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Preparative mass-spectrometry profiling of bioactive metabolites in Saudi-Arabian propolis fractionated by *high-speed countercurrent chromatography* and *off-line* atmospheric pressure chemical ionization mass-spectrometry injection

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ABSTRACT

Propolis is a glue material collected by honeybees which is used to seal cracks in beehives and to protect the bee population from infections. Propolis resins have a long history in medicinal use as a natural remedy. The multiple biological properties are related to variations in their chemical compositions. Geographical settings and availability of plant sources are important factors for the occurrence of specific natural products in propolis. A propolis ethylacetate extract (800 mg) from Saudi Arabia (Al-Baha region) was separated by preparative scale *high-speed countercurrent chromatography* (HSCCC) using a non-aqueous solvent system *n*-hexane–ACN (1:1, v/v). For multiple metabolite detection, the resulting HSCCC-fractions were sequentially injected *off-line* into an atmospheric pressure chemical ionization mass-spectrometry (APCI-MS/MS) device, and a reconstituted mass spectrometry profile of the preparative run was visualized by selected ion traces. Best ion-intensities for detected compounds were obtained in the negative APCI mode and monitored occurring co-elution effects. HSCCC and successive purification steps resulted in the isolation and characterization of various bioactive natural products such as (12*E*)- and (12*Z*)-communic acid, sandaracopimaric acid, (+)-ferruginol, (+)-totarol, and 3β-acetoxy-19(29)-taraxasten-20*a*-ol using EI-, APCI-MS and 1D/2D-NMR. Cycloartenol-derivatives and triterpene acetates were isolated in mixtures and elucidated by EI-MS and 1D-NMR. Free fatty acids, and two labdane fatty acid esters were identified by APCI-MS/MS. In total 19 metabolites have been identified. The novel combination of HSCCC fractionation, and APCI-MS-*target-guided* molecular mass profiling improve efficiency of lead-structure identification.

1. Introduction

Propolis is a regional strongly differing heterogeneous glue material generated by honeybees using plant resins and beeswax. It is used to seal cracks in hives and to protect the bee populations from bacterial and fungal infections [1,2]. Bees collect the material from leaves, leaf buds, bark resins, and exudates [3,4]. Since many different plant sources can be used by the honeybees the chemical profiles are as complex as mixing different medicinal plants to produce a decoction for a phytomedical formulation.

Propolis was already used in the antique by Egyptians, Romans and Greeks as a medicine to treat various diseases, e.g. disinfection and treatment of open wounds [5]. Geographical settings, plant sources, and the collecting season are important factors in the diversity of the propolis samples regarding their chemical profiles, and their multiple biological activities which are of interest to researchers [5].

Up to now, mass spectrometry with ESI- and APCI-MS has been used intensively for metabolite profiling- and fingerprinting

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-studies of propolis. HPLC–DAD–ESI-MS/MS implemented to a study of Italian propolis [6], resulted in 40 metabolites such as phenolic acids, and flavonoids. The investigation of propolis samples from different locations in Brazil using LC–APCI-MS (negative ion mode) led to a large amount of metabolites such as diterpenes and phenolic compounds. For the respective aqueous extracts, dicaffeoyl-quinic acids were identified [7]. Gardana et al. compared the profiles of propolis from Europe, Latin American countries, Russia and China using HPLC–ESI-DAD–MS, and identified mainly flavonoids and phenolic acid derivatives [8]. Volpi and Bergonzini quantified flavonoids in propolis from different regions of the world by LC–ESI-MS [9]. The mass spectrometric fingerprinting could be seen as a reasonable approach to correlate specific geographical origins, regional variations and to prove authenticity of propolis samples [10]. Fingerprinting and chemical profiling has been applied to a set of 49 ethanolic extracts of propolis samples collected worldwide (North and South America, Europe, Asia and Oceania) using ambient sonic-spray ionization mass spectrometry (EASI-MS) in the negative ionization mode [11].

Investigations and analysis of very complex constituted crude extracts (plants, terrestrial microorganism, and marine organism) are strongly dependent on efficient preparative chromatographic separation methodologies. Techniques such as *countercurrent chromatography* (CCC) and *countercurrent partition chromatography* (CPC) using solely liquid chromatographic phases are versatile in its use and increasingly implemented to lab-scale isolation protocols for the recovery of bioactive natural products [12–14]. A specific spectrometric detection of substances is also required for easy and fast tracing of lead- or target-compounds in order to facilitate the isolation process.

In the past we have used a direct *on-line* coupling of *high-speed countercurrent chromatography* (HSCCC) to an ion-trap ESI-MS/MS device or APCI-MS/MS as a fast screening method for acquisition of structural information of compounds eluting from preparative chromatography [15,16]. This method collected essential data to perform accurate preparative fractionations. Furthermore, it facilitated the specific detection of potential target molecules. Co-elution effects of up to 10 compounds could be easily monitored by selective ion traces including the respective MS/MS fragmentation data. In the present study we applied a novel *off-line* APCI-MS monitoring approach to characterize apolar metabolites recovered from the HSCCC fractions [17–19]. Sequential injections of collected fractions to the APCI-MS/MS mass spectrometer resulted in a reconstituted molecular weight profile for the separated and recovered compounds. It is important to recognize the presence of so far unknown metabolites by the acquired molecular weight data. Knowing specific bioactivities for the extracts and the CCC or CPC fractions, bio-assay-guided fractionation could correlate interesting biological data to mass spectrometric profiles.

There are many studies focusing on the metabolite profiles of propolis for elucidation of lead structures responsible of bioactivities (anti-inflammatory, anti-tumor, anti-bacterial) [20–23]. Honeybees harvest from available plant resources. The influence on the chemical constitution of propolis, plant biodiversity in the respective geographical regions is a strong limitation to elucidate or compare compound profiles of materials coming from not intensively investigated geographical areas. Especially, classical honey production areas in Persia, Arabia, and East-Africa will require much more efforts to build up a chemical data base for these regional propolis products.

In the presented study we investigated propolis from Al-Baha region (Saudi Arabia), a well-known honey production area. One principal direction of our investigation was to correlate the elucidated metabolites with existing phytochemical knowledge of local plants in order to trace the origin of the propolis product in this specific area.

A large amount of studies and surveys focused on therapeutic activities of propolis was given by Marcucci [21]. Recent progress in pharmacological research on propolis was summarized by Banskota et al. [1], and recent trends to elucidate the biological lead structures using bio-assays were surveyed by Bankova [22].

One issue regarding propolis is also toxicity due to the possibility that it could contain toxic plant metabolites. This aspect was reviewed by Burdock [23].

Interesting biological activities against parasites of so-called 'neglected tropical diseases (NTDs'), such as *Leishmania tropica* [24] were found. Also strong inhibitory activity of ethanolic extracts of a Bulgarian propolis material against proliferative epimastigotes of *Trypanosoma cruzi* (Chagas disease) was observed [25].

The all-liquid HSCCC study – which has a lower risk of chemisorptive losses – was initiated to open an effective route for fractionation, isolation and the recovery of bioactive metabolites from propolis without the potential loss of the target compounds responsible for observed bioactivities.

2. Materials and methods

2.1. Reagents

For the solvent extraction of the propolis material ethylacetate was used (Chromasolv® for HPLC, Sigma Chemical Co., St. Louis, USA). The preparative HSCCC separation was done with HPLC gradient grade solvents *n*-hexane, acetonitrile (Sigma Chemical, Deisenhofen, Germany). For the make-up solvent mixture being used for the APCI-MS/MS continuous *off-line* injections *tert*.-butylmethylether (Chromasolv®, Sigma, Deisenhofen, Germany), methanol (LC–MS-grade, Fisher Scientific, Loughborough, UK), and water (Nanopure®, Barnstead, USA) were used.

2.2. Propolis material and extraction of metabolites

All propolis material has been collected by the project team from our hives located in Al-Baha region. The total area of the patio area is comprising 10,362 km^2 and is located south-west of Saudi Arabia (coordinates 41°27′ E/20°0′ N) with an altitude range from 1550 to 1900 m including mountains areas up to 2215 m. The local bee colonies were classified as *Apis mellifera jemenitica*.

2.3. High speed countercurrent chromatography apparatus (HSCCC)

The separation of the propolis extract was performed on a preparative triple multilayer coil planet J-type *HSCCC* instrument (model CCC-1000, Pharma-Tech Research Corp., Baltimore, MD, USA). The three preparative separation coil columns were connected in series and were equipped with polytetrafluorethylene (PTFE) tubings: 165 m × 2.6 mm i.d. with 865 mL theoretical total volume (given by manufacturer). The measured total volume was 850 mL. The distance (revolution radius = *R*) of the holder axis of the coils to the central (solar) axis of the instrument was 7.5 cm. The inner β_r-value was measured to be 0.53 at the internal end of the coil and the outer β_r-value was 0.8 (equation: $\beta_r = r/R$; *r* is defined as the distance from the coil (planetary) axis to the nearest and farthest layer of the PTFE tubes wound on the coil system). The *HSCCC* system's direction of rotation determined the *head* locations at the periphery of the three coils.

2.4. HSCCC separation of the ethylacetate extract of propolis – biphasic solvent system

A suitable liquid–liquid separation system for the medium polar to apolar propolis metabolites in the ethylacetate extract was the