ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Hortorum Cultus, 16(5) 2017, 97–108

www.acta.media.pl ISSN 1644-0692 DOI: 10.24326/asphc.2017.5.10

ORIGINAL PAPER

Accepted: 12.05.2017

HISTOCHEMICAL INVESTIGATION OF TRICHOMES AND CHEMICAL COMPOSITION OF ESSENTIAL OIL FROM *Euphrasia stricta* D. Wolff ex J.F. Lehm. (OROBANCHACEAE)

Weronika Haratym✉, Elżbieta Weryszko-Chmielewska

University of Life Sciences in Lublin, Poland

ABSTRACT

Micromorphological and anatomical investigations of trichomes on vegetative and generative organs of the drug eyebright (*Euphrasia stricta* D. Wolff ex J.F. Lehm.) were carried out. Additionally, identification of the main components of the secreted products was conducted. The following types of glandular structures were found: capitate trichomes (i) with a unicellular head, a neck cell, and a 2-celled stalk, (ii) with a unicellular head, a neck cell, and 3-celled stalk, (iii) with a bicellular head and sporadically with a 3- or 4-celled head; moreover, on the surface of corolla unicellular and 2–3-celled clavate trichomes, ribbon-like trichomes, and papillae were present. Using fluorescence microscopy and histochemical tests, various substances such as lipids, phenolic compounds, terpenes containing steroids, terpenoids, sesquiterpenes, tannins, and flavonoids were determined but only in the capitate trichomes. The analysis of the essential oil showed that its content in the dry herb of *E. stricta* was on average 0.257%. Gas chromatography revealed the presence of 28 compounds.

Key words: chemical compounds, drug eyebright, histochemistry, micromorphology, secretory structure

INTRODUCTION

The genus *Euphrasia* L., previously belonging to the family Scrophulariaceae, is now classified as Orobanchaceae due to its semiparasitic biological features [Szafer et al. 1986, Stevens 2001]. The genus has been described to comprise 450 species distributed in Asia, Europe, the northern part of America, Indonesian mountains, New Zealand, and the southern part of South America. In Polish flora, only ten species occur in natural localities such as dry meadows and grassy slopes [Fisher 2004, Mabberley 2008, Posz 2014]. Recently, many eyebright species have been reported to be threatened with extinction, which is a consequence of dramatic landscape changes associated with urbanization and modernization of farming in Europe leading to fragmentation of natural plant habitats [Kolseth et al. 2005, Schmalholtz and Kiviniemi 2007].

The *Euphrasia stricta* is an annual plant growing up to 30 cm. The species possesses an erect, highly branched, and finely haired stem. Its ovate leathery leaves with sharp-pointed teeth on the blade edges have an opposite arrangement. The hermaphrodite

✉ weronikaharatym@gmail.com

zygomorphic white or light purple flowers are located on the apical parts of shoots. The corolla is bilabiate, i.e. the lower part consists of 3 fused petals, whereas the upper lip is formed of 2. On the surface of lower lip, there are 3 purple stripes on each petal. A yellow spot is visible in the central part of the middle corolla petal. Each lobe of the upper lip bears purple stripes. Yellow colouration is visible in the entry of the corolla tube. The flowers possess a pistil with a small green ovary, a long stamen, and a hairy stigma. Four stamens, which are characterised by unequal filaments, are fused with the upper lip. Their anthers equipped with sharp-tipped appendages are also fused. The species is adapted to pollination by small insects. Moreover, the flower is wrapped up by a cylindrical calyx with four sharp teeth. The fruit is a capsule [Weryszko-Chmielewska et al. 2010, Haratym and Weryszko-Chmielewska 2013].

Since *Euphrasia* has been confirmed as an ideal natural remedy for eye problems by the many-centuries long tradition, it is called "eyes of the Mother of God" [Posz 2014]. The eyebright has been widely used in folk medicine, phytomedicine, and homeopathy for many centuries. The raw material is the eyebright herb (Herba Euphrasiae), commonly known as eyebright grass, consisting of stems, leaves, and flowers collected before fruit formation [Kozłowski 1992]. Given the variety of biological active substances such as essential oil, iridoid glycosides, flavonoids, phenolic compounds, and tannins, the eyebright exhibits a wide spectrum of therapeutic activities including antibiotic, antifungal, antihyperglycemic, anti-inflammatory, antioxidant, antitumor, antiviral, astringent, hepatoprotector, and hypotensive properties [Porchezhian et al. 2000, Trovato et al. 2000, Gorji 2003, Ríos and Recio 2005, Blazics and Kéry 2007]. Aqueous tinctures of the eyebright are used for the treatment of eye disorders, e.g. cataract, conjunctivitis, glaucoma, hordeolum, and ocular allergy symptoms [Sharma and Singh 2002, Bielory and Heimall 2003, Petrichenko et al. 2006]. Furthermore, the tinctures found applications in treatment of diseases of the upper respiratory tract and the gastrointestinal tract [Shestakova et al. 2008, Dimitrova et al. 2013]. In homeopathy preparations, aqueous and alcohol extracts of *Euphrasia* are used in patients suffering from corneal ulceration, sharp pain in eyes, and spastic phobia [Petrichenko et al. 2006].

Although there are numerous studies of the medicinal properties of different species of the *Euphrasia* genus, the structure of secretory tissue has been scarcely investigated. The results of our previous microscopic analyses of *Euphrasia stricta* trichomes revealed 10 different types on the flower surface and 2 more types on the leaf and stem [Weryszko-Chmielewska et al. 2010, Haratym and Weryszko-Chmielewska 2013]. This paper reports our further research, which are a continuation of previous analyses. Anatomical and histochemical investigations of various trichomes of the drug eyebright were performed with the use of light, fluorescence and scanning microscopy. Histochemical tests were carried out to evaluate the main classes of chemical compounds occurring in the cells of the trichomes and their secretions. The aim of the present study was also to provide data about the composition of the secreted material of glandular trichomes of the drug eyebright.

MATERIAL AND METHODS

Plant material. The research material comprised flowering shoots of the drug eyebright (*Euphrasia stricta* D. Wolff ex J.F. Lehm.). Plants were collected from their natural habitats on xerothermic grasslands in Czechow district, Lublin (51°15'N, 22°30'E), Poland, between late July and mid-September 2010–2015. For the investigations, vegetative leaves were harvested from the third and fourth nodes of the plant. Flowers were sampled from the apical parts of shoots in the initial bloom stage, full bloom stage, and the end of the flowering period. Fragments of stems were cut from the middle part of the 3rd and 4th internodes.

Microscopy. Trichomes were observed on aboveground organs such as stems, leaves, and flowers using light (LM), fluorescence (FM), and scanning electron microscopy (SEM).

Stereoscopic microscope (SM). Preliminary analyses were performed using a stereoscopic microscope coupled with a Nikon Coolpix 4500 camera.

Fixation of the plant material and light microscopy (LM). To obtain permanent preparations, small leaves and flowers (≤15 mm^2) were fixed without further sectioning, whereas cross-sections (5–7 mm wide) were cut across the centres of larger leaf laminas containing a midrib. The plant material was placed in 2.5% glutaraldehyde/4% formaldehyde in phosphate buffer (pH 7.4) for 2 hours at ambient temperature. After preservation, small fragments (5 mm^2) were prepared. Subsequently, they were washed tree times in phosphate buffer and dehydrated in alcohol series (30, 50, 70, 90, 96%). Afterwards, they were immersed in anhydrous ethanol twice for 30 min. This was followed by progressive embedding in LR White resin (Sigma Aldrich) and polymerization at 60°C for 36 hours. Next, the material was cut into semi-thin sections (0.7 µm thick) with a Leica EM UC7 ultramicrotome, mounted on glass slides by gentle heating at 60°C on a slide warmer, and stained with a 1% aqueous methylene blue – Azure B solution. Observation and photos were made using a Nikon Eclipse 400 light microscope.

Scanning electron microscope (SEM). Aboveground segments (5 mm × 5 mm) of flowers (sepals and petals), leaves, and stems were prepared for SEM examination. They were fixed in a 4% glutaraldehyde solution in 0,1 M phosphate buffer (pH 7.0) for 12 hours at room temperature. Subsequently, the plant material was washed in the same buffer four times at 20-minute intervals and after that dehydrated in ethanol series (30, 50, 70, 90 and 95%), which was followed by application of absolute alcohol three times. Afterwards, the samples were transferred to acetone. After critical drying in liquid CO_2 using Bal-Tec CPD 030, the samples were mounted on a double-sided carbon tape on stubs and coated with 10-µm gold using a Polaron SC 7640 sputter coater. The surface of stems, leaves, and flowers was examined and imaged under a TESCAN/VEGA LMU scanning electron microscope at an accelerating voltage of 30 kV.

Histochemical tests and fluorescence microscopy (FM). The main classes of metabolites in the secreted material were investigated in fresh sections using histochemical tests. The fragments of leaves and flowers were soaked in Sudan III, Sudan IV, and Sudan Red solutions for 10 min. These reagents are universal indicators of lipid compounds [Johansen 1940, Lison 1960, Brundrett et al. 1991]. Some parts were soaked for 2 min in Neutral Red, which facilitated identification of lipids and essential oils [Kirk 1970, Clark 1981]. Other fragments were embedded for 1 min in Nile Blue, which is used to examine neutral and acidic lipids [Jensen 1962]. Moreover, the plant fragments were treated with Ruthenium Red for 5 min to determine polysaccharides other than cellulose [Johansen 1940], 1% potassium dichromate for 10 min to detect tannins, and 2% ferric trichloride for 10 min to identify phenolic compounds [Johansen 1940, Soloway and Wilen 1952, Gabe 1968]. Furthermore, a reaction with concentrated sulphuric acid was performed to detect sesquiterpenes [Cappelletti et al. 1986]. After every reaction, the plant material was washed in distilled water and mounted in a 50% aqueous solution of glycerol. The stained sections were observed and photographed with a Nikon Eclipse 400 light microscope. All stains were matched with controls. For the fluorescence microscopy investigation, fresh material was treated with 10% aluminium trichloride and 10% magnesium acetate for flavonoids [Charrière-Ladreix 1976], vanillin–HCl for terpenoids [Nikolakaki and Christodoulakis 2004], and 1% antimony trichloride for terpenes containing steroids [Mace et al. 1974].

Oil isolation. Essential oils from *Euphrasia stricta* were obtained from dry herb. Twenty grams of powdered aboveground organs of the drug eyebright were submitted to water – distillation in a Clevenger type apparatus with 400 mL water for 30 min according to the Polish Pharmacopoeia VII [2006]. The essential oil yields were measured. Subsequently, the essential oils obtained were dried over anhydrous sodium sulphate and stored at 4°C until gas chromatographic determination of their composition.

Gas chromatography–mass spectrometry (GC-MS). The analysis of the essential oils was performed using the GC-MS instrument ITMS Varian 4000 GC-MS/MS (Varian, USA) equipped with a CP-8410 auto-injector and a 30 m × 0.25 mm i.d. VF-5ms column (Varian, USA). A mass selective detector was operated in an electron impact mode with ionization energy of 70 eV and a mass range

from 40 to 1000 Da. The scan time was 0.80 s. The split ratio was 1 : 100 and the volume of injection was 1 µL. The column temperature was initially kept at 50°C, then gradually increased to 250°C at a 4°C/min rate, and kept at this temperature for 10 min. The injection temperature was 250°C. Helium was used at a flow rate of 0.5 mL/min as carrier gas.

Gas chromatography–flame ionization detection (GC-FID). In our analysis, also a Varian 3800 Series (Varian, USA) instrument with a DB-5 column (J&W, USA), operated under the same condition as GC-MS, was used.

The components were identified based on the comparison of their Kovats retention indices relative to n-alkanes series and mass spectra with those of authentic samples, National Institute of Standards and Technology (NIST), and with data available in the literature [Van Den Dool and Kratz 1963, Adams 2001, Mass Spectral Library 2008].

RESULTS

Morphology and distribution of trichomes. Fresh samples of aboveground organs such as calyces, corollas, leaves, and stems of *Euphrasia stricta* exhibited various trichomes on all their surfaces.

Non-glandular trichomes. *Euphrasia stricta* bears four types of non-glandular trichomes. The first type comprised single, uniseriate, built of two or three cells, pointed, curved downward structures. They covered the surface of the stem. Finely striated ornamentation was observed on their surface (fig. 1a). The second type of non-glandular trichomes was long 1- or 2-celled hairs located especially on the abaxial surface of the upper lip (fig. 1b). Moreover, long 2-celled trichomes with visible wall ornamentation were found on the stamens and both corolla surfaces. The abaxial surface of the sepals was covered by unicellular, hook-shaped, short trichomes which sharp ends pointed towards the apex of the calyx (figs 1c, d).

Glandular trichomes. The following types of glandular trichomes were distinguished:

I – capitate trichomes; this group comprised: (i) capitate trichomes with a unicellular head, a neck cell, and a 2-celled stalk; (ii) capitate trichomes with a unicellular head, a neck cell, and a 3-celled stalk (figs 1e–f); these were mostly located on the adaxial and abaxial surfaces of the upper lip. Only few of them were found on generative organs. Their surface was smooth. In mature hairs, the secretions were accumulated in the subcuticular space (fig. 1g); (iii) – trichomes with a 2-celled head, a neck cell, and a unicellular stalk. Their surface was smooth (fig. 1h). An intensely stained protoplast was observed in the head, especially in mature trichomes (fig. 1i). During secretion, glandular cells undergoing vacuolation were observed. In the postsecretory stage, the largest part of secretory cells was filled with huge vacuoles (fig. 1j). This type of trichomes was irregularly distributed and represented by only few on the surface of the stem. They occurred abundantly on the abaxial surface of the leaf, i.e. they were located on each tooth. On the adaxial side of the leaf, they were found only in the epidermis above the vascular bundles. Furthermore, capitate trichomes with a 3–4-celled head were also observed, but sporadically (figs 1k, l).

II – clavate trichomes; two kinds of trichomes were found within this group: (i) unicellular clavate trichomes; they possessed a cell wall covered by a thick cuticle with sculpture consisting of short striae (figs 2a–e); (ii) 2–3-celled clavate trichomes. Their cell wall was thinner and chromoplasts were visible (figs 2f, g). Both types were located near the yellow spot on the lower lip of the corolla.

III – ribbon-like trichomes with wall thickening. The hairs were white, long, and twisted. They were made up of several cells with irregular thickenings. Moreover, wrinkled striae were observed on their surface. They occurred on the anthers and the upper part of the adaxial surface of the upper corolla lip (figs 2h–k).

IV – papillae – the conical structures with apically convergent striae covered the adaxial surface of the lower corolla lip (figs 2l, m).

Histochemical analyses. Using fluorescence microscopy and histochemical tests, various substances such as lipids, phenolic compounds, terpenes containing steroids, terpenoids, sesquiterpenes, tannins, and flavonoids were determined only in the capitate trichomes (tab. 1).

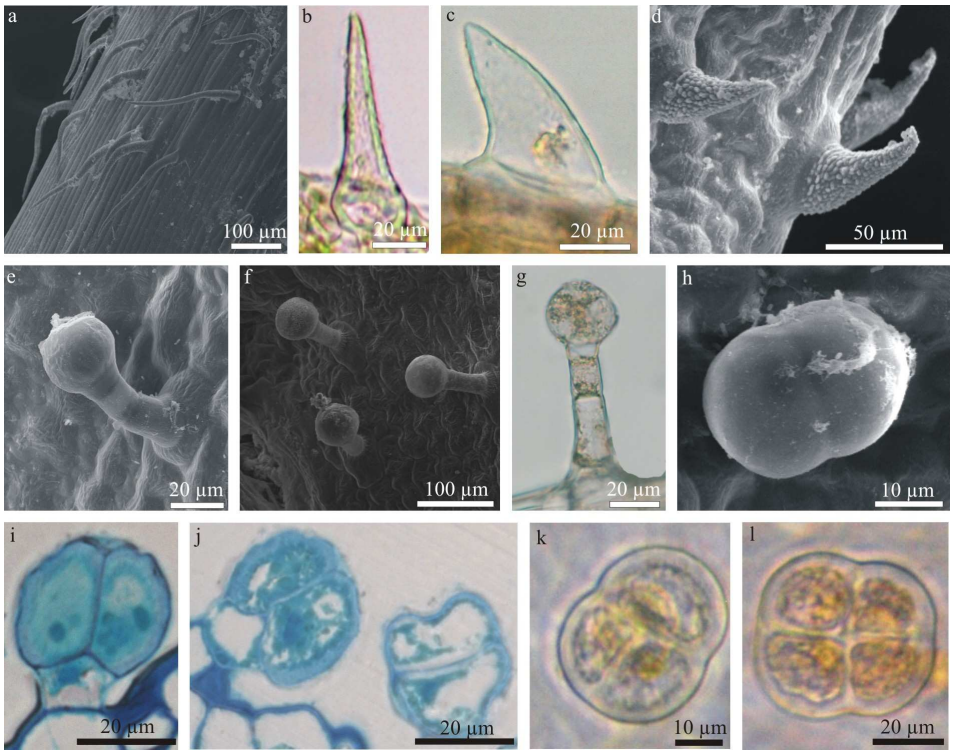

Fig. 1. Protective (a–d) and glandular trichomes (e–l) on the surface of *Euphrasia stricta* vegetative and generative organs (a, d–f, h SEM; b, c, g, i–l LM); a) stem surface with uniseriate, pointed, curved downward trichomes; b) long 1- or 2-celled hairs located on the abaxial surface of the upper lip; c, d) hook-shaped trichomes on the surface of the calyx; e–g) capitate trichomes with a unicellular head on the surface of petals; h–j) capitate trichomes with a bicellular head located on the surface of leaves: remnants of secretion visible on the smooth surface of the trichome in h; i) trichome in the secretory stage; j) trichomes in the postsecretory stage. Note there glandular cells that underwent vacuolation; k) capitate trichome with a 3-celled head; l) capitate trichome with a 4-celled head

The long-stalked capitate trichomes with a unicellular head stored hyaline or yellow, slightly viscous secretion whereas the secretory product accumulated in the head cells of the short-stalked trichomes with a bicellular head was yellowish or brownish (figs 3a, b). The positive reaction with Neutral Red observed under a light microscope confirmed the presence of essential oils (figs 3c, d). After the Sudan III, Sudan IV (figs 3e, f), and Sudan Red (fig. 3g) treatments, orange or reddish stained total lipids were observed to be scattered in the neck and head cells of the capitate trichomes. The presence of lipids was also confirmed by the reaction with Neutral Red, in which all types of the trichomes changed into yellow under the UV light. The blue colour appearing only in the capitate trichomes upon the application of Nile Blue indicated the presence of acid lipids (fig. 3h). Tannins were present in the head cells of the capitate trichomes located on the flowers and in the head, neck, and stalk cells in the trichomes present on the leaves and calyces, which was confirmed by the brown colour when stained with potassium dichromate. The strongest reaction was observed in the bicellular head of the short trichomes located on

Table 1. Compounds identified by histochemical tests and/or fluorescence microscopy in the capitate trichomes with a bicellular head (BC) and capitate trichomes with a unicellular head (UC) of drug eyebright calyces, flowers, leaves, and stems

Test	Compound	Colour observed	Type of trichomes	
			BC	UC
Sudan III	lipids	orange	+	+
Sudan IV	lipids	orange	+	+
Sudan Red	lipids	red	+	+
Neutral Red (under UV)	lipids	yellow	+	+
Nile Blue	acid lipids	blue	+	+
Neutral Red	essential oil	red	+	+
Ruthenium Red	polysaccharides	crimson	–	–
Potassium dichromate	tannins	brown	+	+
Ferric trichloride	phenolic compounds	black	+	+
Aluminium trichloride (under UV)	flavonoids	yellow-greenish	+	+
Magnesium acetate (under UV)	flavonoids	yellow-greenish	+	+
Antimony trichloride (under UV)	terpenes containing steroids	yellow	+	+
Vanillin-HCl (under UV)	terpenoids	yellow	+	+
Conc. sulphuric acid	sesquiterpenes	yellow	+	+

– negative, + positive

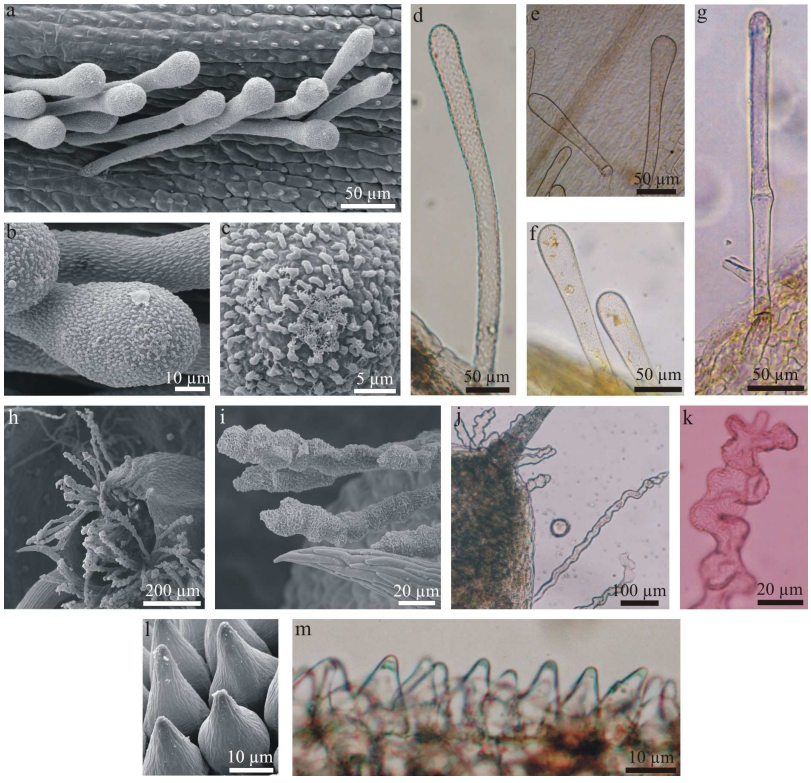

Fig. 2. Various types of trichomes on the corolla of the drug eyebright documented by SEM (a–c, h, i, l) and LM (d–g, j, k, m); a–e) unicellular clavate trichomes; f, g) 2- and 3-celled clavate trichomes; h–k) thickened ribbon-like trichomes on the surface of stamen heads; l–m) papillae with visible striae converging at the trichome apex on the lower corolla lip

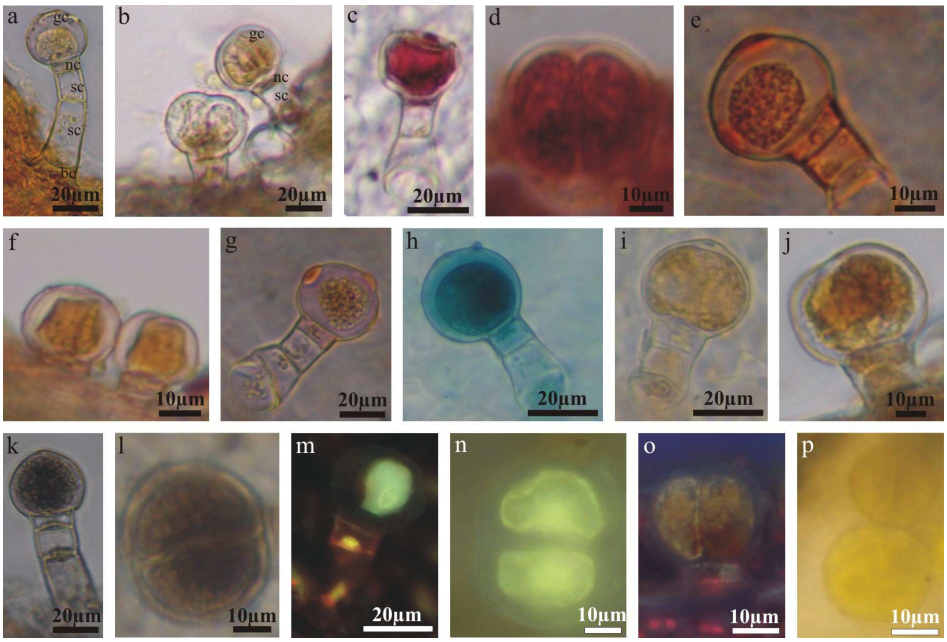

Fig. 3. Histochemical characterisation of the secretions of *Euphrasia stricta* capitate trichomes a, b) – control samples without staining LM; c–l, p – histochemical reactions observed under LM, m–o – samples observed under FM); a) capitate trichome with a visible glandular cell, a neck cell, stalk cells, and a basal cell; b) capitate trichomes with a bicellular head; c, d) trichomes stained with Neutral Red; e, f) trichomes stained with Sudan IV. Droplets of secretion visible on the surface of the head cell in e; g) droplet of secretion produced by a capitate trichome stained red with Sudan Red; h) secretion stored in a secretory cell stained dark blue with Nile Blue; i, j) content of glandular cells stained brown after the potassium dichromate test; k, l) secretory material in the head cells stained black after reaction with ferric trichloride; m, n) yellowish secondary fluorescence stained with $AlCl_3$ under UV light; o) yellow secondary fluorescence visible in the head cells observed after reaction with magnesium acetate under UV light; p) secretion in the head cells stained yellow with concentrated sulphuric acid; gc – a glandular cell, nc – a neck cell, sc – a stalk cells, bc – a basal cell

the calyx and leaf surface (figs 3i, j). The black colour appearing upon the application of $FeCl_3$ indicated the presence of phenolic compounds (figs 3k, l). The yellowish secondary fluorescence was induced in the head cells and secreted exudate after the use of fluorochromes for flavonoid detection, i.e. aluminium trichloride (figs 3m, n) and magnesium acetate (fig. 3o). With concentrated sulphuric acid, the capitate trichomes showed yellow colour, indicating the presence of sesquiterpenes (fig. 3p). The histochemical tests carried out with antimony trichloride to indicate terpenes containing steroids gave positive results in the head cells of capitate trichomes. The yellow fluorescence of capitate trichomes after treatment with vanillin-HCl indicated the presence of terpenoids. Only the test with Ruthenium Red applied to detect polysaccharides gave negative results.

Chemical study. The study showed that the essential oil content in the dry herb of *E. stricta* was on average 0.257%. The chromatographic analysis revealed the presence of 28 compounds, including 24 identified ones (tab. 2). The main substances characterised were as follows: octen-3-ol (71.80%), 1,2-benzenedicarboxylic acid, butyl cyclohexyl ester (3.99%), linalool (3.68%), phytol acetate (2.84%), cembrene (2.79%), benzene 1,2,3-trimethyl (2.53%), and benzene acetaldehyde (1.99%). The identified compounds constituted 99.96% of all the components of the examined oil.

Table 2. Chemical composition of herb essential oil of *Euphrasia stricta* harvested in the generative stage

No.	Compound	RT (min)	RRI	%	±SD
1.	Cumene	8.134	928	1.74	0.01
2.	α-pinene	8.391	936	tr	–
3.	1,2,3-trimethyl benzene	9.642	975	2.53	0.08
4.	Octen-3-ol	9.979	985	71.80	0.88
5.	Benzene acetaldehyde	12.844	1067	1.99	0.05
6.	Linalool	14.366	1109	3.68	0.55
7.	Cis-verbenol	15.908	1152	0.20	0.00
8.	Trans-verbenol	16.087	1157	0.82	0.02
9.	α-terpineol	17.884	1208	0.79	0.28
10.	Pulegone	18.688	1231	0.43	0.11
11.	n.i.	20.858	1293	0.21	0.14
12.	n.i.	23.676	1378	0.51	0.04
13.	E-β-damascenone	24.059	1390	0.65	0.04
14.	E-β-damascone	25.010	1420	0.19	0.08
15.	E-caryophyllene	25.283	1428	0.30	0.21
16.	E-β-ionone	27.276	1492	0.41	0.10
17.	β-curcumene	28.055	1518	0.17	0.02
18.	Chamazulene	34.765	1754	0.13	0.01
19.	3,7,11,15-tetramethyl-2-hexadecen-1-ol	36.981	1839	0.95	0.10
20.	6,10,14-trimethyl-2-pentadecanone	37.226	1848	0.50	0.10
21.	n.i.	37.609	1863	0.25	0.04
22.	1,2-benzenedicarboxylic acid, butyl 2-methylpropyl ester	37.857	1873	0.78	0.06
23.	n.i.	38.079	1882	0.38	0.08
24.	Cembrene	39.737	1949	2.79	0.12
25.	1,2-benzenedicarboxylic acid, butyl cyclohexyl ester	40.226	1969	3.99	0.21
26.	Manool	41.691	2031	0.40	0.02
27.	n-heneicosane	43.345	2102	0.54	0.09
28.	Phyto acetate	43.674	2114	2.84	0.79
	Total 99.96%				

n.i. – non-identified, RT – retention time, RRI – relative retention index, tr – trace (<0.05%)

DISCUSSION

In our previous study, we found capitate trichomes with a 1- and 2-celled head on the leaves and stems of *Euphrasia stricta* [Weryszko-Chmielewska et al. 2010, Haratym and Weryszko-Chmielewska 2013]. In this paper, besides these types, we describe trichomes with a 3- or 4-celled head, which occur in the analysed material relatively infrequently. Using the histochemical assays, we have shown that all the types of capitates trichomes contain the same secondary metabolites, despite their morphological differences.

Histochemical studies of the *Euphrasia stricta* trichomes have shown the presence of flavonoids, lipids, phenolic compounds, sesquiterpenes, tannins, terpenes, and terpenoids. In the drug eyebright, the lipophilic nature of the secretion was demonstrated by positive reactions with Sudan III, Sudan IV, Sudan Red, Nile Blue, and Neutral Red under UV.

Analyses performed by Miladinovic et al. [2014] and Novy et al. [2015] revealed that eyebright herb contained fatty acids (mostly palmitic acid) and their derivatives as main constituents. Similar results of trichome histochemistry in *Calceolaria adscendens* (Scrophulariaceae) [Sacchetti et al. 1999] or *Orobanche ramose* (Orobanchaceae) [Sacchetti et al. 2003] have been reported.

Terpenoid secretion was confirmed by the remarkable yellow colour obtained with vanillin-HCl under UV. The reaction with antimony trichloride revealed the presence of terpenes containing steroids. A positive result was obtained after using concentrated sulphuric acid, which helped identify sesquiterpenes among the secreted products. In addition, our phytochemical analysis confirmed that substances such as pinene, linalool, terpineol, pulegone, caryphyllene, chamazulene, and cembrene, which represent various types of terpenes, were found in the essential oil in the eyebright. The data reported by other authors also showed that these substances are volatile constituents in *Euphrasia stricta* and *E. rostkoviana* [Miladinovic et al. 2014, Novy et al. 2015]. The terpene compounds are responsible for the antioxidant, antiviral, antibacterial, and antifungal properties [Thoppil and Bishayee 2011].

Some literature data have shown that various types of trichomes on a plant can produce different secondary metabolites. As indicated by Combrinck et al. [2007], large peltate trichomes and small capitate trichomes in *Lippia scaberrima* (Verbenaceae) contained terpenoids and phenolic compounds, respectively, as the major components. In two *Calceolaria* (Scrophulariaceae) species, Sacchetti et al. [1999] found a relationship between the type of the glandular trichomes and the class of terpene produced. The presence of a 2-celled secretory head in *C. adscendens* was associated with production of diterpenes, whereas 8-celled head secretory trichomes in *C. volckmanni* produced triterpenes.

In our study, we observed that the numerous trichomes with a 2-celled head in the *E. stricta* leaf epidermis function asynchronously, since besides trichomes containing protoplasts in their heads, there were trichomes with light heads without cellular content. These trichomes had probably released their secretion and the protoplasts had been degraded. In the trichomes with 2-celled heads with coloured cellular content, we observed different colours from yellow to dark orange, which may have been associated with the differences in the amount of each component. Combrinck et al. [2007] found that the numerous glandular trichomes on mature *Lippia scaberrima* leaves exhibited different shades of orange and yellow, which may imply different contents of flavones and flavonols. Flavonoids usually occur as yellow stains dissolved in the cell sap in flowers and leaves, or less frequently in other plant organs. These compounds are regarded to be responsible for the therapeutic properties of most plant raw materials [Kohlmünzer 2007]. Many flavones and a few flavonols have been detected in *Herba Euphrasiae*. In our research, the production of flavonoids by *E. stricta*, reported by Matławska et al. [1998], has thus been confirmed by the histochemical reactions with aluminium chloride and magnesium acetate.

We observed substantial content of phenolic compounds in the head cells of some trichomes. It was confirmed by the ferric trichloride test, which is based on the formation of intensively coloured precipitate from the reaction of iron with orthodihydroxyphenols [Johansen 1940]. In the heads of other trichomes, the compounds usually formed small dark spots, which may suggest that the trichomes were undergoing different stages of metabolic transformations. As shown in literature, some phenol carboxylic acids are precursors of various complexes of secondary metabolites, e.g. flavonoids [Dewick 1977, Combrinck et al. 2007]. Moreover, the potassium dichromate test [Gabe 1968] revealed the presence of tannins. These substances were found in chlorophorm and methanol extracts of the eyebright by Dimitrova et al. [2013]. They are responsible for antioxidant activity [Blazics and Kéry 2007].

SEM images of trichomes of many plant species show secretion on their surface, which confirms their secretory activity. Our previous [Weryszko-Chmielewska et al. 2010, Haratym and Weryszko-Chmielewska 2013] and present observations allow a conclusion that both the capitate trichomes on the *E. stricta* leaves, stems, and flowers and the clavate trichomes on the flowers have a secretory ability. Similarly, other

authors observed secretion remnants on the surface of various trichomes: Sacchetti et al. [1999] in *Calceolaria adscendens*, Weryszko-Chmielewska and Chwil [2006] in *Asphodelus albus*, and Weryszko-Chmielewska and Czernecki [2005] in *Kalanchoë*.

The content of essential oil in the drug eyebright was 0.257%, whereas the level of the oil content in *Euphrasia stricta* reported by Miladinovic et al. [2014] and *E. rostkoviana* analysed by Novy et al. [2015] reached 0.02%. The GC–MS analysis revealed 28 constituents representing 99.96% of total oil content. Miladinovic et al. [2014] indicated the presence of 47 compounds. In turn, Novy et al. [2015] identified 70 different compounds in the essential oil from *E. rostkoviana*. The biosynthesis of essential oils depends on a number of factors such as the cultivation method, fertilization, irrigation, weather conditions, type of soil, or date of harvest. All of them can significantly modify the content and the composition of essential oil [Dubey et al. 2003, Woronuk et al. 2011, Nurzyńska-Wierdak 2013]. This can be a cause of the differences between the results obtained by various scientists.

CONCLUSIONS

Three types of capitate on the surface of vegetative organs and the aforementioned plus two types of clavate glandular trichomes on the surface of generative organs were present.

The histochemical tests which indicated the presence of various compounds such as lipids, phenolic compounds, terpenes containing steroids, terpenoids, sesquiterpenes, tannins, and flavonoids revealed heterogeneous nature of the drug eyebright essential oil.

The analysis of the essential oil showed that its content in the dry herb of *E. stricta* was on average 0.257%. The presence of 28 compounds was determined by using gas chromatography with mass spectrometry.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science and Higher Education of Poland as part of the statutory activities of the Department of Botany, University of Life Sciences in Lublin.

REFERENCES

Adams, R.P. (2001). Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. 3rd ed. Allured Pub. Corp., Carol Stream, Ill.

Bielory, L., Heimall, J. (2003). Review of complementary and alternative medicine in treatment of ocular allergies. Curr. Opin. Allergy Clin. Immunol., 5, 395–399.

Blazics, B., Kéry, Á. (2007). Antioxidant activity of compounds in *Euphrasia officinalis* L. – revaluation of a traditional medicinal plant. Planta Med., 73, 266.

Brundrett, M.C., Kendrick, B., Peterson, C.A. (1991). Efficient lipid staining in plant material with Sudan Red 7B or Fluoral Yellow 088 in polyethylene glycol-glycerol. Biotech. Histochem., 66, 111–116.

Cappelletti, E.M., Caniato, R., Appendino, G. (1986). Localization of the cytotoxic hydroperoxyeudesmanolides in *Artemisia umbelliformis*. Biochem. Syst. Ecol., 14(2), 183–190.

Charrière-Ladreix, Y. (1976). Répartition intracellulaire du secrétat flavonique de *Populus nigra* L. Planta, 129, 167–174.

Clark, G. (1981). Staining procedures. 4th ed. Williams and Wilkins, Baltimore.

Combrinck, S., Du Plooy, G.W., McCrindle, R.I., Botha, B.M. (2007). Morphology and histochemistry of the glandular trichomes of *Lippia scaberrima* (Verbenaceae). Ann. Bot., 99(6), 1111–1119.

Dewick, P.M. (1997). Biosynthesis of pterocarpan phytoalexins in *Trifolium pratense*. Phytochemistry, 16, 93–97.

Dimitrova, M., Hristova, L., Damianova, E., Yordanova, Y., Petrova, N., Kapchina-Toteva, V. (2013). Antioxidant activity and secondary metabolites in different extracts of *Euphrasia officinalis* L. growing in Bulgaria. Sci. Technol. Med., 3(1), 128–132.

Dubey, V.S., Bhalla, R., Luthra, R. (2003). Sucrose mobilization in relation to essential oil biogenesis during palmarosa (*Cymbopogon martini* Roxb. Wats. var *motia*) inflorescence development. J. Biosci., 28(4), 479–487.

Fisher, E. (2004). Scrophulariaceae. In: The families and genera of vascular plants, Kadereit, J.W. (ed.). Springer-Verlag, Berlin–Heidelberg, 7, 332–432.

Gabe, M. (1968). Techniques histologiques. Masson, Paris, p. 204.

Gorji, A. (2003). Pharmacological treatment of headache using traditional Persian medicine. Trends Pharmacol. Sci., 24(7), 331–334.

Haratym, W., Weryszko-Chmielewska, E. (2013). Structural features of flower trichomes in drug eyebright (*Euphrasia stricta* D. Wolff ex J. F. Lehm.). Acta Agrobot., 66(4), 35–44.

Jensen, W.A. (1962). Botanical histochemistry principles and practice. 1st ed. WH Freeman and Company, San Francisco.

Johansen, D.A. (1940). Plant microtechnique. 1st ed. McGraw Hill, New York–London.

Kirk, P.W. jr (1970). Neutral red as a lipid fluorochrome. Stain Technol., 45, 1–4.

Kohlmünzer, S. (2007). Farmakognozja. Wyd. Lek. PZWL, Warszawa.

Kolseth, A.K., Lönn, M., Svensson, B.M. (2005). Genetic structure in two meadow varieties of *Euphrasia stricta* on the Baltic island of Gotland (Sweden) and implications for conservation. Folia Geobot., 40, 163–176.

Kozłowski, J. (1992). Świetliki i ich zastosowanie w lecznictwie. Wiad. Ziel., 4, 17–18.

Lison, L. (1960). Histochemie et cytochemie animals. Principes et méthods, v. 1, 2. Gauthier-Villars, Paris.

Mabberley, D.J. (2008). Mabberley's plant book. A portable dictionary of plants, their classification and uses. 3rd ed. Cambridge University Press, Cambridge.

Mace, M.E., Bell, A.A., Stipanovic, R.D. (1974). Histochemistry and isolation of gossypol and related terpenoids in roots of cotton seedlings. Phytopathology, 64, 1297–1302.

Mass Spectral Library (2008). USA:NIST/EPA/NIH.

Matławska, I., Sikorska, M., Kowalewski, Z. (1998). Związki flawonoidowe w *Herba Euphrasiae*. Herba Pol., 34(3), 97–102.

Miladinovic, D.L., Ilic, B.S., Nikolic, D.M., Markovic, M.S., Nikolic, N.D., Miladinovic, L.C., Miladinovic, M.D. (2014). Volatile constituents of *Euphrasia stricta*. Chem. Nat. Compd., 49(6), 1146–1147.

Nikolakaki, A., Christodoulakis, N.S. (2004). Leaf structure and cytochemical investigation of secretory tissues in *Inula viscosa*. Bot. J. Linn. Soc., 144, 437–448.

Novy, P., Davidova, H., Serrano-Rojero, C.S., Rondevaldova, J., Pulkrabek, J., Kokoska, L. (2015). Composition and antimicrobial activity of *Euphrasia rostkoviana* Hayne essential oil. Evid.-Based Compl. Alt. Med., article ID 734101.

Nurzyńska-Wierdak, R. (2013). Does mineral fertilization modify essential oil content and chemical composition in medicinal plants? Acta Sci. Pol. Hortorum Cultus, 12(5), 3–16.

Petrichenko, V.M., Sukhinina, T.V., Babiyan, L.K., Shramm, N.I. (2006). Chemical composition and antioxidant properties of biologically active compounds from *Euphrasia brevipila*. Pharm. Chem. J., 40, 312–316.

Polish Pharmacopoeia VII (2006). PTFarm, Warsaw.

Porchezhian, E., Ansari, S.H., Shreedharan, N.K.K. (2000). Antihyperglycemic activity of *Euphrasia officinale* leaves. Fitoterapia, 71, 522–526.

Posz, E. (2014). Rodzaj *Euphrasia* L. w Polsce. Taksonomia i rozmieszczenie. Instytut Botaniki Uniwersytetu Jagiellońskiego, Kraków.

Ríos, J.L., Recio, M.C. (2005). Medicinal plants and antimicrobial activity. J. Ethnopharmacol., 100, 80–84.

Sacchetti, G., Ballero, M., Serfaini, M., Muzzoli, M., Tosi, B., Poli, F. (2003). Morphological and histochemical investigation on glandular trichomes of *Orobanche ramose* subsp. *nana* (Orobanchaceae). Phyton, 43(1), 207–214.

Sacchetti, G., Romagnoli, C., Nicoletti, M., Di Fabio, A., Bruni, A., Poli, F. (1999). Glandular trichomes of *Calceolaria adscendens* Lidl. (Scrophulariaceae): histochemistry, development and ultrastructure. Ann. Bot., 83, 87–92.

Schmalholz, M., Kiviniemi, K. (2007). Relationship between abundance and fecundity in the endangered grassland annual *Euphrasia rostcoviana* ssp. *fennica*. Ann. Bot. Fenn., 44, 194–203.

Sharma, P., Singh, G.A. (2002). A review of plant species used to treat conjunctivitis. Review article. Phytother. Res., 16, 1–22.

Shestakova, T.S., Petrichenko, V.M., Sukhinina, T.V. (2008). Elemental composition of *Euphrasia brevipila* herbs and extracts. Pharm. Chem. J., 42(8), 460–462.

Soloway, S., Wilen, S.H. (1952). Improved ferric chloride test for phenols. Anal. Chem., 24(6), 979–983.

Stevens, P.F. (2001). Angiosperm Phylogeny Websitem, version 12, http//mobot.org/Research/APWeb/welcome.html [accessed: July 2012].

Szafer, W., Kulczyński, S., Pawłowski, B. (1986). Rośliny polskie. PWN, Warszawa.

Thoppil, R.J., Bishayee, A. (2011). Terpenoids as potential chemopreventive and therapeutic agents in liver cancer. World J. Hepatol., 3(9), 228–249.

Trovato, A., Monforte, M.T., Forestieri, A.M., Pizzimenti, F. (2000). In vitro anti-mycotic activity of some medicinal plants containing flavonoids. Boll. Chim. Farm., 139, 225–227.

Van Den Dool, H., Kratz, P.D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J. Chromat., 11, 463–471.

Weryszko-Chmielewska, E., Chernetskyy, M. (2005). Structure of trichomes from the surface of leaves of some species of *Kalanchoë* Adans. Acta Biol. Cracov. Bot., 47(2), 15–22.

Weryszko-Chmielewska, E., Chwil, M. (2006). Nutritive for insects attractants in *Asphodelus albus* Miller flowers. Acta Agrobot., 59(1), 155–164.

Weryszko-Chmielewska, E., Matysik-Wozniak, A., Sadowska, D. (2010). The structure and distribution of glandular trichomes on the stem and leaves of drug eyebright (*Euphrasia stricta* D. Wolff ex J. F. Lehm.). Acta Agrobot., 63(2), 13–23.

Woronuk, G., Demisse, Z., Rheault, M., Mahmoud, S. (2011). Biosynthesis and therapeutic properties of *Lavandula* essential oil constituents. Planta Med. 77(1), 7–15.