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Ultrastructural and histochemical analysis of glandular trichomes of *Marrubium vulgare* L. (Lamiaceae)

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ABSTRACT

Anatomical and ultrastructural investigations of the glandular trichomes of aboveground organs of the white horehound (*Marrubium vulgare* L.) were carried out. Additionally, identification of the main components of the secreted products was conducted. The following types of glandular structures were found: peltate with a stalk cell and a large eight-celled head and capitate trichomes, which were divided into three types: I. long-stalked with a 1-celled head, II. short-stalked with a 2-celled head, III. short-stalked capitate with a 4-celled head. Histochemical tests showed that the glands contained various substances such as lipids, polysaccharides, phenolic compounds, terpenes, tannins, and flavonoids. During the secretory stage, the ultrastructure of the bicellular head of the capitate trichome was characterized by a highly developed rough endoplasmic reticulum, numerous plastids, and mitochondria. After exocytosis, the secretion was temporarily stored in the periplasmic space and passed through the cell wall to the subcuticular space.

1. Introduction

For many centuries, natural components, such as mineral, plant, and animal products were the only raw material used for the process of making medicines (De Pasquale, 1984). The development of organic chemistry has led to the production of synthetic pharmaceuticals. The Industrial Revolution in the 18th and 19th centuries allowed obtaining pure compounds quite easily, and modification of their structures gave possibilities for preparation of potentially more active and safer drugs. It was also the time when the economic power of pharmaceutical companies increased (Rates, 2001). Lately, consumers in developed countries have become disillusioned with modern healthcare. They have started seeking alternative therapies and therapeutic use of natural products, especially those derived from plants, which is caused by several factors such as the inefficiency of conventional medicine, abusive and/or incorrect use of synthetic drugs, or their side effects (Rates, 2001; Kong et al., 2003).

The species belonging to the Lamiaceae family are considered one of the most valuable components of herbal treatments for a variety of ailments (Naghibi et al., 2005). Many of them produce, store, and release various secondary metabolites at the plant surface through their external glandular structures, namely trichomes (Naidu and Shah, 1981; Liu and Liu, 2012; Osman, 2012; Choi and Kim, 2013; Jia et al., 2013; Marin et al., 2013). These glandular trichomes are classified into two main types: peltate and capitate. According to Evert (2006) the first type has a basal cell, a short stalk, and a large head consisting of 4–18 cells, whereas the second one has a basal cell and a stalk, which is twice as long as their 1–4-celled head. Among these medicinal species, there is *Marrubium vulgare*, commonly known as the white horehound. Its natural habitats are Western Asia, North Africa, and Europe. Moreover, this plant is cultivated worldwide as a source for food flavouring and medicinal purposes. The white horehound is a perennial growing to 70 cm. The lower leaves are cordate, while the upper ones are oval. Both have a densely crinkled surface covered by silver trichomes. The small white n axillary clusters on the upper part of the main stem. (Szweykowska and Szweykowski, 2003; Senderski, 2007).

The medicinal raw material is the herb (*Marrubi herba*) consisting of whole or crushed flowering aerial parts of *Marrubium vulgare* (Pharmacopoeia, 2008). The main active ingredient produced and accumulated in these organs is a diterpenoid known as marrubiin (Popoola et al., 2013). The white horehound also contains diterpene alcohols, sterols, flavonoids, phenyl propanoids, sesquiterpens, tannins, alkaloids, and others (El-Bardai et al., 2003; Senderski, 2007; Verrna et al., 2012; Abadi and Hassani, 2013; Zawislak, 2015). Given the content of these substances, this plant is reported to possess analgesic, antihypertensive, anti –

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inflammatory, antioxidant, antiedematogenic, hypoglycemic, gastroprotective, vasorelaxant, and many other biological activities (El-Bardai et al., 2001; Senderski, 2007; Vergara-Galicia et al., 2013; Chedia et al., 2014). Moreover, the essential oil and extract from *M. vulgare* have antimicrobial activity against *Aeromonas hydrophila*, *Bacillus cereus, Escherichia coli, Listeria monocytogenes, Proteus vulgaris, Pseudomonas aeroginosa, Salmonella typhimurium, Shigella shinga,* and *Staphylococcus aureus* (Meyre-Silva and Cechinel-Filho, 2010; Rigi et al., 2013; Bokaeian et al., 2014; Chedia et al., 2014).

The healing activities of the horehound have been know since ancient Egypt (Wolski et al., 2007b). The Greek physician Hippocrates and other ancients valued the horehound as a panacea (Senderski, 2007). They used it to treat a lot of diseases such as: tuberculosis, bronchitis, pharyngitis, sinusitis, typhus, and hepatitis. Moreover, it was commonly applied in fertility treatment and lactation problems (Schaffner, 1996; El-Leithy et al., 2013). Nowadays, the horehound is used in herbal medicine as a cure for liver diseases, biliary tract disorders, cholelithiasis and also for increasing the appetite and supporting the function of the stomach. Furthermore, horehound infusions are an expectorant in respiratory system disorders (Wolski et al., 2007a; Senderski, 2007). The alcoholic extract is used as a new therapeutic agent with potential vasorelaxant and antihypertensive effects in the case of hypertension, which is a cardiovascular disease with the highest epidemiological impact in the world and represents a major risk factor for developing other illness such as congestive heart failure, coronary artery disease, diabetes, endothelial dysfunction, metabolic syndrome, renal dysfunction, and stroke (Vergara-Galicia et al., 2012, 2013; Yousefi et al., 2013).

Even though there are numerous studies about the chemical composition of the different species of *Marrubium* secretion, the structure of the secretory tissue has been scarcely investigated. A preliminary study of the secretory elements of *Marrubium cuneatum* was conducted by Baher Nik et al. (2004). The database of the micromorphology of *Marrubium vulgare* L. comprises one publication (Belhattab and Larous, 2006). Also the results of our initial microscopic analyses of *Marrubium vulgare* trichomes reported only the types of trichomes and their dimensions (Dmitruk and Haratym, 2014). A literature search did not reveal any reference about further analyses of these structures. Because of widely use of the biologically active substances produced by *Marrubium vulgare* the aim of this study is to determine the location of glandular trichomes and the composition of their secretion. To do that the appropriate methods were used. We show the various kind of those structures on stems, leaves and flowers in relation to the morphology, distribution, ultrastructure, and histochemistry of the main secreted products. Moreover, we consider the taxonomic value of investigated trichomes.

2. Material and methods

The *Marrubium vulgare* L. plants were collected at the Botanical Garden of the Maria Curie-Sklodowska University in Lublin, Poland (51°15.629′N 22°30.975′E). The investigations were carried out between June and August 2013–2016, when the plants were in full bloom.

The plant material was sampled by using razor blades and tweezers. Trichomes were observed on aboveground organs such as stems (parts from internodes), leaves (collected from 3rd and 4th nodes), and flowers (upper and lower lips of corolla, and calyx). The light (LM), fluorescence (FM), scanning electron (SEM), and transmission electron (TEM) microscopy was used.

2.1. Light microscopy (LM)

The anatomical analysis of the secretory trichomes was based on semi-thin sections (0,7 μm thick) made from transverse parts of leaves (squares from the central part of the leaf blade) and stems (the middle part of the 3rd and 4th internode). They were stained with a 1% aqueous methylene blue-Azur B solution. The material was fixed and embedded in acrylic resin (LR White) with the standard method used in transmission electron microscopy (see below). The observations and photos were made using a Nikon Eclipse 400 light microscope.

2.2. Histochemistry

The main classes of metabolites in the secreted material were investigated in fresh sections, using the following histochemical tests: Sudan III, IV, Red, and Black B for lipids (Johansen, 1940; Lison, 1960; Brundrett et al., 1991), Neutral Red for lipids and essential oils (Kirk, 1970; Clark, 1981), Nile Blue for neutral and acidic lipids (Jensen, 1962), Ruthenium Red for polysaccharides other than cellulose (Johansen, 1940), potassium dichromate for tannins (Gabe, 1968), concentrated sulphuric acid for sesquiterpenes (Cappalletti et al., 1986), Nadi reagent for terpenoids (David and Carde, 1964), magnesium acetate and aluminium trichloride for flavonoids (Charrière-Ladreix, 1976), and antimony trichloride for terpenes containing steroids (Mace et al., 1974). All stains were matched with controls. The stained sections were observed and photographed with a Nikon Eclipse 400 light microscope.

2.3. Fluorescence microscopy (FM)

Hand-made semi-permanent glycerol-mounted cross sections through fresh leaves were examined using a Nikon Eclipse 90i microscope equipped with a fluorescein isothiocyanate-FITC filter (excitation light 465–495 nm) and a barrier filter (wavelength 515–555 nm) (Mabry et al., 1970; Huang et al., 2008). Autofluorescence of the glandular trichomes, which helped identify phenolic compounds (Ascensão et al., 1999), and stain reactions with magnesium acetate, lead acetate, and antimony trichloride were observed using a Cy5 filter (excitation light – 590–650 nm and a barrier filter – wavelength 663–738 nm). Images were obtained with a digital camera Nikon Fi1 and NIS – Elements Br 2 software.

2.4. Scanning electron microscopy (SEM)

Five samples of each part of the stems, leaves, calyces, and corollas (5 mm × 5 mm) were fixed in a 4% glutaraldehyde solution in 0,1 M phosphate buffer (pH 7.0) for 12 h at room temperature. Subsequently, the plant material was washed in the same buffer four times at 20-min intervals and after that dehydrated in ethanol series (30, 50, 70, 90, and 95%), which was followed by application of absolute alcohol three times. When dehydrated, the samples were transferred to acetone. The next stage was drying at critical point in liquid CO_2 using Bal-Tec CPD 030 and coating with gold using the 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.

2.5. Transmission electron microscopy (TEM)

Ten fragments of leaves (3 mm × 3 mm) were fixed in 2,5% glutaraldehyde/4% formaldehyde in phosphate buffer for 2 h at ambient temperature. Afterwards, they were washed three times in phosphate buffer, post-fixed in 1,5% osmium tetroxide and placed in a dark cupboard for 1,5 h and washed in distilled water two times for 5 min. Next, the material was dehydrated in ethanol series