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Identification of lipid fraction constituents from grasshopper (*Chorthippus* spp.) abdominal secretion with potential activity in wound healing with the use of GC–MS/MS technique

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ABSTRACT

In recent years biologically active compounds isolated from insects call special interest of drug researchers. According to some Polish etnopharmacological observations, secretion from the grasshopper's abdomen (*Orthoptera* family) is believed to speed up the process of wound healing.

In the present work we focused on determination of main components of the lipid fraction of material from grasshopper abdomen using GC–MS/MS. Samples were qualitatively analyzed using gas chromatography coupled with mass spectrometry. Both liquid–liquid extraction and solid-phase extraction pretreatment methods were used to concentrate and fractionate the compounds from the insect. In the derivatized fractions *ca.* 350 compounds were identified, including substances of known biological activity. The potential agents affecting wound healing have been indicated.

A set of compounds characteristic for all the studied *Chorthippus* spp., have been identified. Data analysis revealed different lipidomic profiles of grasshoppers depending on the insects origin and collection area.

1. Introduction

Many currently used drugs took their origin from etnopharmacological observations. Folk tales for centuries have been a source of scientific hypotheses regarding therapeutic (healing) properties of various inorganic and organic materials. In the era of diseases of affluence and the pathogens resistant to commonly prescribed medicines, there is a need of constant search for more effective drugs. New biologically active substances are sought, *i.e.* new compounds which could be subjected to research and development process as active substances or as "lead compounds", serving as primary structures for future derivatives with the optimum pharmacodynamic and pharmacokinetic properties. According to the personal experiences in the childhood of one of the authors (R. Kaliszan) as well as on the basis of ethnographic literature, an ointment-like material squeezed out from abdomen of grasshoppers was used by villagers of Poland to facilitate healing of wounds and scars. A characteristic feature of this material is an extraordinary rheological property, which certainly encouraged villagers to apply supposed natural drug.

In the ethnography publication portraying folk medicine practiced in the South East of Poland there is a description of a mixture which was administered to heal skin cuts. Wounds were medicated with a mixture composed of whole wheat bread, cobweb, saliva and "fresh drug", which was a grasshopper's ointment [1]. From there originates a short rhyme, indicating the common usage of grasshopper abdomen secretion in the folk medicine:

"Koniku, koniku, daj mi maści,

To cię puszczę bez napaści" [2].

It could be translated as follows:

"Grasshopper, grasshopper let me have your ointment,

and I'll free you with no harm".

Grasshoppers were also used in the treatment of boils and warts. Boils were treated with a poultice of ground grasshopper legs and some villagers still insist that if one has a grasshopper bite a wart, the brown substance it leaves behind, will cause the offending growth to disappear. There are also etnopharmacological reports indicating that the grasshopper was used to treat fits in the nineteenth century in the state of New York [3]. In Poland, warts were

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treated by pinning a needle in them and then placing grasshopper secretion into the hole [4].

Grasshopper is one of the 20 000 species belonging to the *Orthoptera* genus. In the same group crickets, locusts and katydids are included. Present research focuses on grasshopper, which is one of the most common insects on the Polish territory. The primary aim of the study was to determine the main compounds in the lipidome of grasshopper abdominal secretion. The main goal of the research lays in the grasshopper eggs protective secretion compounds identification, as this secretion, most probably, possesses the wound healing properties. So far its protective and nourishing role was described. Moreover, the secretion takes important part in eggs cluster formulation and is used as a thermoisolation biomaterial [5].

In the study of insect's lipids, the most commonly used separation techniques are high performance liquid chromatography and gas chromatography. Zhao et al. [6] as well as Gołębiowski et al. [7–11] reported biochemical studies on insects with the use of HPLC combined with laser light scattering detector and GC combined with mass spectrometry. The reported lipidomic profiles contained both cuticular and internal lipids: fatty acids, sterols, carbohydrates, hydrocarbons, aldehydes, alcohols, and methyl and ethyl esters of fatty acids [7–13]. In a recent publication by Zhao et al. [6], 319 metabolites were detected in locust with the use of such techniques as GC-MS and HPLC-MS: 67 of them, analyzed by GC-MS, were identified as lipids, fatty acids, sterols, carbohydrates, hydrocarbons and amino acids. Gołębiowski et al. [7] used GC, GC–MS and HPLC–LLSD for identification and quantification of internal and cuticular lipids of the blow-fly (*Lucilla sericata*) male and female specimens [7]. Among the internal lipids, 23 free fatty acids, 5 alcohols and cholesterol were identified, while among the cuticular lipids, 16 free fatty acids, 7 alcohols and cholesterol were detected and quantified [7].

In another study on cuticular and internal lipids of an insect *Calliphora vomitoria*, the GC–MS technique was used. Authors of this study identified some fatty acids specific for the male cuticular lipidome [9].

Howard et al. [12] applied GC–MS for the analysis of cuticular lipidomic profile of *Liposcelis bostrychophila*.

Buckner et al. [13] used such techniques as GC and GC-MS for identification of cuticular lipids of the bellows and perring (*Bemisia argentifolii*) nymphs and exuviae. Analysis of the cuticular lipids demonstrated the presence of wax esters (86%), long-chain aldehydes (7%), hydrocarbons (3–4%) and long-chain alcohols (3–4%) at similar levels for both nymphs and exuviae forms of the insects.

The GC–MS and GC–FID analysis of both sexes of beetle (*Acanthoscelides obtectus*) showed the presence in cuticula of hydrocarbons, aldehydes, methyl- and ethyl-esters of fatty acids, triacylglycerols, free fatty acids, alcohols and sterols [10].

Gołębiowski et al. [8] used GC–FID, GC–MS for determination of qualitative and quantitative profiles of cuticular fatty acids from three insect species: *Calliphora vicina*, *Dendrolimus pini* and *Galleria mellonella.* Fatty acids, containing from 5 to 20 carbon atoms, were identified at varied amounts (from trace to 44%). The major compounds were the C16–C18 congeners. The lipidomic profiles of the *C. vicina* extract were significantly different for *D. pini* and *G. mellonella* subspecies. In another publication of Gołębiowski et al. [11] an analysis of epicular profile of western flower thrips (*Frankliniella occidentalis*) was carried out by GC–MS and GC–FID. Two groups of compounds: hydrocarbons and free fatty acids, were found in adult and larval forms of the insect.

The study presented in this paper is to our knowledge the first one reporting identification of lipidomic compounds contained in abdomen secretion of grasshopper *Chorthippus* spp. We report here a preliminary identification of lipidomic profile of grasshoppers abdominal secretion determined with the use of the gas chromatography–mass spectrometry technique.

2. Materials and methods

2.1. Collection and identification of insect material

The insects of *Chorthippus* spp. were collected from Starogard Gdański and Łubianka meadows (the north and middle part of Poland). The grasshoppers were macroscopically and microscopically identified. The study was performed with the use of abdominal secretion obtained from female grasshoppers. Gender identification was based on the anatomical differences of the insects wings (width to the length ratios) as well as on differences in anatomy of ovipositor between male and female specimens. Microscopic identification was carried out with the use of stereoscopic microscope (MST 132 LAB TK PZO, Warsaw, Poland). Insects were dipped for 30 s in liquid nitrogen in order to inactivate proteolytic enzymes. The insects were kept at −80 °C till the day of abdominal secretions preparations.

2.2. Chemicals

Oleic acid, cholesterol, cinnamic acid, octanal and pentadecanoic acid standards were purchased from Sigma Aldrich, St. Louis, MO, USA and benzyl alcohol from Fluka Chemie, Steinheim, AG, Switzerland. The derivatizing agents, *bis-N-O*-trimethylsilyl trifluoroacetamide (BSTFA) and chlorotrimethylsilane (TMCS) were purchased from Sigma Aldrich, St. Louis, USA. Hexane, isopropanol and methanol were purchased from J.T. Baker, Deventer, Netherlands, and chloroform from POCH, Gliwice, Poland. Deionized water was obtained with Milli-RO and Milli-QPlus instrumentation from Millipore, Switzerland.

2.3. Preparation of grasshoppers' abdominal secretion

The secretion was obtained from grasshopper abdomen (Fig. 1). Each sample weighted 30–40 mg and was prepared by combination of grasshopper abdominal secretion obtained from 3 to 6 insects. Lipids were extracted from the secretion with a chloroform:methanol (1:2, v/v) mixture, according to Bligh and Dyer method [7,8,14], adapted to the sample mass. The extraction resulted in obtaining two fractions: an aqueous fraction, rich in proteins and other compounds soluble in water, and organic fraction, composed of lipid compounds.

Compounds included in the lipid fraction were purified and separated by solid phase extraction (SPE) with Bond Elut Plexa 1 mL cartridges (Varian, Palo Alto, CA, USA). Cartridges were conditioned with 1 mL of methanol, followed by washing with 1 mL of water. After addition of 300 μL of sample, the cartridges were washed with 1 mL of 5% methanol in water and eluted with six different eluents, 1 mL each: hexane, hexane:chloroform (in ratio 1:2, v/v), chloroform, chloroform:methanol (in ratio 1:2, v/v), isopropanol. The solution was evaporated to dryness in a vacuum concentrator (Genevac Inc., Valley Cottage, NY, USA) for 20 min at temperature of 30 °C. The derivatization process was carried out with the use of a 25 μL portion of mixture of BSTFA with TMCS. After the reagent addition, the sample was heated in a thermo-block for 60 min at 97 °C. Derivatized mixtures were separately evaporated to dryness (30 °C, 3 min) in a vacuum centrifuge. Each of the dry residues was dissolved in 600 μL of chloroform and vortex-mixed for 2 min. The obtained samples were transferred to vials and 1 μL of extract was injected into the gas chromatograph–mass spectrometer system. Blank samples were included in each sample sequence. Blank comprised