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Identification of potential markers of elevated anticandidal activity of propolis extracts

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ABSTRACT

Ethnopharmacological relevance: For centuries, propolis has been one of the most important and popular antimicrobial (antibacterial and antifungal) agents used in traditional medicine worldwide, including Central and Eastern Europe. Despite centuries of use of this product, the molecular mechanisms of its activity remain not fully recognized, and the components that determine its biological activity have not been identified.

Aim of the study: Hence, the main goal of the present study was to identify propolis ingredients that are crucial for the antifungal activity of this product.

Materials and methods: A serial two-fold microdilution method was applied to evaluate the activity of 83 ethanolic extracts of propolis (EEP) samples collected in different regions of Poland. The chemical composition of all EEPs was determined using UHPLC-DAD and UHPLC-QqTOF-MS methods. Advanced chemometric analysis of the correlation between antifungal activity and chemical composition was performed to identify the components related to the increased antifungal potential of propolis. Subsequently, the antifungal activities of pure "active ingredients" and their combinations were determined.

Results: Only seven extracts (8.4 %) exhibited high anticandidal potential with MIC (Minimum Inhibitory Concentration) values between 32 and 256 μg/mL. The identified most important potential markers related to increased antifungal activity of propolis collected in East Europe are: pinocembrin, pinobanksin-3-acetate, chrysin, galangin, pinobanksin, techtochrysin, genkwanin, pinostrobin and sakuranetin isomer. However, the pure compounds did not inhibit the growth of Candida spp. up to a concentration of 256 μg/mL (MIC >256 μg/mL). Much better activity was observed for combinations of these ingredients. The highest activity was observed for a mixture of five compounds: chrysin, galangin, pinocembrin, pinobanksin, and pinobanksin-3-acetate, with MIC and MFC (Minimal Fungicidal Concentration) values 64 and 128 μg/mL (summary concentration of all compounds – 12.8 or 25.6 of each μg/mL), respectively.

Conclusions: The relatively low number of propolis samples collected in Poland exhibit considerable activity against *Candida* spp. Markers of elevated antifungal potential have been identified. Moreover, it has been proved,

Abbreviations: CLSI, Clinical Laboratory Standard Institute; DAD, Diode Array Detector; DMSO, dimethyl sulfoxide; EEP, ethanolic extract of propolis; ESI, Electrospray Ionization; HRMS, High-Resolution Mass Spectrometry; LC-MS, Liquid Chromatography - Mass Spectrometry; MBEC, Minimum Biofilm Eradication Concentration; MFC, Minimum Fungicidal Concentration; MIC, Minimum Inhibitory Concentration; MOPS, 3-N-morpholinopropanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; OPLS-DA, Orthogonal Partial Least Squares - Discriminant Analysis, also known as Orthogonal Projections to Latent Structures; PBS, phosphate-buffered saline; PCA, Principal Component Analysis; PTFE, polytetrafluoroethylene; QqTOF-MS, Quadrupole Time-of-Flight Mass Spectrometry, rDA, retro Diels-Alder reaction; RT, retention time; SD, standard deviation; SPE, solid-phase extraction; UHPLC, Ultra-high Performance Liquid Chromatography; UV, ultraviolet radiation.

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that only the composition of these compounds (not pure ingredients alone) is effective in the treatment of *Candida* spp. Mixtures of these ingredients can be considered as potential antifungal agents (artificial propolis). Moreover, UHPLC-DAD and UHPLC-QqTOF-MS methods of determining the chemical composition of EEPs have been optimized.

1. Introduction

Infections with *Candida* species in humans, commonly referred to as candidiasis, have become more common in recent decades and this increase has coincided with an elevated number of immunocompromised patients (Arendrup, 2010; Katsipoulaki et al., 2024; Pfaller and Diekema, 2010). Globally, fungal infections are thought to affect tens of millions with mucosal candidiasis, 150 million individuals with severe illnesses, and roughly a billion with cutaneous infections (Bongomin et al., 2017). Most cases of candidiasis are endogenous, meaning that they arise from immunosuppressive and cytotoxic drugs, the use of broad-spectrum antibiotics, or an underlying medical condition (Pfaller and Diekema, 2010). *Candida albicans* is the most common fungal pathogen of humans, however, an increasing incidence of systemic candidiasis is caused by other *Candida* spp., such as *Candida glabrata*, *Candida auris*, *Candida krusei*, *Candida tropicalis* and *Candida parapsilosis* (Arendrup et al., 2023; Hernández-Pabón et al., 2024; Papon et al., 2013). Furthermore, according to the World Health Organization report from 2022 (WHO, 2022), most of the mentioned *Candida* species have been classified as immediate attention-requiring microorganisms. Treatments for *Candida* spp. infections are currently scarce and ineffective despite the high prevalence and severity of these diseases. Only a few groups of drugs – polyenes, triazole derivatives, echinocandins, allylamines, and 5-fluorocytosine – offer antifungal therapy alternatives, however, none of them fulfills all the necessary conditions (Sanglard et al., 2009). The side effect of using a limited number of medicines for the treatment of candidiasis and other fungal infections is a selection of resistant strains (Arendrup et al., 2023; Arendrup and Patterson, 2017; Vitiello et al., 2023). Thus, it is necessary to look for new, effective, safe for patients and inexpensive antifungals. Diverse natural products, for instance plant extracts (Dong et al., 2023; Hsu et al., 2021; Sun et al., 2021), essential oils (Gucwa et al., 2018a; Shala et al., 2022; Silva et al., 2021) and bee products (Gucwa et al., 2018b; Ożarowski et al., 2022) represent a promising, but yet undervalued group of potential antifungal agents.

Bee products are a great source of biologically active compounds (Bava et al., 2024; Giampieri et al., 2022), among which propolis is of particular interest regarding antimicrobial potential. For centuries, propolis was one of the most important and popular antimicrobial agents used in traditional medicine worldwide, including Central and Eastern Europe (Kuropatnicki et al., 2013; Rojczyk et al., 2020). It is a mixture of bee saliva, beeswax, and exudates from flowers, leaf buds, or other botanical sources (sap flows, etc.). Bees use this adhesive, resinous product as a form of protection against dangerous microorganisms and predators, to create an aseptic environment for larvae or to seal and thermally insulate the hive (Silva-Carvalho et al., 2015). Propolis typically contains 50 % resin, 30 % bee, and vegetable wax, 10 % essential oils, 5 % pollen, and 5 % additional components, such as organic pollutants (Burdock, 1998). Extracts of this product are a rich source of diverse classes of compounds, such as flavonoids, polyphenols, phenylpropanoids, terpenes, stilbenes, lignans, coumarins, and their prenylated derivatives (Bankova, 2005; Huang et al., 2014). Several variables affect its complex chemical composition and biological activities, such as the source apiary's geographical location, the species of plants that the bees can feed on, and the environmental conditions (Huang et al., 2014; Marcucci, 1995; Ristivojević et al., 2015). Extracts derived from propolis have shown numerous health-promoting qualities. These include broad antimicrobial (antibacterial, antifungal, and antiviral), antioxidant, carcinostatic, diastolic, anti-inflammatory, and anesthetic properties (Bava et al., 2024; Burdock, 1998; de Groot, 2013; Sforcin, 2007). Interesting reviews on the antifungal potential of propolis collected in various geographical regions have been recently presented by Ożarowski et al. (2022) and Cerqueira et al. (2022). Despite centuries of use of this product and significant progress in research on the biological activities of propolis, substances crucial for its antimicrobial activity have still not been identified, and the exact molecular mechanism of the antibacterial/antifungal activity of this product remains unknown. Reports presented by several research groups, including ours (Gucwa et al., 2018b; Grecka et al., 2019) suggest that the concentration of some fractions of flavonoids: flavonols (e.g. galangin), flavones (e.g. apigenin and chrysin), flavanones (e.g. pinocembrin and pinobanksin) decide about antimicrobial (including antifungal) potential of propolis samples collected in Western and Central European region. However, this hypothesis has not been finally proven to date. Another important gap in our knowledge that had to be filled was the assessment and comparison of the antifungal activity of pure compounds identified as markers of elevated anticandidal activity and their mixtures. This would make it possible to determine whether the antifungal potential of propolis is a consequence of the presence of some individual ingredients of high anticandidal activity or is the effect of positive/synergistic interactions between constituents of these products.

This study aimed to assess the antifungal activity of propolis samples gathered from different parts of Poland against planktonic cultures of three *Candida* species: *C. albicans*, *C. glabrata*, and *C. krusei*. The chemical composition of ethanolic extracts of propolis (EEPs) was thoroughly analyzed to identify particular components responsible for the antimicrobial/antifungal properties. The antifungal potential of these compounds and their mixtures was also investigated.

2. Materials and methods

2.1. Chemicals

Reagents: resazurin sodium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DMSO (dimethyl sulfoxide), PBS (phosphate-buffered saline), D-(+)-glucose, MOPS (3-N-morpholinopropanesulfonic acid), acetonitrile (both gradient grade and LC-MS grade), LC-MS grade water as well as formic acid and absolute ethanol were purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol and isopropanol (propan-2-ol) were obtained from POCH (Gliwice, Poland). Standard compounds for evaluation of antimicrobial activity: apigenin, chrysin, galangin, kaempherol, pectolinarigenin, pinobanksin, pinobanksin-3-acetate, pinocembrin, pinostrobin, quercetin, sakuranetin were obtained from ChemFaces (Wuhan, China), while phenolic acids: *p*-anisic, *p*-coumaric, caffeic and *p*-ferulic acid from Sigma-Aldrich (Steinheim, Germany). Analytical standards of caffeic acid, *p*-coumaric acid, pinobanksin, kaempferol, chrysin, pinocembrin, sakuranetin, galangin, pinostrobin, pinocembrin dihydrochalcone, techtochrysin were purchased from Extrasynthese (Genay, France). Analytical standards of 2′,6′-dihydroxy 4′-methoxydihydrochalcone were purchased from Merck (Darmstadt, Germany). Ultrapure water (<0.06 μS/cm) was obtained from Hydrolab HLP20UV (Hydrolab, Straszyn, Poland) purification system.

2.2. Fungal strains and media

The antifungal activity of propolis was investigated against four *Candida* reference strains: *Candida albicans* ATCC 10231, *Candida*

albicans SC 5314, *Candida glabrata* DSM 11226 and *Candida krusei* DSM 6128. The strains were routinely grown on Sabouraud Dextrose Agar (Merck KGaA, Darmstadt, Germany). All assays aiming at evaluation of antifungal activity (fungistatic, fungicidal, and biofilm eradication) were performed using RPMI 1640 medium Sigma-Aldrich (Steinheim, Germany) with neutral pH (7.0), supplemented with 2 % glucose, and buffered with the MOPS (3-*N*-morpholinopropanesulfonic acid). The pH of the medium was adjusted with solid NaOH.

2.3. Preparation of ethanolic extracts of propolis (EEPs), pure compounds solutions and mixtures

Eighty-three samples of *Apis mellifera* propolis were provided by apiaries located across various regions in Poland (Fig. 1). All samples were collected by beekeepers in autumn, between September and October 2022. Before further processing, samples were stored at room temperature, in the absence of lights, and in dry conditions. In each case, 5 g of raw propolis underwent extraction using 50 mL of 70 % ethanol according to the procedure presented in our previous article (Grecka et al., 2019). Briefly, the extraction process was conducted in darkness for 100 h at room temperature with gentle shaking (50 RPM). Following extraction, the ethanol extract solutions were subjected to centrifugation at 9000 RPM and filtration through Millipore filters with a pore size of 0.22 μm. The resulting filtrates were then evaporated to dryness at 40 °C using a rotary vacuum evaporator. The obtained resinous substance was weighed and subsequent stock solutions of the extracts were prepared at a concentration of 81.92 mg/mL in 70 % ethanol.

To create the stock solutions of pure flavonoids and phenolic acids, 100 % DMSO was used, and the final concentration of these solutions was 10.24 mg/mL. All the experimental compositions were made by mixing their constituents in a 1:1 ratio (w/w).

2.4. Fractionation of propolis extracts

Fractionation of the selected propolis extracts was performed using the SPE (solid-phase extraction) technique in the following manner. A C18 (E) SPE column (55 μm, 70 Å; 10 g/60 mL) Strata® (Phenomenex, Torrence, CA, USA) was preconditioned by rinsing with 100 mL methanol and 100 mL ultrapure water. Afterward, 50 mL of ultrapure water acidified with formic acid (0.1 % v/v) and 2.5 mL of ethanolic propolis solution (81.92 mg/mL) were loaded into the column. The column was rinsed subsequently with 100 mL of ultrapure water, 40 % aqueous methanol, 80 % aqueous methanol, and 100 % methanol. The obtained extracts were collected and evaporated under a vacuum.

2.5. UHPLC-DAD and UHPLC-QqTOF-MS analysis

For Ultra-high Performance Chromatography (UHPLC) analyses, Thermo Scientific™ UltiMate™ 3000 system (Thermo Scientific™ Dionex™, Sunnyvale, CA, USA) with an autosampler, Diode Array Detector (DAD), and/or Compact Quadrupole Time-of-Flight Mass Spectrometry (QqTOF-MS) detector (Bruker, Darmstadt, Germany) was used. Chromatographic separation was performed similarly as in previous research (Jenny et al., 2024) on Kinetex® C18 polar 2.6 μm, 100 Å, 150 × 2.1 mm analytical column with guard-column (Phenomenex, Torrence, CA, USA) thermostated at 20 ± 1 °C. The injection volume of the sample was

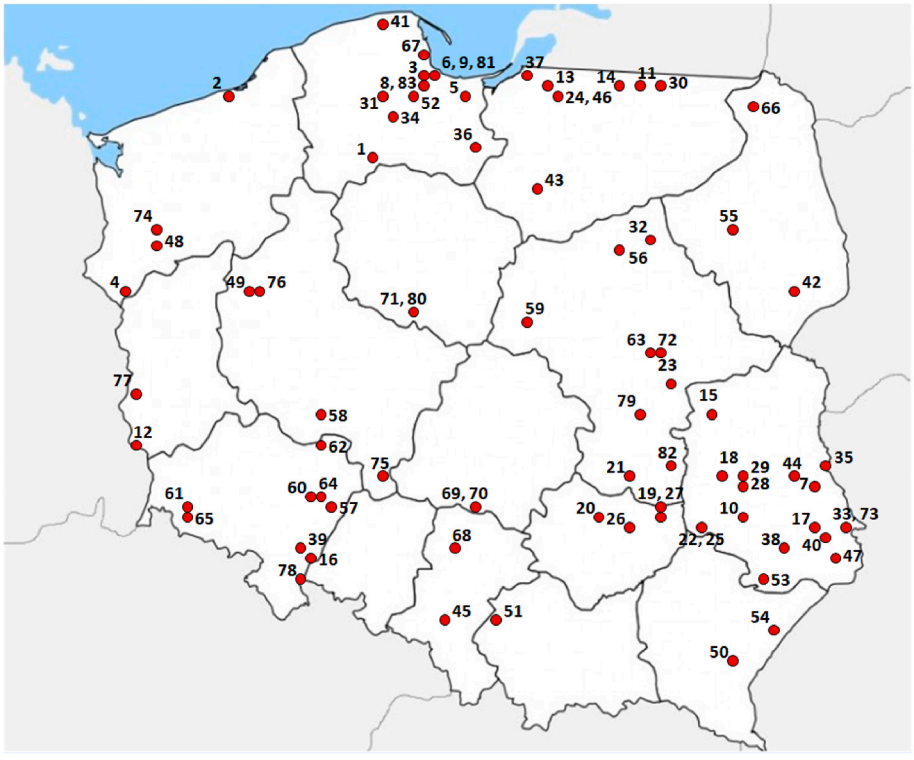

Fig. 1. Map of Poland indicating the geographic origin of the propolis samples and the place where they were collected. Locations: 1-Brusy, 2-Miłogoszcz, 3-Skrzeszewo, 4-Cychry, 5-Malbork, 6-Gdańsk, 7-Chełm, 8-Pruszcz Gdański, 9-Gdańsk, 10-Modliborzyce, 11-Sochodoły, 12-Łęknica, 13-Pieniężno, 14-Sępopol, 15-Potok, 16-Jagielno, 17-Grabowiec Góra, 18-Zwoleń, 19-Ostrowiec Świętokrzyski, 20-Zagnańsk Świętokrzyski, 21-Radom, 22-Wólka Szczecka, 23-Warka, 24-Lidzbark Warmiński, 25-Wólka Szczecka, 26-Ociesęki, 27-Szczucice, 28-Palanówka, 29-Lublin, 30-Wydutki, 31-Stężyca, 32-Modzele, 33-Hrubieszów, 34-Kozłowiec, 35-Włodawa, 36-Łasin, 37-Braniewo, 38-Trzęsiny, 39-Zarzyca, 40-Miączyn, 41-Perlin, 42-Bielsk Podlaski, 43-Prusy, 44-Spiczyn, 45-Łaziska Górne, 46-Lidzbark Warmiński, 47-Kryszyn, 48-Krzęcin, 49-Miłkowo, 50-Sielnica, 51-Chrzanów, 52-Liniewo, 53-Biszcza, 54-Lubaczów, 55-Łomża, 56-Maków Mazowiecki, 57-Nadolice Wielkie, 58-Krobia, 59-Płock, 60-Wrocław, 61-Olszyna, 62-Świętoszyn, 63-Warszawa, 64-Brzezia Łąka, 65-Mlądz, 66-Olecko, 67-Gdynia, 68-Kłobuck, 69-Miedźno, 70-Miedźno, 71-Polanowice, 72-Boryszew, 73-Hrubieszów, 74-Choszczno, 75-Kępno, 76-Połajewo, 77-Sękowice, 78-Paczków, 79-Kozinki, 80-Polanowice, 81-Gdańsk, 82-Lipsko, 83-Pruszcz Gdański.

set to 1 μL. The mobile phase used for chromatographic separation consisted of 0.1 % formic acid solutions in water (solvent A) and acetonitrile (solvent B). The flow rate was set at 0.4 mL/min. For a detailed analysis of propolis extracts the separation was obtained using the following gradient: 95 % of solvent A isocratic for 10 min, decreasing to 80 % within 1 min, and held isocratic for another 10 min, decreasing to 65 % A within 1 min and held isocratic for 8 min, decreasing to reach 40 % A within 15 min and isocratic for another 15 min. Subsequently, the elution solvent increased to 100 % B, the column was rinsed and then the solvent returned to 95 % A. Before the next analysis, the system was stabilized. Spectral data was recorded in the range of 200–600 nm as well as at 280, 320, and 360 nm. For LC–MS-based untargeted metabolomics, the same settings as above were used except the gradient that was as follows: 90 % of solvent A decreasing to 20 % within 27 min, to 0 % A within another 2 min, and held isocratic for another 5 min. Then, the solvent returned to 90 % A and before the next analysis, the system was stabilized. Several pooled-QC injections were analyzed at the beginning, end, and every 10 samples.

High-Resolution Mass Spectrometry (HRMS) detector was used in Electrospray Ionization (ESI) negative and positive mode, ion source temperature was set at 100 °C, nebulizer gas pressure at 2.0 bar, dry gas flow at 0.8 L/min, and temperature at 210 °C. The capillary voltage was set at 2.20 kV (negative mode) or 4.50 kV (positive mode) and collision energy at 8.0 eV. Internal calibration was obtained by injection of 10 mM solution of sodium formate clusters. For ESI-MS/MS experiments, collision energy was set at 35 eV and nitrogen was used as collision gas. Before the analysis, all the extracts were filtered through PHENEX™ 0.2 μm, Ø 25 mm, PTFE syringe filter (Phenomenex, Torrence, CA, USA). Standard solutions were prepared in absolute ethanol and the working standard solutions were diluted in ethanol or ultrapure water. The calibration curves were prepared in the concentration range of 6.25–200 μg/mL and the correlation values were 0.9996–1.0000. The content of derivatives of caffeic acid, *p*-coumaric, and pinobanksin were calculated as their equivalents. Additionally, the results were corrected using molecular mass.

2.6. Determination of MIC and MFC values

The MIC values were determined by the two-fold broth microdilutions according to the Clinical Laboratory Standard Institute (CLSI) standard guidelines (M27-A2, 2002). Yeasts were plated on Sabouraud solid medium and incubated for 24 h at 37 °C. One loop of pure culture was taken directly from the plate and transferred into PBS solution. The fungal suspensions were adjusted to the optical density (OD, λ = 660 nm) of 0.1 and further diluted in RPMI 1640 medium at a ratio of 1:50 (v/v) to the cell count of approximately 1.0×10^4 CFU/mL. 100 μl of such suspension was transferred to each well of a 96-well microtiter plate (Nest Biotechnology, Wuxi, China) containing 100 μl of dilutions of analyzed samples in the RPMI 1640. After the inoculation, the final concentration of tested ethanolic extracts of propolis, fractions, pure compounds (DMSO solutions), or pure compound mixtures (also prepared in DMSO) ranged from 256 to 8 μg/mL. Before the experiments each solvent used for tested solutions was confirmed not to have any antifungal activity in the applied amounts. Agent-free wells served as a growth control and agent- and cell-free wells as sterility controls. Plates were incubated stationary for 24 h at 37 °C. Most of the EEPs and pure compound samples, especially in higher concentrations, are poorly soluble in water and create sediment in contact with the medium. To overcome the difficulty of interfered growth measurements, the resazurin test was used. After incubation, resazurin sodium salt solution (0.015 % in PBS buffer) was added to all wells in the volume of 30 μL and further incubated for 2 h at 37 °C in the dark. The lowest concentration with no color change was recognized as MIC value (blue resazurin color remained unchanged). The MFC value was determined by transferring each dilution used for MIC assay on Sabouraud solid medium using a sterile 48-well microtiter plate replicator. The plates were then incubated for 24 h at 37 °C. Concentrations, for which no growth was observed, were recognized as minimum fungicidal concentrations.

2.7. Chemometric analysis of the LC-MS fingerprints

Before the statistical analyses, the obtained LC-MS profiles were combined in datasets using MetaboScape® 2021b software (Bruker). The obtained data were combined with microbiological results. The samples were divided based on MIC values determined for *Candida albicans* ATCC 10231 reference strain in three groups: MIC ≤128 μg/mL, MIC = 256 μg/mL, and MIC ≥256 μg/mL. Using Simca® v. 17.02.34594 software (Sartorius Stedim Data Analytics AB) the data were analyzed by applying multivariate data analysis tools. The obtained data were evaluated by Principal Component Analysis (PCA) to observe chemical variability within the dataset comparing different options of data preprocessing techniques which resulted in the selection of no-scaling or centering (when indicated). For the determination of propolis quality type and related chemical markers based on LC-MS fingerprints, datasets containing extracts with MIC ≤128 μg/mL and MIC ≥256 μg/mL as highly active and less active classes, respectively were used for Orthogonal Partial Least Squares - Discriminant Analysis (OPLS-DA) analyses after appropriate data pre-treatment. S-line and S-plot were used for the identification of putative biomarkers (pairs of retention time and exact mass) related to highly active and less active propolis extracts. Identification of the compounds was based on retention time, exact mass, MS fragmentation, UV spectra, and comparison with analytical standards.

2.8. Biofilm formation and determination of $MBEC_{50}$ of EEPs

The biofilm cultivation and determination of $MBEC_{50}$ values were performed in accordance with the method described by Gucwa et al. (2018b) with minor modifications. From *C. albicans* cultured on solid Sabouraud medium for 24 h at 37 °C, cell suspension of $OD_{660} = 0.1$ was prepared in sterile PBS, similarly to the above-mentioned MIC assay. Subsequently, the cell suspension underwent 1:100 (v/v) dilution in RPMI 1640 medium and was transferred to a 96-well microtiter plate so that each well contained 200 μL. One row of the plate remained free of fungal inoculum and served as a sterility control. Biofilm structure was formed for 24 h at 37 °C in stationary conditions. Following the removal of inoculums, wells were gently washed with PBS and then filled with 200 μl of EEP solutions in RPMI 1640 at concentrations ranging from 64 to 2048 μg/mL. A row free of EEP addition was used as growth control (untreated biofilm/cells). After 24 h incubation at 37 °C, wells were washed with PBS once more. To assess the activity of EEPs against biofilm formed by *C. albicans*, 200 μL of MTT solution (5 mg/mL in PBS) was added to the wells and mixed. After 2 h incubation at 37 °C in the dark, the MTT was replaced with isopropanol to dissolve formed formazan crystals. The absorbance (OD_{540}) of the obtained solutions of formazan was measured at 540 nm using a Victor3 microplate reader (PerkinElmer, Waltham, USA). $MBEC_{50}$ values were defined as the lowest concentration of propolis that caused the eradication of at least 50 % of biofilm (living cells, that have the ability to metabolize MTT and produce formazan crystals) in comparison to the biofilm/cells growing in the RPMI media not supplemented with EEPs (untreated control). The biofilm eradication percentage was calculated using the following formula:

$$\text{Biofilm eradication } [\%] = \left(1 - \frac{OD_{540} \text{ of treated biofilm/cells}}{OD_{540} \text{ of untreated biofilm/cells}}\right) \times 100$$

2.9. Time-kill assay

Selected EEP samples with high (EEP 8, 33, 74, and EEP 33 Met80 Fraction) and low (EEP 18 and 21) antifungal activity were subjected to a Time-kill assay against *C. albicans, C. glabrata,* and *C. krusei* using a

similar method as described by Gucwa et al. (2018b). Yeast suspensions in PBS with an OD_{660} of 0.1 were prepared using cells that had been cultured for 24 h at 37 °C on Sabouraud agar plates. Following dilution in RPMI 1640 medium at a ratio of 1:50 (v/v), EEPs were added to the suspensions at concentrations equivalent to 64, 128, or 256 μg/mL. Samples containing fungal suspensions without the addition of EEP served as control. Prior to the experiments, solvents used in tested solutions were confirmed not to have any antifungal activity in the applied amounts. Incubation was carried out at 37 °C for 0, 2, 4, 8, and 24 h while shaking (140 RPM). After each predefined time interval, samples were serially diluted in PBS buffer (from 10^{-1} to 10^{-4}) and spotted onto Sabouraud agar plates in the volume of 10 μL. After incubation for 24 h at 37 °C, colonies were counted and the number of cells in 1 mL was estimated.

2.10. Growth kinetics analysis

To ascertain the growth kinetics of *C. albicans, C. glabrata,* and *C. krusei* in the presence of varying concentrations of EEPs, flavonoids, or their mixtures, a microtiter plate-based assay was employed analogous to the one used by Grecka and Szweda (2021). Growth curves were generated for five EEPs and their Met80 fractions, seven mixtures of flavonoids and phenolic acids, as well as pure flavonoids, namely chrysin, galangin, pinobanksin, pinobanksin-3-acetate, pinocembrin, pinostrobin and sakuranetin, that were found to be the most abundant compounds in the EEP samples with the highest antifungal activity. Two-fold dilutions of flavonoids were prepared in the RPMI 1640 medium. Subsequently, to 100 μL of each dilution present in the wells, an inoculum containing approximately 1.0×10^4 CFU/mL was then added, resulting in a final volume of 200 μL. Microbial growth kinetics were monitored over 24 h using the SPARK® multimode microplate reader (Tecan, Männedorf, Switzerland). At hourly intervals, the turbidity of the culture was read by measuring absorbance at 660 nm, with agitation for 10 s preceding each optical density measurement.

2.11. Data analysis

In the case of Time-kill assay and Growth kinetics analysis, all experiments were performed in triplicate and the data was expressed as the means ± SD. XY graphs were constructed using GraphPad Prism® 8.0.2 (GraphPad Software, Inc., La Jolla, USA). The values of MIC, MFC, and $MBEC_{50}$ were determined in three independent experiments. If any differences in the values were observed between experiments the values were determined in two additional experiments. The value that repeated at least three times was finally presented as MIC, MFC, or MBEC.

3. Results and discussion

3.1. Determination of MIC and MFC values of produced EEPs

The detailed results of the investigation of the antifungal potential of all prepared EEPs are presented in supplementary materials (Table S1). Even at the highest tested concentration (256 μg/mL) 53 products (63.9 %) did not exhibit even fungistatic activity against at least one of the investigated reference strains of *Candida* spp. and exhibited low or lack of activity against other strains tested. Thus, the activity of these EEPs was classified as very low. The activity of another 16 extracts was classified as low – the MFC values of these products for three tested strains (except *C. albicans* SC 5314) were higher than 256 μg/mL. MIC values for these EEPs were in the range of 128–256 μg/mL. EEPs (n = 7) of medium activity effectively killed at least both *C. albicans* reference strains at a concentration of 256 μg/mL. The seven most active EEPs effectively killed the cells of all strains tested at a concentration of 256 μg/mL or lower. The values of MIC and MFC of these products are also presented in Table 1. The EEP number 33 was found as the most effective, with MIC and MBC values in the range 32–128 and 128–256 μg/mL, respectively. In most cases (except *C. albicans* SC 5314), achieving the fungicidal effect required a concentration twice as high as the concentration necessary to inhibit the growth of yeast cells. Interestingly, considering only MIC values, *C. glabrata* DSM 11226 seemed to be the most sensitive to the activity of EEPs' constituents. However, MBC values for this strain were the same or even higher compared to other strains tested. The effectiveness of EEPs investigated herein was similar to the activities of the extracts of propolis samples collected in other European countries. For instance, Al-Ani et al. (2018) evaluated the antifungal effectiveness of propolis collected in Ireland, the Czech Republic, and Germany (two samples), and the MIC values against *C. albicans* ATCC 90028 were 600, 600, 2500, and 5000 μg/mL, respectively. Activity against other *Candida* spp. reference strains were at the same level and similar to the activity of the majority of our products (MIC >256 μg/mL). Boisard et al. (2015) reported that extracts of French propolis prepared with 70 % ethanol effectively inhibited the growth of *C. albicans* and *C. glabrata* at a concentration of 31.25 μg/mL. However, those authors presented the activity of produced extracts as MIC_{80}, not MIC_{90}. The group led by Fernández-Calderón (2021) studied the activity of ethanolic extract of Spanish propolis against twelve strains of *C. glabrata*. The extract had good antifungal activity, with MIC values within the range of 0.1–0.4 % (60–240 μg/mL). However, the authors used a different methodology (dilution in agar) for the determination of activity. Our previous reports have confirmed a significant anticandidal activity of Polish propolis (collected in another time period), MFC values for the analyzed products were in the range of 0.08–1.25 % (*v/v*) (Gucwa et al., 2018b). Unfortunately, a slightly different method of preparing extract was applied in this study, thus it was difficult to present a detailed comparison of the results of these two investigations. Propolis collected in other geographical areas, particularly in Brazil, also can be considered as a potential agent for candidiasis treatment. Freires et al. (2016) stated that extracts produced from Brazilian propolis (types 3 and 13) showed MIC values ranging from 0.2 to 125 μg/mL and MFC values between 125 and 500 μg/mL against *Candida* spp. Another research group found that fungicidal activities of Brazilian red propolis were in the range of concentrations of 64–512 μg/mL for *C. albicans* and 64–256 μg/mL for *C. glabrata* (Siqueira et al., 2015). A considerably lower fungicidal potential was stated for green propolis collected in Brazil (Tobaldini-Valerio et al., 2016). Quite high activity of Iranian propolis against *C. albicans* ATCC 10231 was observed by Gavanji and Larki (2017), with MIC_{90} and MFC equal to 39 and 65 μg/mL. Using disk diffusion assay, Polish researchers (Okińczyc et al., 2020) revealed promising antifungal activity of propolis collected in another Asiatic country – Nepal. The extracts of Cameroonian propolis also showed considerable antifungal potential with MICs ranging from 250 to 500 μg/mL on *C. albicans, C. krusei,* and *C. glabrata* (Tamfu et al., 2022). Saleh et al. (2023) obtained hydrogels functionalized with ethanolic extract of Egyptian propolis that exhibited an antimicrobial potential against *Escherichia coli, Salmonella typhimurium, Streptococcus*

Table 1
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the most active ethanolic extracts of propolis (EEP) samples against *Candida* genus representatives.

No. EEP	MIC and MFC (μg/mL) against different strains of *Candida*							
	Candida albicans ATCC 10231		*Candida albicans* SC 5314		*Candida glabrata* DSM 11226		*Candida krusei* DSM 6128	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
8	128	256	256	256	64	256	128	256
33	64	128	128	128	32	256	64	128
39	128	256	256	256	128	256	128	128
60	128	256	128	128	256	256	64	128
74	128	256	256	256	64	256	128	256
76	128	256	256	256	128	256	128	256
83	128	128	128	128	64	128	64	128

mutans, and C. albicans. MFC values of the extracts of Mexican propolis determined against *C. glabrata, C. krusei*, and *C. albicans* ranged from 312 to 1250 μg/mL (Rivera-Yañez et al., 2022). Interestingly, Massaro et al. (2015) stated that propolis from eastern Australia was not active against *C. albicans* ATCC 10231.

Another, and a bit worrisome conclusion from this part of our research was that only about 10 % of tested samples of propolis collected in different regions of Poland exhibit elevated antifungal activity. This result is consistent with our previous observations (Gucwa et al., 2018b; Grecka et al., 2019). It is probably a consequence of some negative changes in biodiversity and the composition of plant species that are available for bees in natural ecosystems. It has been proved that the antimicrobial potential of propolis is strongly associated with plant species that are a source of raw materials (resins) for preparing this product. The antimicrobial activity of propolis collected in West and Central Europe strongly depends on availability of the trees of the *Populus genus* (e.g. *Populus nigra*). Unfortunately, within several decades the number of these trees in natural ecosystems is systematically decreasing.

Considering the application of EEPs as antifungal agents it must be stated that each product would have to be tested for its antimicrobial activity (eventually content of active ingredients). On the other hand, this results support our idea of using compositions/mixtures of active ingredients of propolis of confirmed activity instead of EEPs.

3.2. LC-MS fingerprinting

For a better understanding of observed differences in the antifungal potential of produced extracts and also for identification of ingredients that are crucial for fungistatic/fungicidal effect, all 83 extracts were subjected to detailed analysis of their chemical composition with the UHPLC technique. A representative chromatogram of propolis extract is presented in Fig. 5. The obtained datasets included 7391 and 29964 variables for ESI- and ESI+, respectively. Most relevant compounds were identified in the propolis extracts by UHPLC-QqTOF-MS and UHPLC-DAD. The most abundant peaks in ESI-, in terms of signal intensity, were those of several flavonoids and chalcones (pinocembrin, pinobanksin-3-acetate, pinobanksin-3-propanoate, chrysin, pinobanksin, galangin and 2′,6′-dihydroxy-4′-methoxydihydrochalcone) as well as phenylpropanoids, mostly esters thereof (*p*-coumaric acid benzyl ester, caffeic acid benzyl ester, pentyl *p*-coumarate, *p*-coumaric acid, *p*-coumaric acid methylbutenyl ester, caffeic acid 3-methyl-2-butenyl ester, caffeic acid 2-methyl-2-butenyl ester, *p*-coumaric acid cinnamyl ester. In ESI+ the most abundant were: chrysin, techtochrysin, genkwanin, pinocembrin, pinobanksin 3-acetate, pinostrobin, 2′,6′-dihydroxy-4′-methoxydihydrochalcone, sakuranetin isomer, and several unidentified compounds.

A relevant number of components, including *p*-coumaric acid, kaempferol, pinocembrin, pinobanksin, chrysin, galangin, 2′,6′-dihydroxy-4′-methoxydihydrochalcone, genkwanin, techtochrysin, and pinostrobin were identified based on comparison with reference compounds. Different *p*-coumaric acid and caffeic acid esters characteristic of propolis were identified based on exact mass, MS^2 fragmentation, and UV spectra and data from previous research (Okińczyc et al., 2021; Gardana and Simonetti, 2011). For example, *p*-coumaric acid methylbutenyl esters exhibited absorption maxima at 313 nm and m/z 231.10242 ([M-H]-), calculated for $C_{14}H_{16}O_3$, 232.10994. The MS^2 experiment at 35 eV in negative mode resulted in fragments: 119 corresponding to decarboxylated *p*-coumaric acid or loss of methylbutenyl and carbonyl moiety ([M - H]$^-$- C_5H_9-CO), 145 corresponding to dehydrated *p*-coumaric acid or loss off methylbutenyl moiety and water ([M - H]$^-$- C_5H_9-H_2O), 163 corresponding to *p*-coumaric acid or loss of methylbutenyl moiety ([M - H]$^-$- C_5H_9). The ions 163/145 can be considered diagnostic ions for *p*-coumaroyl. The determination of other phenylpropanoid - caffeoyl derivatives was confirmed by the presence of appropriate losses and diagnostic ions (179/161) in MS^2 spectra of their esters. The compound characterized by retention time (RT) 16.0 min and pseudomolecular ion [M-H]$^-$ = 247.0986 corresponding to molecular formula $C_{14}H_{15}O_4^-$ and MS^2 fragments m/z 133 as well as 179 and 161 was identified as caffeic acid 3-methyl-2-butenyl ester. On the other hand, in positive ionization the compound was unstable and the base peak was m/z 163.0389 accompanied by m/z 181.0496 corresponding to caffeate and very weak m/z 249.1121 corresponding to pseudomolecular ion $[M+H]^+$.

Some minor components, highlighted by OPLS-DA analysis as significant for the characterization of propolis extracts with high antifungal activity are less commonly reported or not previously reported in propolis, and therefore their tentative identification was further described.

Compounds characterized by retention time (RT) of 17.6 min, 20.9 min, and pseudomolecular ion $[M+H]^+$ = 285.0760 corresponding to molecular formula $C_{16}H_{13}O_5^+$ may be attributed as methylated trihydroxyflavones. This is supported by the fact, that their MS^2 fragmentation spectra in positive mode are very similar to galangin-5-methyl ether (RT = 14.5). Production in ESI + at m/z 270 corresponds to loss of methyl radical and m/z 242 of further loss of carbonyl group. Compound eluting at 20.9 min produced a characteristic fragment (167.0339$^+$, $C_8H_7O_4^+$) that may be identified as the product of retro Diels–Alder fragmentations (rDA reaction) - $[A^{1,3}]^+$ methylated fragment of A ring. The corresponding fragment was also present in pinostrobin (Okińczyc et al., 2024) which may suggest a similar structure of the A-ring in these components. Apart from $[A^{1,3}]^+$ methylated fragment of the A ring, also fragments 179 and 105 were present. They may be identified as rDA $[A^{0,2}]^+$ and $[B^{0,2}]^+$ fragments, respectively. Generally, in positive mode, the production of rDA $[A^{1,3}]^+$, $[A^{0,2}]^+$, and $[B^{0,2}]^+$ fragments were described as characteristic of flavonols, while flavones should rather produce $[A^{1,3}]^+$, $[B^{1,3}]^+$ rDA fragments (Ma et al., 1997). Moreover, also neutral CO loss (fragment [M + H-CH_3-CO]$^+$ = 242.0582$^+$) suggested the rDA fragmentation pathway. Additionally, for this compound, the ionization in ESI- was very poor. It is known, that the presence of a more acidic hydroxyl group makes ionization in negative mode easier (Nikolic and van Breemen, 2004). If hydroxyl groups are absent or blocked, ionization in negative mode is more difficult. In extreme cases, pinostrobin (flavanon) and techtochrysin (flavone) ionization in negative mode were not observed (Okińczyc et al., 2024). Both flavonoid aglycones have only two hydroxyl groups and position 7 of the A-ring is methylated, which suggests that methylation of the 7-hydroxyl group in the A ring prevents ion production in negative mode. Own comparison of fragmentation of more structures, based on reference compounds, showed that the presence of additional free hydroxyl group in the 4' position in the B ring (sakuranetin) allows ionization in negative mode. For these reasons, it may be suspected, that the discussed compound was rather methylated in position 7 and the B-ring was not hydroxylated. As a result, RT = 20.9 may be tentatively identified as galangin-7-methyl ether. In the case of a similar compound (RT = 17.6 min) in ESI+, the main fragmentation pattern is comparable, however, fragment m/z 285 dominates, suggesting more difficult demethylation and thus methoxy group in C ring with possible identification as galangin-3-methyl ether. For the second compound (RT = 20.9 min) fragment 270 dominates suggesting a possible methylation site in A ring and supports the identification as galangin-7-methyl ether. He and others reported that the methoxy group at the C ring is more easily demethylated (−15 Da) than that of the A ring (He et al., 2017). Characteristic MS^2 fragments for compound at RT = 20.9 min in both ESI+ (285, 270, 242) and ESI- (283, 268, 239, 211) and UV maxima correspond to those in the literature for izalpinin (galangin-7-methyl ether) (Agüero et al., 2010). Similarly, MS^2 fragments for compound at RT = 17.6 corresponded to literature data of galangin-3-methyl ether (Bojilov et al., 2023; Erusappan et al., 2021) and its UV maximum corresponded to previously reported data (Alday et al., 2019). Those compounds were previously found in different propolis samples e.g. from the Netherlands, China, and Spain (Banskota et al., 2002; García-Viguera et al., 1992; Greenaway et al., 1991; Usia

et al., 2002).

The compound characterized by a retention time of 17.3 min and pseudomolecular ion $[M+H]^+ = 301.0714$, corresponding to $C_{16}H_{13}O_6^+$, was tentatively identified as kaempferol methyl ether. Demethylation of components in ESI+ was rather difficult, which resulted in a higher presence of protonated molecular ions, than of its demethylated fragment ($[M + H\text{-}CH_3]^+ = 286.0411^+$) and ($[M + H\text{-}CH_3\text{-}CO]^+ = 258.0530^+$) in MS^2. Negative MS^2 ionization gave characteristic fragments at m/z 284, 255, 211, 227, and 151 (Gu et al., 2012).

Another compound identified among compounds highlighted by OPLS-DA as related to elevated anticandidal activity was characterized by mass 252.17262 corresponding to molecular formula $C_{15}H_{24}O_3$ and retention time (RT) of 15.9 min. The observed pseudomolcular ions in ESI+ were 275 $[M+Na]^+$ and 235 $[M\text{-}H_2O + H]^+$ automatically detected as separate variables and in ESI- 251 $[M\text{-}H]^-$ was present. Similar behavior was observed for some sesquiterpenes, e.g. ilicic acid (Mamoci et al., 2011), however, it is not consistent with fragmentation reported by Yuan et al. (2024). The observed MS^2 fragments in negative mode were: *m/z* 233, 205, 191, 165, 135, 117, 99 and MS^2 fragments in positive mode: *m/z* 235, 217, 189, 161, 147, 133, 119, 107, 91. The fragment *m/z* 233 corresponds to a loss of water $[M\text{-}H\text{-}H_2O]^-$, *m/z* 205 to successive loss of water, and CO $[M-H_2O\text{-}CO]^-$, *m/z* 217 $[M\text{-}H\text{-}2H_2O]$ which are characteristic ions occurring in sesquiterpene lactone fragmentation patterns and *m/z* 191 $[M\text{-}H\text{-}4CH_3]^-$ (Zengin et al., 2023). Successive loss of water and CO is present in sesquiterpene lactones with hydroxyl and acetyl substitution (El-Sabagh et al., 2021), thus, the compound could be tentatively identified as acetylated sesquiterpene lactone.

The compound characterized by RT 22.9 min, UV maximum of 281 nm, and m/z 317.2078 is accompanied by m/z 295, and 277 and probably corresponds to $[M+Na]^+$ adduct of 295.2271 $[M+H]^+$ ($C_{18}H_{31}O_3^+$). The latter is fragmented in MS^2 giving characteristic fragmentation ions with m/z 277, 249, 205 in ESI+ and *m/z* 275, 249, 197, 185, 125 in ESI- which is consistent with data for oxylipin: 9-oxo-10 (*E*),12(*Z*)-octadecadienoic acid (9-*oxo*-ODE) (Hu et al., 2022; Kim et al., 2011). This compound was identified as a poplar propolis component and had relevant activity against bacteria and fungi (Bilikova et al., 2012; Hu et al., 2022; Prost et al., 2005). It was found in *Populus nigra* and is particularly abundant in *Populus canadensis* bud exudates (Wang et al., 2017) and the concentration in poplar bud exudate was elevated in the autumn and winter period (Hu et al., 2022).

3.3. Principal component analysis (PCA)

The PCA was conducted separately using datasets obtained in negative (ESI-) or positive (ESI+) ionization, as described previously, that included data from 83 samples and 7391 and 29964 variables (pairs of retention time and exact mass). The data was used with no scaling before analysis and the PCA scores plot revealed natural clustering (Fig. 2) according to the activity expressed as MIC values determined for *C. albicans* ATCC 10231 reference strain. The two first factors explained 90.0 % (ESI-) or 86.4 % (ESI+) of variance between the samples. The group of samples characterized by MIC ≤128 was clearly separated from those with MIC >256 and those with MIC = 256 partially overlapped

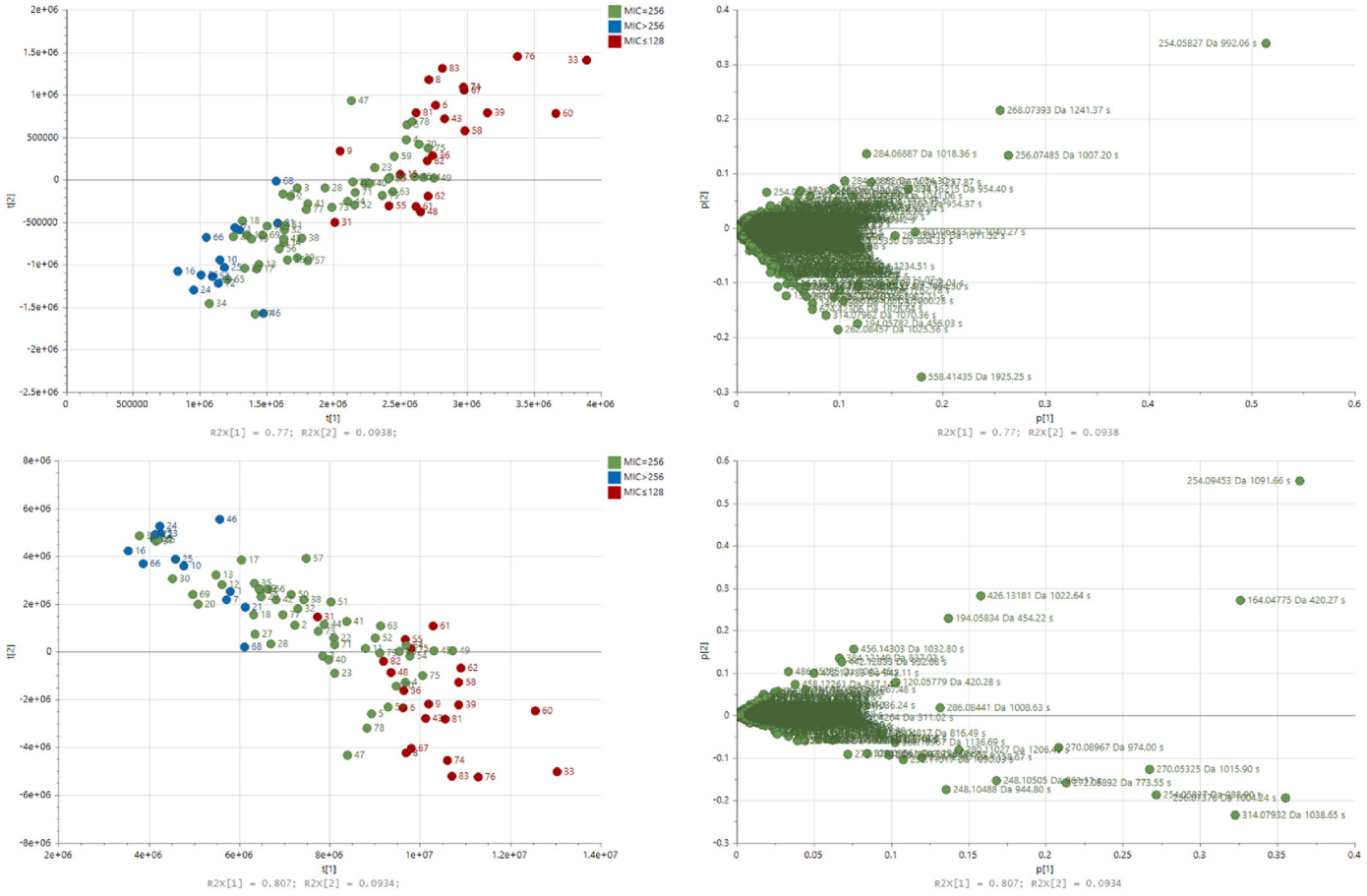

Fig. 2. Principal component analysis (PCA) scores and loading plots (not scaled) based on LC-MS fingerprints recorded in ESI+ (top) and ESI- (bottom) for 83 propolis samples characterized by different anticandidal activity (MIC determined for *C. albicans* ATCC 10231). MIC values ≤ 128, = 256, >256 are marked in red, green or blue, respectively. Numbers correspond to specific samples and variables are marked as pairs of retention time and exact mass. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

both groups which was expected, considering the gradual nature of MIC values. The corresponding loading plot indicated four variables as correlated with lower MIC: 254.05827 Da 992.06 s; 268.07393 Da 1241.37 s; 256.07485 Da 1007.20 s; 284.06887 Da 1018.36 s.

3.4. Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA)

To evaluate the discrimination between the active and non-active propolis extracts and to further identify metabolites responsible for the differentiation of those two groups, an OPLS-DA classification model was developed using selected 34 samples with MIC $\leq$128 as active samples and MIC >256 as inactive samples. Before analyses, the data was centered.

Fig. 3 shows the OPLS-DA score and loading plot for discrimination of active class (blue) and less active class (green) of propolis extracts along the predictive component (t[1]) characterized with a 47.8 % or 53.8 % variation (R2X = 0.478), (R2X = 0.538) based on data obtained in positive and negative ionization, respectively. Both models (based on data obtained in positive and negative ionization, respectively) have high R2Y (0.912; 0.907) and Q2 (0.836; 0.875) values indicating good fit, predictive capability, and reliability of the models. Different groups of propolis were classified correctly giving a success rate of 100 % in both cases. This confirms differences between extracts that possess higher and lower antifungal activity. To validate the models, a permutation test was conducted with 200 random permutations in an OPLS-DA model. According to the results, the OPLS-DA models were proved to have good robustness without overfitting (Fig. S1). To explore variables and specific compounds correlated with elevated antifungal activity, an S-line and S-plot were generated to investigate metabolites related to elevated activity by visualizing variable contributions and correlations of metabolites in each OPLS-DA. Potential markers with significant contributions were marked in the S-plot/S-line (Fig. 4). The variables present on extreme ends of the S-plot demonstrate high reliability and magnitude propolis class discrimination. The selected variables (pairs of retention time and exact mass) with significant contributions were plotted at the bottom left and top right and highlighted in blue (top right - related to high antimicrobial activity) or red (bottom left - correlated to low antimicrobial activity). The selected potential markers related to high antimicrobial activity were evaluated with ANOVA $p \leq 0.05$ and were characterized by max fold change $\geq$2.4. The potential markers were identified by comparison with reference compound and/or exact mass, MS^2 fragmentation, and UV spectra are presented in Table 2. The analyses resulted in the selection of 18 compounds related to elevated activity picked from a model based on ESI+ and 19 others picked from a model based on ESI-. Among them, 11 overlapped and others were different. In particular, in negative ionization large group of *p*-coumaric and caffeic acid esters were present, e.g. pentyl *p*-coumarate. On the other hand, in positive ionization several additional compounds, mainly flavonoids were highlighted. This may be related to different susceptibilities to ionization that affect the final MS profile. For example, it is known that phenolic acids and their esters ionize much better in ESI- while e.g. techtochrysin or pinostrobin ionize well in ESI + while do not ionize or ionize poorly in ESI- (Okińczyc et al., 2024).

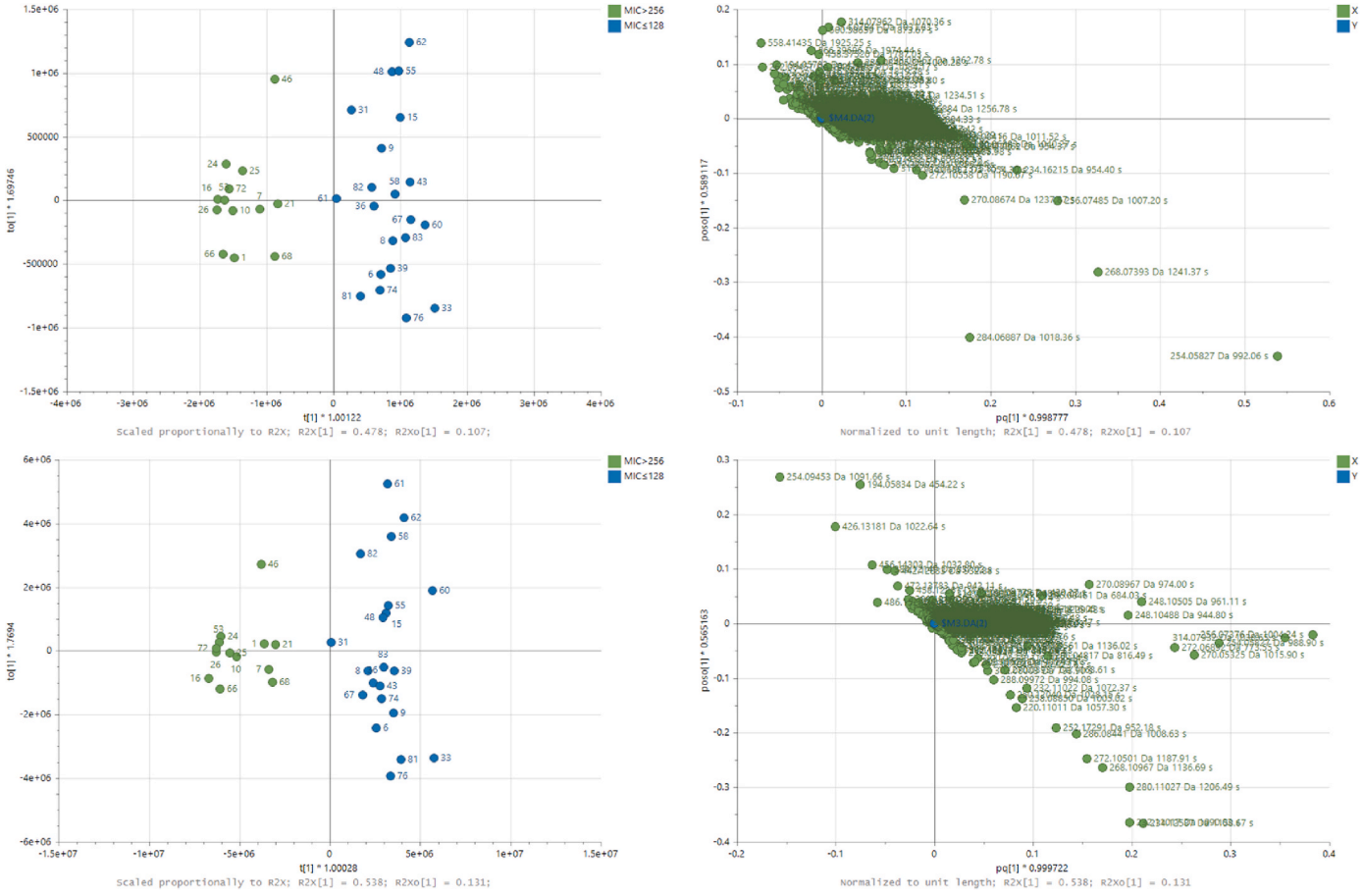

Fig. 3. Orthogonal Partial Least Squares - Discriminant Analysis (OPLS-DA) scores and loading plots (cantered) based on LC-MS fingerprints recorded in ESI+ (top) and ESI- (bottom) for 34 propolis samples characterized by different anticandidal (*C. albicans* ATCC 10231) activity. MIC values $\leq$ 128 and > 256 are marked in blue or green, respectively. Numbers correspond to specific samples and variables are marked as pairs of retention time and exact mass. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

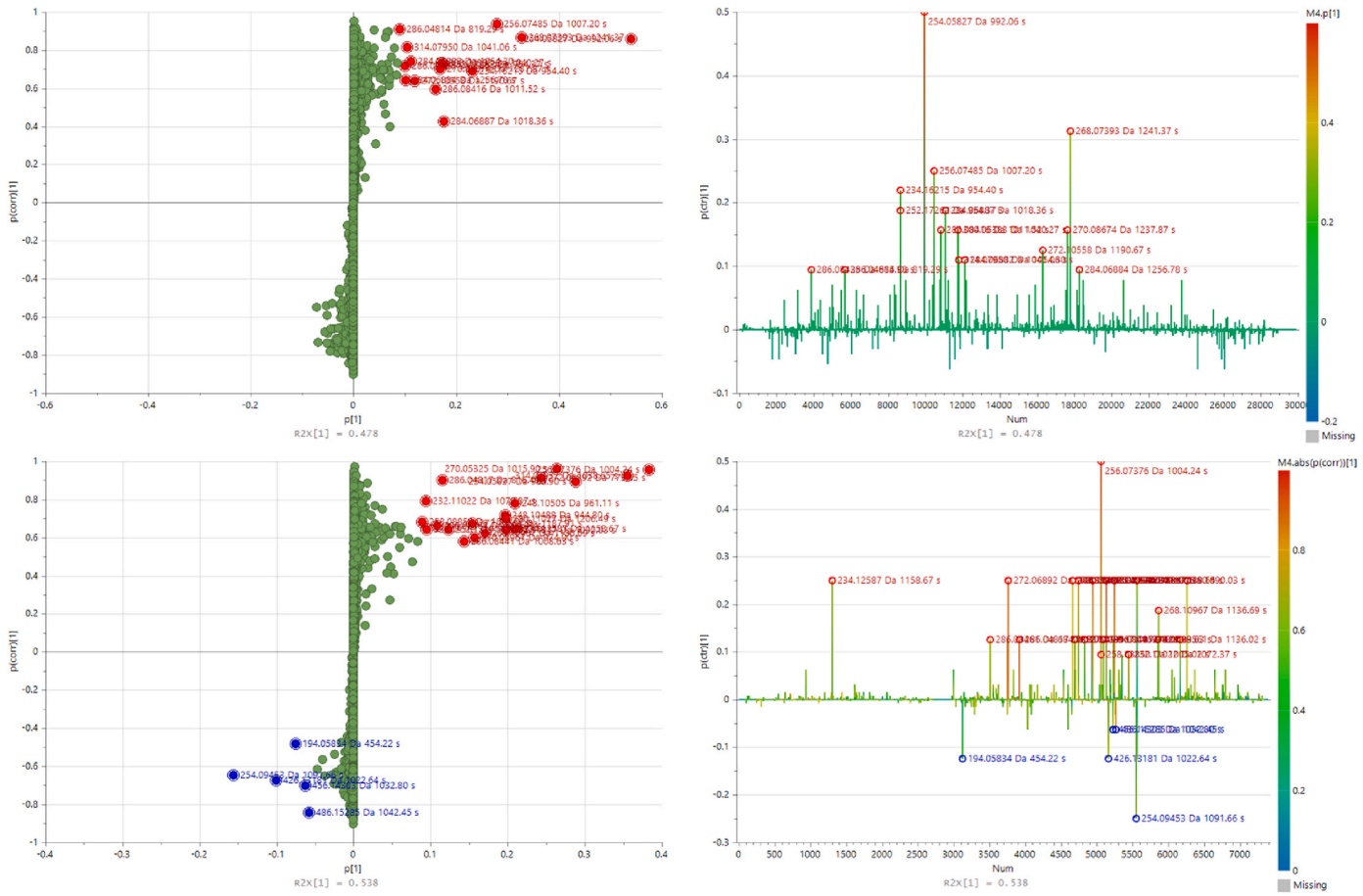

Fig. 4. Orthogonal Partial Least Squares - Discriminant Analysis (OPLS-DA) S-plots and S-lines based on LC-MS fingerprints recorded in ESI+ (top) and ESI- (bottom) for 34 propolis samples characterized by different anticandidal activity. Selected variables are marked as pairs of retention time and exact mass and highlighted in red or blue as potential markers related with high or low anticandidal activity, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

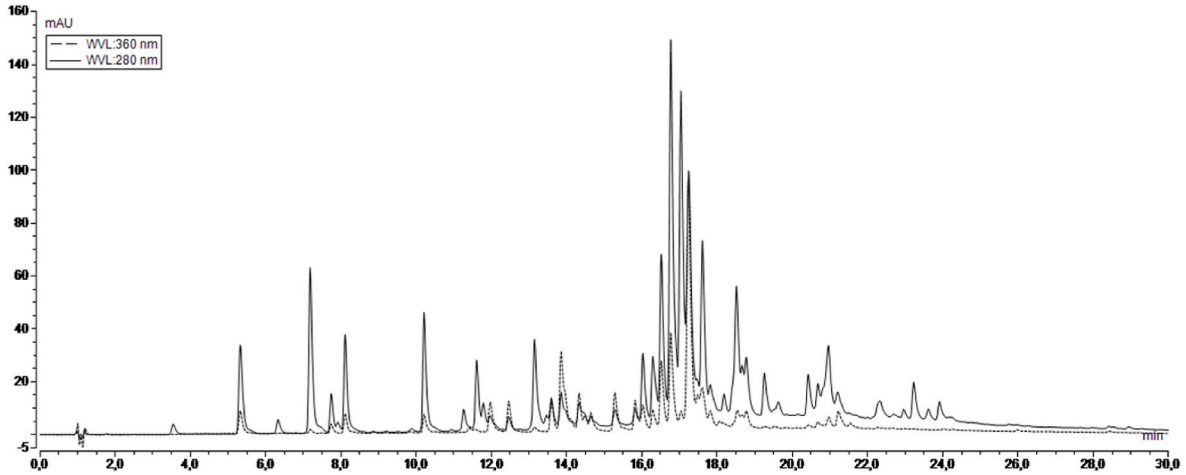

Fig. 5. Representative UHPLC chromatogram of propolis extract recorded at 280 and 360 nm.

The most important potential markers related to increased antifungal activity, characterized by elevated reliability and high magnitude were: pinocembrin, pinobanksin 3-acetate, chrysin, galangin, pinobanksin, techtochrysin, genkwanin, pinostrobin as well as sakuranetin isomer. All of them belonged to the group of most abundant peaks identified as major compounds in the investigated propolis extracts. Therefore, it is likely that samples more rich in these compounds may exhibit elevated antifungal activity. According to our best knowledge, it is the most detailed analysis of the correlation between chemical composition and antifungal activity of propolis samples collected in Central Europe (Poland) that was presented in the literature to date. The chemical composition of all 83 extracts was determined with modern UHPLC-DAD and UHPLC-QqTOF-MS techniques, antifungal activity was evaluated against the three most important pathogenic species of *Candida* spp.

Table 2
The most relevant potential marker compounds related to the increased or low antifungal activity. Chemical structures of the most notable compounds are presented in Fig. S6.

OPLS-DA (ESI+)				OPLS-DA (ESI-)			
positive correlation with activity				*positive correlation with activity*			
Compound	**Mass (Da)**	**UV**	**RT (min)**	**Compound**	**Mass (Da)**	**UV**	**RT (min)**
Chrysin[a]	254.05827	312sh, 268	16.5	Pinocembrin[a]	256.07376	290	16.8
Techtochrysin[a]	268.07393	213, 269	20.7	Pinobanksin-3-O-acetate[b]	314.07932	295	17.4
Pinocembrin[a]	256.07485	290	16.8	Chrysin[a]	254.05827	312sh, 268	16.5
Unidentified sesquiterpene [ab]	252.17262	–	15.9	Galangin[a]	270.05325	358, 266	17.0
Genkwanin[a]	284.06887	336, 280sh,267	17.0	Pinobanksin[a]	272.06892	292	12.9
Kaempferol methyl ether [ab]	300.06383	–	17.3	Pentyl *p*-coumarate[b]	234.12587	309	19.3
Pinostrobin[a]	270.08674	289	20.6	Caffeic acid 3-methyl-2-butenyl ester[b]	248.10505	325	16.0
Sakuranetin isomer[b]	286.08416	290	16.9	*p*-Coumaric acid methylbutenyl ester[b]	232.11017	325	18.2
2′,6′-Dihydroxy-4′-methoxydihydrochalcone[a]	272.10558	286	19.8	*p*-Coumaric acid cinnamyl ester[b]	280.11027	313	20.1
Galangin-3-methyl ether [ab]	284.06882	267	17.6	Caffeic acid 2-methyl-2-butenyl ester[b]	248.10488	325	15.7
Pinobanksin-3-O-acetate[b]	314.07950	295	17.4	*p*-Coumaric acid phenethyl ester[b]	268.10967	310	18.9
Galangin-7-methyl ether [ab]	284.06884	267, 352	20.9	Caffeic acid benzyl ester[b]	270.08967	326	16.2
Pinobanksin-5-methyl ether[b]	286.08435	287	11.4	2′,6′-Dihydroxy-4′-methoxydihydrochalcone[a]	272.10501	286	19.8
Kaempferol[a]	286.04814	366, 295sh, 265	13.7	Sakuranetin isomer[b]	286.08441	290	16.9
9-oxo-10(*E*),12(*Z*)-octadecadienoic acid [ab]	294.21927	281	22.9	Unidentified sesquiterpene[ab]	252.17291	–	15.9
Caffeic acid 3-methyl-2-butenyl ester[b]	248.10450	217, 326	16.0	Kaempferol[a]	286.04817	366, 295sh, 265	13.7
Galangin[a]	270.05320	358, 266	17.0	Pinobanksin-5-methylether[b]	286.08461	287	11.4
Pinobanksin[a]	272.06880	292	12.9	Pinobanksin-3-O-propanoate[b]	328.09561	295	18.9
				p-Coumaric acid 3-methyl-3-butenyl ester[b]	232.11022	313	17.9

OPLS-DA (ESI-)			
negative correlation with activity			
Ferulic acid[b]	194.05834	325	7.6
2-Acetyl-1,3-di-*p*-coumaroyl glycerol[b]	426.13181	312, 296	17.0
p-Coumaric acid benzyl ester[b]	254.09453	312	18.2
2-Acetyl-3-*p*-coumaroyl-1-feruloylglycerol[b]	456.14303	316	17.2
2-Acetyl-1,3-di-feruloylglycerol[b]	486.15285	324	17.4

[a] – tentatively identified; - – not recorded/overlapping with other compound; a – confirmed by a reference compound; b – identified based on literature data.

genus and advanced chemometric tools have been used for the investigation of correlation between chemical composition and antifungal potential.

Pinocembrin and pinostrobin significantly inhibited *C. albicans* in a concentration-dependent manner during the biofilm development stage (Kanchanapiboon et al., 2020). Similarly, chrysin and galangin exhibited relevant antifungal activity. Because of their low number of hydroxyl groups, they more easily penetrate membranes than other flavonoids (Candiracci et al., 2011). Pinobanksin, pinobanksin-3-acetate, pinocembrin, caffeic acid prenyl esters, and other compounds are known to derive from *Populus nigra* and *Populus canadensis* bud exudates (Okińczyc et al., 2024; Wang et al., 2017). Besides pinocembrin, prenyl caffeates were also found as potent anticandidial agents (Boisard et al., 2015). Some other minor compounds correlated with active samples may rather have limited relevance or act synergistically. For example, izalpinin was found to be less active against *Candida albicans* ATCC 10231 and *Candida tropicalis* C 131 with MIC >250 μg/mL (Agüero et al., 2010), so it may have only some potential rather than being an analytical marker, considering their relatively low abundance. Similarly, galangin-3-methyl ether showed antifungal effects against *C albicans* with MIC of 500 μg/mL (Hernández Tasco et al., 2020).

The oxylipin compound correlated with active samples of propolis - 9-oxo-10(*E*),12(*Z*)-octadecadienoic acid (9-*oxo*-ODE) was previously identified as poplar propolis component active against bacteria and fungi (Bilikova et al., 2012; Hu et al., 2022; Prost et al., 2005). It was previously found in *Populus nigra* and was particularly abundant in *Populus canadensis* bud exudates (Wang et al., 2017). Its concentration in poplar bud exudate was elevated in the autumn and winter period.

Only in the model based on ESI- profiles it was possible to select potential markers related to the low anticandidal activity. Among them, the most relevant were ferulic acid, 2-acetyl-1,3-di-*p*-coumaroyl glycerol, *p*-coumaric acid benzyl ester, 2-acetyl-3-*p*-coumaroyl-1-feruloylglycerol and 2-acetyl-1,3-di-feruloylglycerol that are known as compounds from *Populus tremula* and as markers of aspen-type propolis (Isidorov et al., 2014, 2016).

In conclusion, the observations suggest that elevated activity is related to the botanical origin of propolis associated with *P. nigra* or *P. canadensis* while lower activity is related to the contribution of *P. tremula.* This is consistent with previous observations of anticandidal activity for corresponding bud exudates where high activity was observed for black poplar while no activity was observed for Eurasian aspen (Isidorov et al., 2016). Moreover, observed seasonal changes in the phytochemical composition of the bud exudates suggest that the antifungal activity of propolis may vary depending on the time of its collection by the bees (Hu et al., 2022).

3.5. Antifungal potential and chemical composition of produced fractions

Three extracts found as most active, namely 8, 33, and 74 as well as two extracts (18 and 21) of low activity were selected for fractionation with SPE technique. The C18 (E) SPE column (55 μm, 70 Å; 10 g/60 mL) Strata® (Phenomenex, Torrence, CA, USA) was applied for this purpose.

The produced fractions eluted from the column with methanol Table 3 dilutions (40, 80, and 100 %) were evaporated. The obtained resinous product was weighed and dissolved in 70 % ethanol (v/v) to the final concentration of 81.92 μg/mL. Activity against *Candida* spp. strains was observed only in the case of the fractions that were eluted from the column with 80 % methanol (Table 4). As expected, fractions produced from extracts 8, 33, and 74 revealed substantially higher fungistatic and fungicidal activity compared to fractions obtained from extracts 18 and 21.

This observation can be explained by important differences in the chemical compositions of produced fractions (Table 3; Fig. 6). The compounds identified in this study as the most important potential markers of biological activity were eluted from the column mostly with 80 % methanol, which resulted in increased activity of this fraction (Table 4). Concentrations of these constituents in extracts 8, 33, and 74 (produced from the fractions Met80) were significantly higher than in products with numbers 18, and 21. This is a crucial observation particularly when preparing different formulations of propolis. For instance, raw extract used in ointments or wound materials could be replaced by fractions containing the active ingredients of this product (concentrate) without ballast substances (e.g. non-polar waxes or polar organic acids). The non-polar waxes present in extracts immediately precipitate in contact with water (or polar biotic/abiotic surfaces – e.g. with skin) which may affect the availability of active compounds of propolis. In the case of wound dressings containing EEPs precipitation of non-polar ingredients clogs pores and hinders gas exchange which may importantly affect the process of wound healing. Moreover, polar organic acids and non-polar waxes can affect the stability of some formulations – ointments or gels. Thus, optimization of the process of EEPs fractionation and separation and concentration of active ingredients is also important from the point of view of propolis application in clinical scenarios.

Fungistatic activities of these five extracts as well as Met80 fractions derived from them were also compared by the analysis of growth curves - monitoring of the growth kinetics of the yeast cells (measured as turbidity of the cultures at OD 600 nm) in the media supplemented with different concentrations of the extracts/fractions. The results presented in Fig. S2 confirmed the significantly higher activity of the extracts 8, 33, and 74 (and Met80 fractions produced from them). In the case of the most resistant *C. albicans* SC 5314 strain two other products did not effectively inhibit the growth of yeast cells even at the highest used concentration – 256 μg/mL. Moreover, considerably higher activity of extract 33 was observed when compared to 8 and 74. Extract 33 inhibited the growth of *C. albicans* at a concentration of 128 μg/mL whereas two times higher concentrations of extracts 8 and 74 were necessary to achieve the same effect. Interestingly, Met80 fractions of the products 33 and 74 exhibited comparable activity against *C. albicans* SC 5314. This experiment also confirmed a higher ability of propolis extracts for inhibition of *C. glabrata* and *C. krusei* growth compared to *C. albicans*.

Time-kill assay confirmed the highest fungicidal effectiveness of the

Table 4
Comparison of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values determined for ethanolic extracts of propolis (EEP) and different methanolic fractions derived from corresponding EEPs against *Candida* genus representatives.

No. EEP.		MIC and MFC (μg/mL) against different strains of *Candida*					
		Candida albicans SC5314		*Candida glabrata* DSM 11226		*Candida krusei* DSM 6128	
		MIC	MFC	MIC	MFC	MIC	MFC
8	Base extract	256	256	64	256	128	256
	40 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
	80 % Methanolic Fraction	256	256	64	256	128	256
	100 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
18	Base extract	>256	>256	128	>256	>256	>256
	40 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
	80 % Methanolic Fraction	>256	>256	128	>256	>256	>256
	100 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
21	Base extract	>256	>256	128	>256	>256	>256
	40 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
	80 % Methanolic Fraction	>256	>256	128	>256	>256	>256
	100 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
33	Base extract	128	128	32	256	64	128
	40 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
	80 % Methanolic Fraction	128	128	64	256	128	256
	100 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
74	Base extract	256	256	64	256	128	256
	40 % Methanolic Fraction	>256	>256	>256	>256	>256	>256
	80 % Methanolic Fraction	128	128	64	256	128	256
	100 % Methanolic Fraction	>256	>256	>256	>256	>256	>256

Table 3
The content of major compounds, relevant as potential markers of elevated anticandidial acitivity, in different propolis extracts and fractions determined by UHPLC-DAD (data expressed as mg/g of propolis extract or fraction).

No.	Compound	RT [min]	8	18	21	33	74	8 Met80 %	18 Met80 %	21 Met80 %	33 Met80 %	74 Met80 %
1	Pinobanksin	26.61	10.5	2.5	3.1	10.8	12.22	15.9	4.7	4.4	10.4	tr
2	Caffeic acid 2-methyl-2-butenyl ester [a]	31.42	7.8	4.0	3.3	3.8	7.15	14.2	6.3	3.5	3.9	3.41
3	Caffeic acid 3-methyl-2-butenyl ester [a]	32.15	5.7	1.3	1.3	2.9	9.05	11.3	3.8	2.3	2.9	7.03
4	Chrysin	33.23	27.5	8.7	8.5	21.4	22.90	49.6	11.9	8.5	25.0	52.90
5	Pinocembrin	34.16	29.7	8.7	8.8	45.7	25.34	53.6	12.5	9.5	51.7	34.06
6	Sakuranetin isomer	34.51	2.1	2.4	2.5	26.7	6.03	7.2	2.3	2.0	29.8	5.44
7	Galangin	34.82	18.3	5.5	5.4	28.6	22.91	31.5	9.0	6.7	29.6	53.69
8	Pinobanksin 3-O-acetate [c]	35.97	19.0	6.7	6.7	32.1	38.10	49.2	8.9	7.3	38.0	53.69
9	*p*-Coumaric acid 3-methyl-3-butenyl ester [b]	37.81	1.8	1.4	1.4	18.4	2.85	3.2	1.5	1.4	22.4	6.82
10	2′,6′-Dihydroxy-4′-methoxydihydrochalcone	41.15	1.4	1.5	1.5	8.8	1.88	2.7	2.8	2.0	8.2	2.79
11	*p*-Coumaric acid cinnamyl ester [b]	41.91	3.8	3.0	4.0	26.4	6.97	6.2	3.9	2.9	30.9	17.46
12	Techtochrysin	42.2	4.06	2.0	1.4	8.77	5.82	4.27	2.88	1.96	8.67	7.94
13	Pinostrobin	42.39	7.30	4.8	6.5	31.35	13.45	12.34	3.54	2.53	28.45	32.38

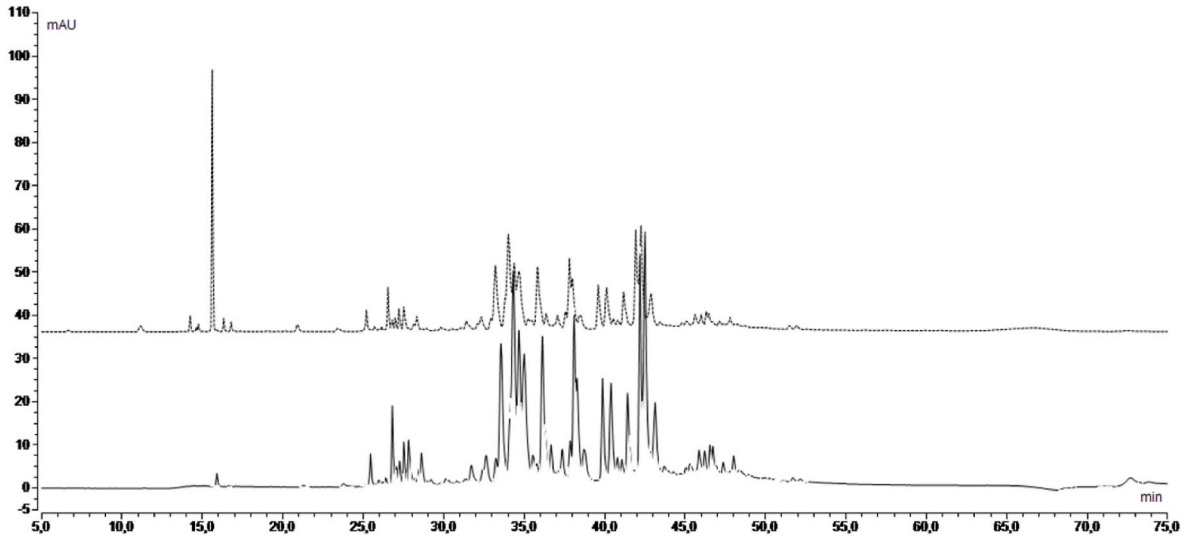

Fig. 6. Representative comparison of chromatographic profiles (recorded at 280 nm) of propolis extract (top) and its 80 % methanolic fraction (bottom) obtained by SPE.

extract 33 and Met80 fraction of this product (Fig. S5). At a concentration of 256 μg/mL complete elimination of *C. albicans* and *C. krusei* cells was achieved after 2 h incubation with extract and 4 h treatment with Met80 fraction. *C. glabrata* exhibited considerably higher resistance, 8 h of incubation with the extract (at a concentration of 256 μg/mL) was necessary for the complete elimination of living cells and only fungistatic effect was achieved for the Met80 fraction. At the concentration of 256 μg/mL extracts 8 and 74 effectively inhibited the growth of all strains tested but the fungicidal effect was not achieved. As expected extracts 21 and 18 exhibited significantly lower antifungal potential in this assay.

The exact molecular mechanism of the antifungal activity of propolis remains unknown. However, several reports indicate that components of EEPs can affect different targets within fungal cells (Ożarowski et al., 2022). D'Auria et al. (2003) showed that an extract of propolis can inhibit the activity of extracellular fungal phospholipases, Gucwa et al. (2018b) and Corrêa et al. (2020) revealed that cell membrane may be the target of EEPs. Stahli et al. (2021) observed that an ethanolic extract of propolis caused a loss of the cell wall integrity of *C. albicans* and decreased the metabolic activity. These pleiotropic mechanisms of activity importantly reduce the risk of selection of resistant strains which is an important benefit of propolis.

Based on revised literature, Cerqueira et al. (2022) concluded that propolis is a highly effective antifungal agent suggesting that it could be considered as an alternative skin treatment against a broad spectrum of pathogenic fungi, including dermatophytes (filamentous fungi belonging to the *Epidermophyton*, *Trichophyton,* and *Microsporum* genera) and different yeasts species of the genus *Candida* and *Malassezia*. Moreover, publications revised by the authors confirm that propolis can be used as a cosmeceutical component or as a source of bioactive ingredients. All these conclusions fully support the results of our study.

3.6. Activity against biofilm

The extracts 8, 33, and 74, as well as fractions (Met80 %) produced from them, exhibited significantly higher potential in the eradication of *C. albicans* biofilm compared to two other investigated products (EEP 18 and 21, along with their corresponding fractions) (Table 5). However, it is worth highlighting that observed values of $MBEC_{50}$ parameters are the same or only slightly higher than the MIC/MFC of these products. It is an important advantage of propolis extracts (and fractions of these extracts) to show this strong antibiofilm activity. In the case of most classical antifungals (and also antibacterial antibiotics) much higher (from 10 to 1000 times) concentration of the agent is required to efficiently eradicate biofilms in comparison to the concentration that is sufficient to obtain a fungistatic or fungicidal effect against planktonic cells. The possible mechanisms of bacterial or fungal biofilm resistance to antimicrobial agents include impeded drug penetration through the extracellular structure, phenotypic switching, and induction of the expression of resistance genes (Douglas, 2002; Massey et al., 2023; Nett & Andes, 2020). Several authors (Bezerra et al., 2020; Fernández-Calderón et al., 2021; Tamfu et al., 2022) have confirmed the ability of propolis extracts to inhibit biofilm formation by different species of the genus *Candida*. On the other hand, assessment of the effectiveness of propolis extracts in eradication of already formed – mature biofilm of *Candida* spp. was much less frequently examined and reported to date. Freires et al. (2016) revealed that EEPs (produced from type 3 and 13 of Brazilain propolis) disrupted the biofilm structures of *Candida* spp. at a concentration of 500 μg/mL, which is close to the $MBEC_{50}$ values of EEPs investigated herein. In another study, green Brazilian propolis effectively disrupted *Candida* biofilm, wherein a reduction of approximately 3.5 log in the number of CFUs was observed, at a concentration of 1400 μg/mL (Gavanji and Larki, 2017). Barros et al. (2022) also reported excellent activity of the extracts produced from green propolis against the 7-day preformed biofilm and were not toxic to Vero cells at concentrations compatible with the antifungal and antibiofilm activities. High potential of propolis extract for eradication of *Candida* spp. biofilm was also stated in our previous reports: Gucwa et al. (2018b) and Grecka et al. (2019, 2020). They revealed a high ability of EEPs produced from Polish propolis for eradication of *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms.

Table 5
Comparison of minimum 50 % biofilm eradication concentration ($MBEC_{50}$) values determined for ethanolic extracts of propolis (EEP) and their corresponding 80 % methanolic fraction against *Candida albicans* SC 5314 biofilm.

No. EEP.	$MBEC_{50}$ [μg/mL]	
	Base extract	80 % Methanolic Fraction
8	256	256
18	2048	1024
21	2048	1024
33	256	128
74	256	128

3.7. Antifungal activity of pure compounds and their mixtures

The performed statistical analysis of the correlation between antifungal activity and the chemical composition of EEPs investigated in this study led to the identification of potential markers of elevated anticandidal activity (Table 2). In order to verify their contribution to the activity of the extracts, the MIC and MFC values of pure compounds and their mixtures were determined. Up to the concentration of 256 μg/mL, none of the pure compounds were able to inhibit the growth of the reference *Candida* spp strains (Table 6). The analysis of the results presented in Table 3 led us to the conclusion that active extracts and fractions are characterized by higher concentrations of flavonoids and some derivatives of *p*-coumaric acid. It suggests that the observed activity of the extracts or fractions must be the result of synergistic activity between individual compounds. Thus, in the next step, the activity of 15 different combinations of two of these agents, and 4 combinations of three compounds was evaluated (Table 7). Activity (at total concentration 256 μg/mL – 125 μg/mL of each compound) was observed for combinations of pinocembrin with three other compounds: pinobanksin 3-acetate (alone active against all strains tested MIC and MFC = 256 μg/mL), galangin (only *C. glabrata* was resistant against this compound used alone) and pinobanksin (alone inhibited growth of only *C. albicans*). The only composition of three compounds that exhibited activity at the highest tested concentration (256 μg/mL, 85.3 μg/mL of each compound) was composed of galangin, pinocembrin, and pinobanksin-3-acetate (mixture number IV in Table 8). Supplementation of this mixture with two other compounds – chrysin and pinobanksin led to a significant increase in antifungal potential, MIC and MFC values against all strains tested were 64 and 128 μg/mL (12.8 and 25.6 μg/mL of each compound), respectively. Interestingly, adding to this mixture *p*-coumaric acid caused the MIC value for all strains to be 128 μg/mL (25.6 μg/mL of each compound - twice higher), MFC value for *C. glabrata* was also two times higher. In the course of our study, activities of two other mixtures (number VII and VIII in Table 8) composed of eight and nine ingredients were also tested. Apigenin, sakuranetin, and pinostrobin were used for preparing these compositions. Mixture VIII did not contain *p*-coumaric acid. In fact, apigenin was not recognized as a potential marker of elevated anticandidal activity, but this flavonoid is well-known as a common component of bee propolis. Thus, it was included in our study. MIC and MFC values for both these compositions against all strains tested were exactly the same as in the case of composition VI (Table 8).

Table 6
Summary of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values determined for different flavonoids and phenolic acids against *Candida* genus representatives.

Chemical compound	MIC and MFC (μg/mL) against different strains of *Candida*					
	Candida albicans SC5314		*Candida glabrata* DSM 11226		*Candida krusei* DSM 6128	
	MIC	MFC	MIC	MFC	MIC	MFC
Pinobanksin	>256	>256	>256	>256	>256	>256
Pinobanksin-3-O-acetate	>256	>256	>256	>256	>256	>256
Pinocembrin	>256	>256	>256	>256	>256	>256
Pinostrobin	>256	>256	>256	>256	>256	>256
Galangin	>256	>256	>256	>256	>256	>256
Chrysin	>256	>256	>256	>256	>256	>256
Sakuranetin	>256	>256	>256	>256	>256	>256
Apigenin	>256	>256	>256	>256	>256	>256
Quercetin	>256	>256	>256	>256	>256	>256
Kaempferol	>256	>256	>256	>256	>256	>256
Pectolinarigenin	>256	>256	>256	>256	>256	>256
p-Coumaric acid	>256	>256	>256	>256	>256	>256
Caffeic acid	>256	>256	>256	>256	>256	>256
p-Ferulic acid	>256	>256	>256	>256	>256	>256
p-Anisic acid	>256	>256	>256	>256	>256	>256

Table 7
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values determined for two-component flavonoids and phenolic acid mixtures against *Candida* genus representatives.

Chemical compound mixture (1:1 ratio w/w)	MIC and MFC (μg/mL) against different strains of *Candida*					
	Candida albicans SC5314		*Candida glabrata* DSM 11226		*Candida krusei* DSM 6128	
	MIC	MFC	MIC	MFC	MIC	MFC
Galangin + Chrysin	>256	>256	>256	>256	>256	>256
Galangin + Pinocembrin	256	256	>256	>256	256	256
Galangin + Pinobanksin	>256	>256	>256	>256	>256	>256
Galangin + Pinobanksin-3-O-acetate	>256	>256	>256	>256	>256	>256
Galangin + *p*-Coumaric acid	>256	>256	>256	>256	>256	>256
Chrysin + Pinocembrin	>256	>256	>256	>256	>256	>256
Chrysin + Pinobanksin	>256	>256	>256	>256	>256	>256
Chrysin + Pinobanksin-3-O-acetate	>256	>256	>256	>256	>256	>256
Chrysin + *p*-Coumaric acid	>256	>256	>256	>256	>256	>256
Pinocembrin + Pinobanksin	256	>256	>256	>256	>256	>256
Pinocembrin + Pinobanksin-3-O-acetate	256	256	256	256	256	256
Pinocembrin + *p*-Coumaric acid	>256	>256	>256	>256	>256	>256
Pinobanksin + Pinobanksin-3-O-acetate	>256	>256	>256	>256	>256	>256
Pinobanksin + *p*-Coumaric acid	>256	>256	>256	>256	>256	>256
Pinobanksin 3-O-acetate + *p*-Coumaric acid	>256	>256	>256	>256	>256	>256

Similarly, as in the case of extracts and Met80 fractions, the fungistatic activity of pure compounds and selected compositions was also evaluated by the analysis of growth kinetics in the media supplemented with investigated agents (pure substances or their compositions). The detailed results are presented in Figs. S3 and S4. Among pure compounds inhibitory effect was caused only by pinocembrin at a concentration of 256 μg/mL, it also partly inhibited the growth of all strains at a concentration of 128 μg/mL. The considerably inhibitory effect was also caused by pinobanksin-3-acetate and pinobanksin (*C. glabrata* was not susceptible). The assay also confirmed satisfactory activity (compared to most active EEPs) of prepared mixtures V-VIII. The most satisfactory effect observed for mixture V, and most sensitive to the activity of prepared compositions was *C. glabrata* (only growth inhibition – fungistatic effect was analyzed). The results of this part of our study clearly indicate that the antifungal potential of propolis extracts is the consequence of synergistic interactions between components of this product, not the presence of one particular ingredient of high antifungal activity. On the other hand, important differences in the antifungal activity of the collected samples are observed which suggest that the presence of certain specific compounds (and their interactions) determine the final antifungal potential of the product. Herein, ingredients that are crucial for the antifungal activity of propolis collected in Polish apiaries have been identified. However, it could be assumed that this result is also valid for propolis collected in most Western and Central European countries, where bees collect their products from similar plant species. The importance of synergistic interactions of propolis constituents was also observed/studied by other authors. Suleman et al. (2015) revealed that the antimicrobial activity of South African propolis is possibly attributed to its flavonoid content. Chrysin, pinocembrin, galangin, and pinobanksin-3-O-acetate were identified as bioactive constituents. Subsequently, pinocembrin, galangin, and chrysin were investigated for interactive antimicrobial activity. Detailed analysis of MIC values demonstrated that combinations of compounds showed better inhibitory

Table 8
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values determined for three- and multi-component flavonoids and phenolic acid mixtures against *Candida* genus representatives.

Mixture No.	Chemical compound mixture (1:1 ratio w/w)	MIC and MFC (μg/mL) against different strains of *Candida*					
		Candida albicans SC5314		*Candida glabrata* DSM 11226		*Candida krusei* DSM 6128	
		MIC	MFC	MIC	MFC	MIC	MFC
I	Chrysin + Pinocembrin + Pinobanksin	>256	>256	>256	>256	>256	>256
II	Chrysin + Pinocembrin + Pinobanksin-3-O-acetate	>256	>256	>256	>256	>256	>256
III	Galangin + Pinocembrin + Pinobanksin	>256	>256	>256	>256	>256	>256
IV	Galangin + Pinocembrin + Pinobanksin-3-O-acetate	256	>256	256	256	256	256
V	Chrysin + Galangin + Pinocembrin + Pinobanksin + Pinobanksin- 3-O-acetate	64	128	64	128	64	128
VI	Chrysin + Galangin + Pinocembrin + Pinobanksin + Pinobanksin 3-O-acetate + *p*-Coumaric acid	128	128	128	256	128	128
VII	Chrysin + Galangin + Pinocembrin + Pinobanksin + Pinobanksin- 3-O-acetate + Apigenin + Sakuranetin + Pinostrobin	128	128	128	256	128	128
VIII	Chrysin + Galangin + Pinocembrin + Pinobanksin + Pinobanksin- 3-O-acetate + Apigenin + Sakuranetin + Pinostrobin + *p*-Coumaric acid	128	128	128	256	128	128

activity than single compounds (Kharsany et al., 2019). Ożarowski et al. (2022) also concluded that the synergy of action of all components of propolis extract determines the antifungal activity. In this work, it was proven that synergy between some of these components is important for antimicrobial effect.

The outcomes of this part of our study are important not only from the scientific point of view. In our opinion, they open a window for investigation of possibilities of application of identified compositions of propolis ingredients (mostly flavonoids) as antifungal agents (as artificial propolis) e.g. for treatment or prophylaxis of topical *C. albicans* spp. infections of skin, wounds, or mucous membranes. Of course, this hypothesis has to be verified by *in vivo* studies aiming at the evaluation of EEPs (and also mixtures of selected ingredients) efficiency in candidiasis eradication using animal models of fungal infections.

4. Conclusions

The most important conclusions from our study are presented below:

First – a significant majority of propolis samples collected in Polish apiaries exhibited low antifungal potential. Only about 10 % of investigated herein products were characterized by high fungistatic/fungicidal activity. This result is in line with our previous reports on the antimicrobial activity of Polish propolis.

Second – only propolis samples with high antimicrobial activity should be considered for therapeutic applications. An important achievement of this study was the identification of 18 compounds related to the elevated antifungal activity. These markers of high anticandidal potential can be used for the development of fast analytical methods of identification of highly active propolis samples. In this study, *UHPLC-DAD* and *UHPLC-QqTOF-MS* methods of detailed analysis of EEPs composition have been optimized.

Third – the antifungal potential of propolis extracts can be attributed to the synergy between the components of this product.

Fourth – mixtures of the substances recognized as markers of elevated antifungal activity exhibited anticandidal potential comparable to or even higher than EEPs. Therefore, the possibility of using these mixtures for the treatment and prevention of candidiasis should be considered.

Fifth – yeasts of the genus *Candida* belong to the most important etiological factors of skin and mucosal infections. The results of the above studies confirmed the validity of the use of propolis in traditional medicine as an effective drug in treating topical fungal infections. Nevertheless, the antifungal potential of propolis varies and is impossible to be evaluated by traditional organoleptic analysis. Therefore, chemical or biological standardization is essential to assure consistent efficacy.

CRediT authorship contribution statement

Piotr Bollin: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Data curation, Conceptualization. **Piotr Marek Kuś:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Piotr Okińczyc:** Writing – original draft, Software, Methodology, Investigation. **Patrick Van Dijck:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Piotr Szweda:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability

All data generated or analyzed during this study will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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