollinators. They were also identified in other Orchidaceae, such as: *Acacallis superba* Reich., *Catasetum viridiflavum* Hook., *Miltonia schroederiana* O'Brien (Kaiser, 1993), *Acacallis cyanea* Lindley (Gerlach, 1991) in *Epipactis* species (Jakubska et al., 2005a, 2005b; Jakubska-Busse and Kadej, 2011) and *Vanilla planifolia* Jacks. ex Andrews (Orchidaceae) (Podstolski et al., 2002).

### *LC-ESI-MS analysis*

The set of the identified compounds is strongly dependent on the analytical technique used. This is why we used two chromatographic techniques combined with mass spectrometry for the identification of individual compounds. To reveal the chemical compositions of *Epipogium aphyllum,* LC-ESI-MS analysis was carried out with both ionization modes (negative and positive). In both ionization modes, the methylene chloride extracts of *Epipogium aphyllum* flowers contained the riches chemical composition. Taking into account the obtained MS spectra, a total of 23 compounds were characterized. All the compounds were tentatively identified according to their mass spectral data (Table. 2).

It is not surprising that disaccharides and fatty acids as well as flavones were identified as major constituents by this technique. The large amount of disaccharide may support the thesis suggested by Rohrbach (1866) that the sweet juice secreted by *Epipogium* plays an important role in attracting insects and is easily accessible for them. It is assumed that the inner spur wall contains sweet juice, which is sucked by the visitor. The small content of some indole and morphine alkaloids was also observed. The presence of such compounds as 7,8-didehydro-4,5-epoxy-3,6-*D*-morphinian as well as 3-{2-{3-{3-(benzyl)propyl}-3-indole can be strictly related to the so-called effect of "drunken insects" that have been earlier observed in the case of those feeding on *Epipactis helleborine* (Jakubska et al., 2005a, 2005b). Both species grow in dark habitats, with difficult access to pollinators, which validates the potential significance of these compounds for attracting insects. They cause dizziness and sluggishness, which greatly increases the plant's chances of being accidentally pollinated; unfortunately in the case of *Epipogium aphyllum* the typical "sluggish pollinators effect" was not observed, which may result from the fact that it does not produce as high amounts of alcohols as *Epipactis*. It confirmed the suggestions of Claessens and Kleynen (2011) that *E. aphyllum* is reputed to have a very low pollination rate and is hardly pollinated at all in many sites. It is quite interesting as the taxon produces many chemical attractants and our findings have proved that its low pollination is not related to indigence in attractants

The identification of phenolic aldehydes, such as vanillin and acetovanillone, confirm the that *Epipogium aphyllum* belongs to the Orchidaceae family possessing an intensive, characteristic vanilla scent.

## CONCLUSIONS

*Epipogium aphyllum*, a very rare orchid on a European scale, despite its short period of flower attractiveness (up to a few days), secretes a set of interesting insect attractants. Therefore, the view that obligate autogamy in this taxon is the result of very low attractiveness for pollinators is unjustified, even though it does not produce as many strong attractants as other orchids, such as *Epipactis* or *Serapias*.

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