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Glycyrrhiza glabra hydroethanolic extract and manuka honey alone and in combination inhibit bacterial and fungal planktonic cells and biofilms

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ABSTRACT

Background: The natural tendency of polymicrobial infections and co-habitation of bacteria and *Candida* are currently well-recognized and reported as highly fatal. This kind of interaction is usually associated with drug tolerance, infection relapse, persistence and high mortality. The interest in natural products has emerged and their potential applicability as therapeutic strategies is being constantly explored.
Purpose: The main goal of this work is to evaluate the effect of the hydroethanolic extract of *Glycyrrhiza glabra*, and Manuka honey, in *Staphylococcus aureus* and *Candida tropicalis* planktonic cells and single and mixed biofilms.
Methods: The effect of both the *G. glabra* extract and the honey was evaluated by determination of the minimum inhibitory/bactericidal/fungicidal concentrations and by determination of their antibiofilm activity (alone and in combination) on single and mixed-species biofilms. In addition, phenolic compounds were characterized in the hydroethanolic extract with high performance liquid chromatography and mass spectrometry.
Results: Among the phenolic compounds identified in the *G. glabra* extract, flavonoids were the most abundant (≈75 % of the total phenolic compounds), with liquiritigenin being the most representative. *S. aureus* and *C. tropicalis* were susceptible to both antimicrobials acting alone. When tested in combination, a significative synergism was observed against *C. tropicalis* single biofilm formation. Instead, the combination showed an additive effect against mixed biofilms of *S. aureus* and *C. tropicalis*. In general the combination *G. glabra*+honey showed a broader antibiofilm effect than each partner alone.
Conclusion: The results showed the good anti *S. aureus* and *C. tropicalis* effect of *G. glabra* and manuka honey against both pathogens tested and highlighted their potential use in combination as therapeutic strategy to combat *S. aureus, C. tropicalis* and mixed species infections particularly when growing as biofilms.

Introduction

The incidence of antimicrobial resistance has been increasing over the past decade (Li and Webster, 2017) and bacteria such as *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter* species are among the most notorious resistant bacteria causing high levels of morbidity and mortality (Li and Webster, 2017). Their associated infections are threatening modern health care (Cassini et al., 2019) being about 65 % of the infections caused by antibiotic-resistant bacteria in the EU and European Economic Area (EEA) associated with health care (Cassini et al., 2019).

Although less common than those caused by bacteria, fungal

Abbreviations: CFU, colony forming unit; EEA, European Economic Area; EUCAST, European Committee on Antimicrobial Susceptibility Testing (Eucast); FCT, Foundation for Science and Technology; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentrations index;MBC, minimal bactericidal concentration; MFC, minimal fungicidal concentration; MIC, minimal inhibitory concentration; MS, mass spectrometer; RPMI, Roswell Park Memorial Institute; SDA, Sabouraud Dextrose Agar; SDB, Sabouraud Dextrose Broth; TSA, Tryptic Soy Agar; TSB, Tryptic Soy Broth.

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infections are usually associated with high morbidity and mortality rates, particularly those infections caused by opportunistic pathogenic fungi, as *Candida* species (Fernandes et al., 2020). *Candida albicans* remains as the most prevalent species of these infections, but a clear rise in the proportion of non-*albicans* species has been noted (Fernandes et al., 2020). Among them, *Candida tropicalis* has been associated with high mortality mainly in biofilms associated infections (Sasani et al., 2021).

Both bacteria and fungi have the ability to form biofilms, as an important virulence factor. A biofilm is a structured microbial community attached to a substrate embedded in a self-produced matrix composed by extracellular polymeric substances. In several environments namely in the human body, biofilms tend to be polymicrobial (involving more than one pathogenic agent), and namely cases of co-habitation and interaction of fungi, including *Candida* spp, and bacteria within polymicrobial biofilms were already reported (Rodrigues et al., 2020). In recent years, polymicrobial infections are becoming increasingly recognized, becoming a great challenge due to their enhanced virulence and increased drug resistance (Van Dyck et al., 2021).

Therefore, there is an urgent need to find new and broad spectrum antimicrobials, preferably from natural sources (plants, animal and microorganisms) (Stan et al., 2021) as alternatives to the traditional agents, against bacteria and fungi, mainly when growing under biofilm form.

Plants used for centuries in folk medicine are nowadays seen as an alternative source for natural antimicrobial compounds and can constitute a promissory substitute to the traditional antimicrobial agents. *Glycyrrhiza glabra* L. (licorice), a plant of the Fabaceae family, has been previously reported as antitussive, mucolytic, expectorant, antiulcer, anti-inflammatory, antimicrobial, cytostatic, immunostimulant, and hepatoprotective (Martins et al., 2016). Although tested in different conditions, microorganisms and types of extraction, licorice extracts previously showed promissory antimicrobial activity (Martins et al., 2016). The antimicrobial potential of *G. glabra* rich in phenolic compounds, among other bioactive phytochemicals, has been increasingly recognized. Licorice contains more than 20 triterpenoids and nearly 300 flavonoids. Among them, glycyrrhizin, 18β-glycyrrhetinic acid, liquiritigenin, licochalcone A, licochalcone E and glabridin are the main active components which possess antimicrobial activities (Wang et al., 2015).

Honey is recognized worldwide for its antioxidant, antitumor, anti-inflammatory, and antimicrobial properties. Regarding antimicrobial activity, it demonstrated to be effective against several pathogenic agents (bacteria and fungi) and has been formerly pointed as a potential alternative treatment for *Candida*-associated infections (Fernandes et al., 2021). Additionally, it also presents good antibacterial activity against several bacteria in single and polymicrobial cultures (Almasaudi, 2021; AL-Waili et al., 2012). Osmolarity, low pH, H_2O_2, phenolic acids and flavonoids content are important factors contributing to its antimicrobial activity (Morroni et al., 2018). Furthermore, synergy was already reported when using honey in combination with antibiotics, namely against biofilms (Almasaudi, 2021; Lu et al., 2014). Manuka honey, a medical-grade honey with a well-known chemical composition (Wang et al., 2024), widely investigated and currently approved for some clinical applications (Morroni et al., 2018), has been reported to exhibit antibacterial and antifungal activity (Fernandes et al., 2020, 2021; Lu et al., 2014). Thus, honey is currently increasingly recognized as a powerful antimicrobial agent with a wide range of effects and as a potential, reliable and safe alternative as therapeutic strategy in the medical setting (Almasaudi, 2021).

To the best of our knowledge there are no previous studies reporting the effect of *G. glabra* against polymicrobial biofilms. Thus, the novelty of this study is based on the search of effective strategies against polymicrobial biofilm infections involving interkingdom microbial communities (fungi and bacteria) based on natural products such as a *G. glabra* extract and its possible synergy with honey. In fact, *G. glabra* and Manuka honey have been pointed as promising antimicrobial and therapeutic options in cases of fungal and bacterial infections. Thus, the aim of this work was to investigate and compare their bioactivity, alone and in combination, against planktonic cells of *S. aureus* and *C. tropicalis* and on biofilm formation and on preformed single and mixed biofilms.

Material and methods

Microorganisms and culture conditions

S. aureus ATCC 25,923 and *C. tropicalis* ATCC 750 were stored at –80 ± 2 °C in broth medium with 20 % (v/v) glycerol. Prior to each assay, *S. aureus* and *C. tropicalis* strains were subcultured from the frozen stock preparations onto Tryptic Soy Agar (TSA) plates and Sabouraud Dextrose Agar (SDA), respectively. TSA and SDA were prepared from Tryptic Soy Broth (TSB; Liofilchem, Roseto degli Abruzz, Italy) or Sabouraud Dextrose Broth (SDB; Liofilchem, Roseto degli Abruzzi, Italy), supplemented with 2 % (w/v) of agar (Liofilchem, Roseto degli Abruzzi, Italy). The plates were then incubated aerobically at 37 °C for 18–24 h. *S. aureus* and *C. tropicalis* were inoculated into 15 ml of TSB and SDB, respectively, and grown overnight at 37 °C. Then, *S. aureus* cells were harvested by centrifugation (4 °C, 9500 g, 5 min), resuspended in TSB and adjusted to an optical density (OD_{640nm}) equivalent to 2×10^6 cells/ml. *C. tropicalis* cells were harvested by centrifugation (twice at 4 °C, 3000 g, 10 min), resuspended in SDB and adjusted to 2×10^7 cells/ml by cell counting by using a Neubauer chamber.

Plant material and extract preparation

Rhizomes and roots of *G. glabra* were supplied by Soria Natural (Garray - Soria, Spain). The samples were obtained in the autumn 2012 (after reaching three years). Before extraction procedures, the samples certified as clean products (monitored parameters for pesticides, herbicides, heavy metals and radioactivity) were dried and powdered. Hydroethanolic extractions were performed by stirring 1 g of the plant material with 30 ml of ethanol/water (80:20, v/v), at 30 × *g* and 25 °C during 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 30 ml portion of the hydroalcoholic mixture. The combined extracts were evaporated at 35 °C under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland). The extract obtained was frozen, lyophilized and further redissolved in sterile water in order to prepare a stock solution of 50 mg/ml to be used in the subsequent assays.

Honey

Commercially available Manuka (*Leptospermum scoparium*) honey (Medihoney ®, Derma Sciences) was used in this study. Honey was used as raw and unprocessed and stored in the dark at 4 °C. Dilutions were prepared with Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco® by Life Technologies TM, NY, USA). Manuka honey was used as a reference (Fernandes et al., 2021).

Analysis of phenolic compounds

The phenolic profile of hydroethanolic extract of *Glycyrrhiza glabra* was determined by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA). These compounds were separated and identified as previously described by Bessada et al. (2016). A double online detection was performed using a DAD (280 and 370 nm as preferred wavelengths) and a mass spectrometer (MS). The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. The identification of the phenolic compounds was performed based on their chromatographic behaviour and UV–vis and mass spectra by comparison with standard compounds, when available, and data

reported in the literature giving a tentative identification. Data acquisition was carried out with Xcalibur® data system. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV–vis signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. The following standard calibration curves were used for quantification: apigenin-6-C-glucoside ($y = 107025x + 61,531$, $R^2 = 0.9989$; LOD = 0.19 μg/ml; LOQ = 0.63 μg/ml); apigenin-7-O-glucoside ($y = 10683x - 45,794$, $R^2 = 0.996$, LOD = 0.10 μg/ml; LOQ = 0.53 μg/ml); formononetin ($y = 37800x + 185,255$, $R^2 = 0.999$; LOD = 0.30 μg/ml; LOQ = 0.93 μg/ml); isoliquiritigenin ($y = 42820x + 184,902$, $R^2 = 0.999$; LOD = 0.18 μg/ml; LOQ = 0.54 μg/ml); and naringenin ($y = 18433x + 78,903$, $R^2 = 0.999$, LOD = 0.17 μg/ml; LOQ = 0.81 μg/ml). The results were expressed as mg/g of extract.

Evaluation of the antimicrobial activity

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC)

Minimal inhibitory concentration (MIC) was determined according to standard European Committee on Antimicrobial Susceptibility Testing (EUCAST), through the microbroth dilution technique (EUCAST, 2003, 2008). MIC values were determined by serial two-fold dilutions, at concentrations ranging from 0.012 to 6.25 mg/ml (*G. glabra*) and 1.17 to 18.75 % (w/v) (2.34×10^4 to 3.75×10^5 μg/ml) (honey), adjusting final cellular concentration to 5×10^5 cells/ml. Positive (cells grown without extracts) and negative growth controls (medium) were also included. The 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium) were incubated at 37 °C during 24 h. After incubation, plates were visualized and MIC values corresponded to the concentrations in which no visible growth was observed. Then, the number of viable cells was assessed by determination of the number of colony forming units (CFUs) as follows: serial 10-fold dilutions of samples were prepared in physiological saline and 10 μl were plated on solid medium and incubated for 24 - 48 h and at 37 °C. The number of colonies formed was counted and the MBC/MFC values were considered the concentrations in which no CFUs were counted. conVancomycin and Amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) were used as standard positive drug controls. Experiments were performed in triplicate and in three independent occasions being the results presented as Log (CFUs).

Checkerboard assay

Checkerboard assay was carried out to determine the fractional inhibitory concentration indices (FICIs) of honey and *G. glabra* hydroethanolic in combination against *S. aureus* and *C. tropicalis*. Honey and *G. glabra* dilutions in the vertical and horizontal direction were prepared in a 96-well plate by two-fold dilution method. Then, one component of the combination was diluted along rows of the plate while the other was diluted down the columns, enabling the creation of a variety of mixtures with different concentrations of tested antimicrobial agents. A volume of 100 μl of cell suspension was added to all wells to bring the total volume to 200 μl and final cell concentration of 5×10^5 CFU/ml. Antimicrobial-free wells were used as control of microbial growth. The plates were incubated at 37 °C for 24 h. The experiments were carried out in triplicate and in three different occasions. The fractional inhibitory concentrations (FICs) were calculated as follows: FIC of honey = MIC of honey in combination/MIC of honey alone, and FIC of *G. glabra* = MIC of *G. glabra* in combination/MIC of *G. glabra* alone. The fractional inhibitory concentrations index (FICI) was defined as the FIC of honey plus the FIC of *G. glabra*. The combination effect of both antimicrobials was interpreted as follow: synergy – FICI $\leq$ 0.5, no interaction – 0.5 $\langle$ FICI $\leq$ 4.0, and antagonism – FICI $\rangle$ 4.0 (Odds, 2003).

Effect of G. glabra and honey alone and in combination on biofilm formation and preformed single and mixed biofilms

Inhibition of biofilm formation - Cell suspensions were prepared in RPMI and cells (final concentration: *S. aureus* - 1×10^6 cells/ml and *C. tropicalis* - 1×10^7 cells/ml, alone and in combination) were added into 96-well microtiter plates along with the antimicrobial agents to be tested (*G. glabra* at 1.5 mg/ml and honey at 37.5 % (w/v) (7.5×10^5 μg/mL), alone and in combination). Plates were incubated at 37 °C during 24 h. After incubation, the biofilm cells were determined by CFU. For that, the biofilm cells were thoroughly scraped and resuspended in 1 ml of 0.9 % NaCl. Viable cells were determined by performing 10-fold serial dilutions in saline solution and plating in TSA (*S. aureus*) and SDA (*C. tropicalis*). Cells were counted after 24 h incubation at 37 °C.

Inhibition of preformed biofilms - Biofilms were grown for 24 h before being exposed to *G. glabra* hydroethanolic and honey alone and in combination. Biofilm formation was performed by transferring 200 μl of cells suspension (*S. aureus, C. tropicalis, S. aureus* + *C. tropicalis*) (final concentration: *S. aureus* - 1×10^6 cells/ml and *C. tropicalis* - 1×10^7 cells/ml) into 96-well plates in quintuplicate (for each condition tested). The plates were incubated at 37 °C during 24 h. Then, non-adhered cells were removed, and biofilms washed with sterile distilled water. The antimicrobial agents, *G. glabra* at 1.5 mg/ml and honey at 37.5 % (w/v) (alone and in combination) were added to biofilm cells and plates incubated again at the previous conditions. After biofilms exposure to the antimicrobial agents tested, cells were collected and CFU determined.

Regarding mixed biofilms, selective agar media were used for CFU determination of *C. tropicalis* (SDA supplemented with 30 mg/l gentamycin, to suppress the growth of *S. aureus*) and *S. aureus* (TSA supplemented with 0.250 mg/l voriconazole to suppress the growth of *C. tropicalis*). The interaction of treatments in biofilms is described as synergistic when the biofilm reduction in combinations is higher than the sum of individual treatments of antimicrobials and additive when the biofilm reduction in combinations is equal to the sum of individual treatments of antimicrobials (Vaou et al., 2022).

Statistical analysis

Data were analyzed using the Prism software package (GraphPad Software version 9.2.0 for Windows). One-way analysis of variance (ANOVA) tests were performed, and means were compared by applying Tukey's multiple comparison test. The statistical analyses performed were considered significant when $p < 0.05$. For all assays, at least three independent experiments were carried out in triplicate.

Results

Analysis of phenolic compounds

The chromatographic data obtained by HPLC-DAD/ESI-MSn, tentative identification and quantification (mg/g extract) of the phenolic compounds present in the hydroethanolic extract of *Glycyrrhiza glabra*, are showed in Table 1.

The phenolic profile, recorded at 280 and 370 is shown in Fig. 1. Nineteen peaks were tentatively identified in the sample, 13 O-glycosylated flavonoids, 4 C-glycosylated flavonoids, 1 isoflavone and 1 chalcone. The most abundant phenolic compounds were flavonoids, constituting approximately 75 % of total phenolic compounds present in the hydroethanolic extract of *G. glabra*. Liquiritigenin was the major flavonoid identified.

Susceptibility testing of planktonic populations of C. tropicalis and S. aureus

The MIC, MBC and MFC of *G. glabra* and Manuka honey against planktonic populations of *S. aureus* and *C. tropicalis* were determined.

Table 1
Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, tentative identification, quantification (mg/g extract), and reference used for identification of the phenolic and other type of compounds present in hydroethanolic extract of *Glycyrrhiza glabra* (Mean±SD).

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g extract)	Reference
1	6.67	277	209	183(2),165(100),133(5)	Dihydrochalcone	13.663±0.094	Wu et al. (2021)
2	9.5	336	593	473(25),383(12),353(23)	Apigenin-6,8-di-*C*-glycoside	6.2 ± 0.116	Martins et al. (2015)
3	13.52	336	563	503(35),473(96),443 (41),413(10),383(29),353 (30)	Apigenin-6-*C*-pentosyl-8-*C*-hexoside	5.817±0.024	Ferreres et al. (2018)
4	13.9	336	563	503(5),473(87),443 (100),413(4),383(19),353 (20)	Apigenin-6-*C*-hexosyl-8-*C*-pentoside	5.387±0.111	Ferreres et al. (2011)
5	16.2	272,sh316	549	429(23),417(15),255(29)	Liquiritigenin apiosyl-glucoside isomer	17.379±0.225	Martins et al. (2015)
6	16.49	272/320	577	559(5),503(12),415(5)	(Iso)violanthin	9.804±0.354	Martins et al. (2015)
7	16.63	334	445	283(100),268(10)	Methyl apigenin-*O*-hexoside	9.942±0.23	Martins et al. (2015)
8	16.90	276,sh316	549	429(3),417(15),255(29)	Liquiritigenin apiosyl-glucoside isomer	15.548±0.657	Martins et al. (2015)
9	17.04	276,sh318	549	429(5),417(11),255(55)	Liquiritigenin apiosyl-glucoside isomer	18.623±0.241	Martins et al. (2015)
10	20.7	284,sh336	565	271(100)	Naringenin-7-*O*-apiosylglucoside	8.374±0.133	Martins et al. (2015)
11	25.57	252,sh300	561	267(100),252(10)	Formononetin-7-*O*-apiosylglucoside	3.846±0.02	Martins et al. (2015)
12	26.6	362	549	417(5),255(100)	(Neo)licuroside	6.562±0.036	Martins et al. (2015)
13	27.8	250, sh292,372	549	417(15),255(100)	(Neo)licuroside	12.02±0.097	Wang et al. (2013); Martins et al. (2015)
14	28.20	229/275/ 313	695	441(32),365(5),301(5), (301)	Licorice glycoside B, D_2 or D_1	2.785±0.164	Ferreres et al. (2011), Wang et al. (2014); Huang et al. (2018); Chen et al. (2019)
15	28.57	229/276/ 321	725	549(25),531(100),423 (5),255(5)	Licorice glycoside A, C_1 or C_2	4.372±0.239	Ferreres et al. (2011), Wang et al. (2014); Huang et al. (2018); Chen et al. (2019)
16	29.19	229/275/ 313	695	441(32),365(5),301(5), (301)	Licorice glycoside B, D_2 or D_1	3.491±0.165	Ferreres et al. (2011), Wang et al. (2014); Huang et al. (2018); Chen et al. (2019)
17	29.39	230/281/ 320	725	549(25),531(100),423 (5),255(5)	Licorice glycoside A, C_1 or C_2	2.793±0.096	Ferreres et al. (2011), Wang et al. (2014); Huang et al. (2018); Chen et al. (2019)
18	29.56	232/281/ 322	725	549(25),531(100),423 (5),255(5)	Licorice glycoside A, C_1 or C_2	1.931±0.023	Ferreres et al. (2011), Wang et al. (2014); Huang et al. (2018); Chen et al. (2019)
19	34.21	229/275/ 313	695	441(32),365(5),301(5), (301)	Licorice glycoside B, D_2 or D_1	7.785±0.09	Ferreres et al. (2011), Wang et al. (2014); Huang et al. (2018); Chen et al. (2019)
Total *C*-glycosylated flavonoids						**27.209±0.373**	
Total *O*-glycosylated flavonoids						**94.226±0.838**	
Total isoflavone						**3.846±0.02**	
Total Chalcones						**13.663±0.094**	
Total Compounds						**164.871±0.323**	

Standard calibration curves used for quantification: apigenin-6-*C*-glucoside ($y = 107025x + 61{,}531$, $R^2 = 0.9989$; LOD = 0.19 μg/ml; LOQ = 0.63 μg/ml, peaks 2, 3, 4, and 6); apigenin-7-*O*-glucoside ($y = 10683x - 45{,}794$, $R^2 = 0.996$, LOD = 0.10 μg/ml; LOQ = 0.53 μg/ml, peak 7); formononetin ($y = 37800x + 185{,}255$, $R^2 = 0.999$; LOD = 0.30 μg/ml; LOQ = 0.93 μg/ml peaks 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20); isoliquiritigenin ($y = 42820x + 184{,}902$, $R^2 = 0.999$; LOD = 0.18 μg/ml; LOQ = 0.54 μg/ml, peak 1); and naringenin ($y = 18433x + 78{,}903$, $R^2 = 0.999$, LOD = 0.17 μg/ml; LOQ = 0.81 μg/ml, peak 10).

Accordingly, Table 2 presents the antimicrobial activity of hydroethanolic *G. glabra* roots extract and Manuka honey in *S. aureus* and *C. tropicalis* evaluated by microbroth dilution assay.

Planktonic cells of *S. aureus* and *C. tropicalis* were susceptible to both antimicrobial agents tested. However, *G. glabra* and Manuka honey presented a higher antimicrobial effect against *S. aureus*, with the plant extract being the most effective, as the MIC concentration exhibited the greatest reduction in the logarithm of CFUs (3 log) (Data not shown).

In order to evaluate a possible synergistic effect, *G. glabra* and honey were tested in combination using the checkerboard assay. However, none of the different combinations tested showed synergy on planktonic cells of *S. aureus* and *C. tropicalis* presenting in general no interaction ($0.5 < FICI \leq 4.0$) (Fig. 2).

Effect of G. glabra and honey alone and in combination in S. aureus and C. tropicalis single biofilms

The antibiofilm effect of *G. glabra* hydroethanolic extract (1.5 mg/ml) and Manuka honey (37.5 % (w/v)) alone and in combination, was assessed on *S. aureus* and *C. tropicalis* single biofilms (Figs. 3 and 4), by determining biofilm viable cells. As it can be seen in Figs. 3A and 4A, both drugs by themselves, prevented biofilm formation (total inhibition) and inhibited preformed *S. aureus* biofilms. *G. glabra* and honey showed a significant killing on preformed biofilms corresponding to a 2.38 and 2.89 log reduction in culturable cells, respectively (Fig. 4A). No synergy was observed with the combination of both antimicrobial agents. Analyzing the results obtained for *C. tropicalis* biofilms, *G. glabra* and honey displayed a similar effect on biofilm formation (0.5 log reduction) ($p < 0.05$) but both in combination exhibited synergy (log reduction > 3) (Fig. 3B). Regarding preformed biofilm, a significant reduction was observed for *G. glabra* (0.5 log) and the combination treatment (1 log) ($p < 0.05$), being observed synergy. No antibiofilm activity was observed after treatment of *C. tropicalis* preformed biofilms with honey (Fig. 4B).

In general, honey on its own and its combination with *G. glabra* were the most effective on *S. aureus* biofilms, while on *C. tropicalis* biofilms the best antimicrobial activity was obtained with the antimicrobial combination strategy.

Effect of G. glabra and honey alone and in combination in S. aureus and C. tropicalis mixed biofilms

The co-culture of *S. aureus* and *C. tropicalis* was evaluated showing

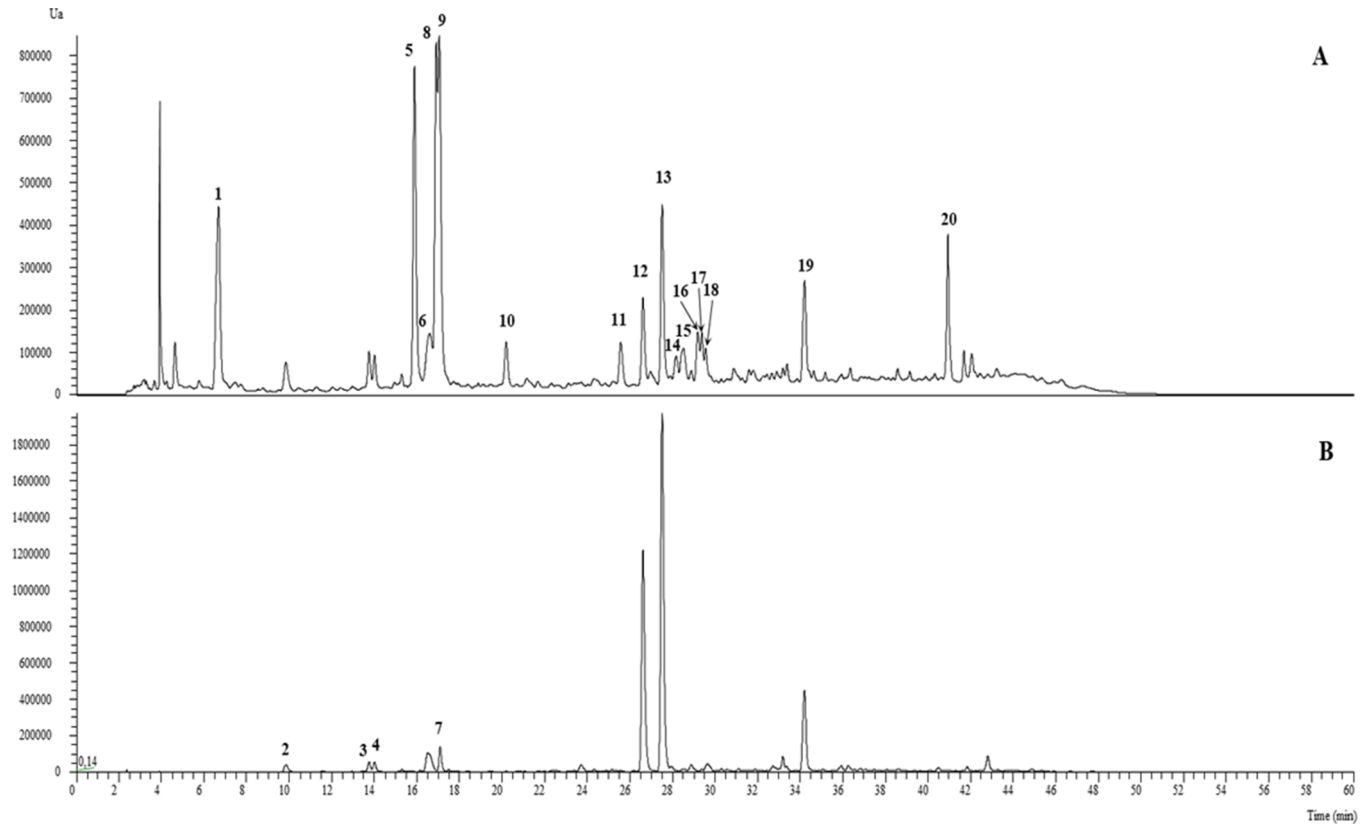

Fig. 1. Exemplificative phenolic and other type of compounds profile of hydroethanolic extract of *Glycyrrhiza glabra* recorded at 280 (A) and 370 (B) nm.

Table 2
Antimicrobial activity of hydroethanolic *Glycyrrhiza glabra* extract and Manuka honey in planktonic cells of *Staphylococcus aureus* ATCC 25,923 and *Candida tropicalis* ATCC 750.

	MIC[1]	MBC[2]/MFC[3]
***Staphylococcus aureus* ATCC 25,923**		
G. glabra	0.0975 mg/ml	0.19 mg/ml
Honey	9.375 % (w/v)	18.75 % (w/v)
Vancomycin	0.001 mg/ml	0.002 mg/ml
***Candida tropicalis* ATCC 750**		
G. glabra	0.78 mg/ml	> 0.78 mg/ml
Honey	37.5 % (w/v)	>37.5 % (w/v)
Amphotericin B	0.005 mg/ml	0.010 mg/ml

[1] minimal inhibitory concentration,.
[2] minimal bactericidal concentration,.
[3] minimal fungicidal concentration; Vancomycin and Amphotericin B were used as standard positive drug control.

that this interaction promoted a statistically significant reduction on *C. tropicalis* population ($p < 0.05$) and, therefore, *S. aureus* has a suppressive effect on *C. tropicalis* growth, namely in biofilm growth. The efficacy of the *G. glabra* and honey alone and, in combination, was further inspected on biofilm formation and on preformed dual-species biofilms of *S. aureus* and *C. tropicalis* (Figs. 5 and 6, respectively). All the antimicrobial strategies tested exhibited a good inhibitory effect on biofilm formation of *S. aureus* population of the mixed biofilm ($p < 0.05$). Honey and its combination with *G. glabra* presented the best and similar inhibitory effect ($p > 0.05$). Biofilm formation of *C. tropicalis* population in mixed biofilm was affected by both antimicrobials with an additive effect in case of combined treatment (Fig. 5) ($p < 0.05$).

Regarding preformed biofilms, the treatment of mixed biofilms with *G. glabra* and honey promoted a log reduction of 3.5 and 2 log, respectively, in *S. aureus* population. With regard to *C. tropicalis* population in mixed biofilms, *G. glabra* promoted a log reduction of approximately 1 log ($p < 0.05$) and in turn the honey did not cause any cell reduction.

Discussion

In this work, the MIC and MBC/MFC of *G. glabra* hydroethanolic and Manuka honey were determined for *S. aureus* and *C. tropicalis*. Analyzing the results obtained (Table 2) it was verified that both species studied were susceptible to both antimicrobial agents.

Synergistic effect between antimicrobials has been pointed as a potential option to improve the efficacy of antimicrobial agents and prevent the emergence of the development of resistance. In fact, some studies showed honey' synergy with other antimicrobials, namely propolis in single and polymicrobial cultures of *S. aureus, E. coli* and *C. albicans* (AL-Waili et al., 2012); crude methanolic extracts of Herba Ocimi Basilici on human bacterial pathogens (Khalil et al., 2012); different cranberry extracts (Prince, 2019) and antibiotics, for planktonic and biofilm cells (Almasaudi, 2021). Accordingly, the checkerboard assay was performed but no synergy was observed for any of the combinations tested (Fig. 2).

More than 65 % of nosocomial infections are due to biofilm-associated infections (Assefa and Amare, 2022). *S. aureus*, the major bacterial human pathogen and *C. tropicalis*, an emerging opportunistic fungal pathogen associated with high mortality rates, are known by their good biofilm formation ability. Mixed biofilms of *Candida* and bacteria are, frequently, responsible by infections and this co-habitation is increasingly frequent and usually associated with chronic infections (De Alteriis et al., 2018). In fact, the possible co-existence between *C. tropicalis* and *S. aureus* forming dual-species biofilms was previously referred by Alteriis et al. (2018).

G. glabra hydroethanolic extract and honey were tested alone and in combination on biofilm formation and single-preformed biofilms of *S. aureus* and *C. tropicalis*. *G. glabra* and honey alone exhibited a good

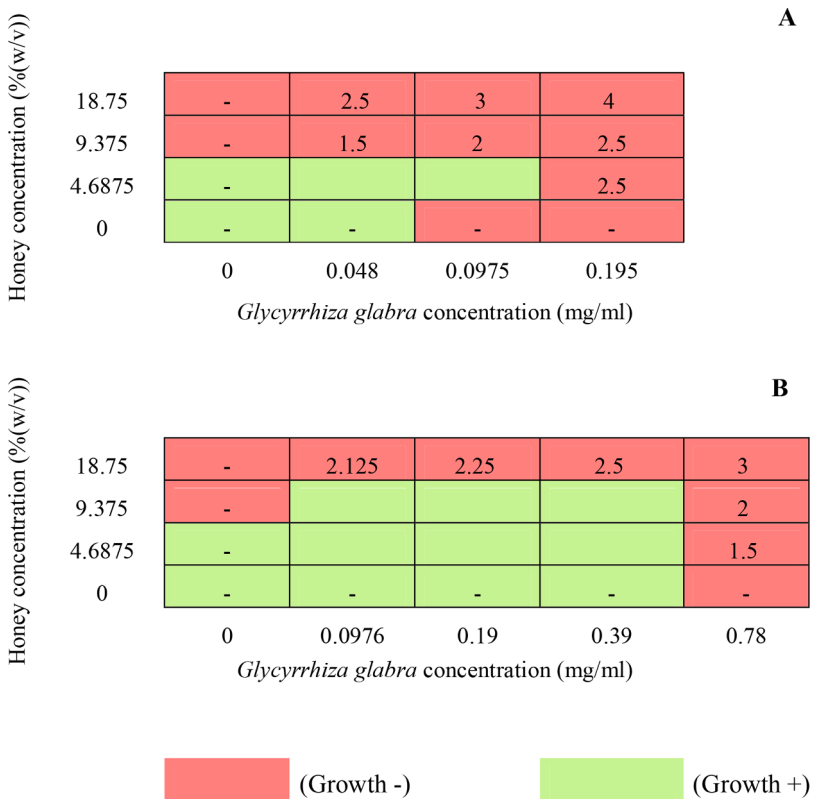

Fig. 2. Checkerboard assay results of the combination of *Glycyrrhiza glabra* Hydroethanolic and Manuka honey tested against planktonic cells of *Staphylococcus aureus* ATCC 25,923 (A) and *Candida tropicalis* ATCC 750 (B). (-) represent the antimicrobial agents tested alone; Data represent the FICI values.

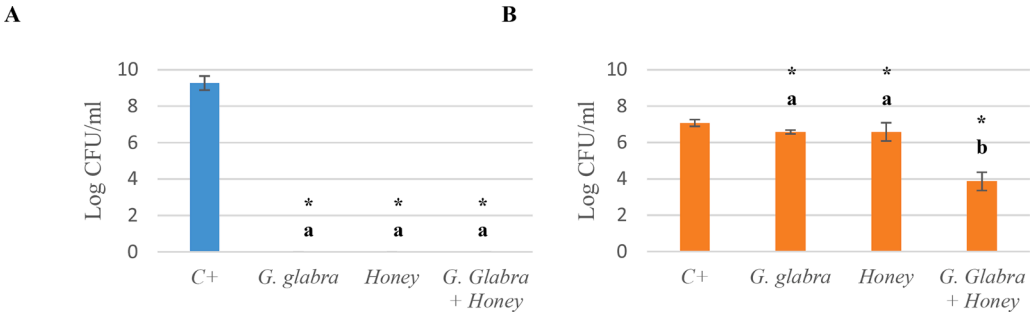

Fig. 3. Antibiofilm effect of *Glycyrrhiza glabra* hydroethanolic extract (1.5 mg/ml) and honey (37.5 %) alone and in combination on biofilm formation of single biofilms of *Staphylococcus aureus* ATCC 25,923 (A) and *Candida tropicalis* ATCC 750 (B), at 24 h. C+ positive growth control. Data represent the mean ± Standard deviation. * $p < 0.0001$ indicates statistical significance as compared to control. Different letters mean significant differences from the different treatments ($p < 0.0001$).

antibiofilm effect on *C. tropicalis* and *S. aureus* biofilms (Figs. 3 and 4). This was not observed for preformed *C. tropicalis* single biofilms, since Manuka honey at 37.5 % (w/v) did not cause any cell reduction (Fig. 4B). Similar results were obtained by Fernandes et al. (2020) with concentration of 25 % (w/v) demonstrating no antifungal activity on single biofilms of *C. tropicalis*. In this work, it was demonstrated that in preformed biofilms Manuka honey has a higher antibacterial activity than antifungal effect. This is in accordance with the bibliography that reported that fungi are more tolerant to honey than bacteria since it exhibited a higher resistance to osmotic pressure (Irish et al., 2006; Fernandes et al., 2020). In *C. tropicalis* biofilms a synergistic effect was observed when combining *G. glabra* and honey (Figs. 3B, 4B). It must be due to the effect of honey on the biofilm matrix, compromising the biofilm structure and enhancing antimicrobial penetration, potentiating the effect of *G. glabra*. The presence of secondary metabolites, namely saponins, alkaloids and flavonoids with their various mechanisms of action including decreased gene expression, growth inhibition and reduction of toxin production, prevention of the yeast-hyphal transition may have contributed to the antimicrobial activity of *G. glabra* (Wang et al., 2015; Pastorino et al., 2018). In turn, some honey components such as metilglioxal, aromatic acids and proteins (flavonoids, polyphenols and defensin-1) and the production of H_2O_2, its acidic pH (3.5) and osmotic pressure, responsible by shrinkage and disruption among the cells, gives it antimicrobial properties (Fernandes et al., 2020). Cell dehydration, and in cases of biofilms, disruption of cell membrane integrity and the reduction of the production of extracellular polysaccharide matrix are some of the known mechanisms of action of honey (Irish et al., 2006; Fernandes et al., 2020).

The phenolic composition of hydroethanolic extract of *G. glabra* obtained are in accordance with the previously published by Chen et al. (2019); Huang et al. (2018); Martins et al. (2015); Qiao et al. (2012) and Wu et al. (2021). From the nineteen phenolic compounds identified,

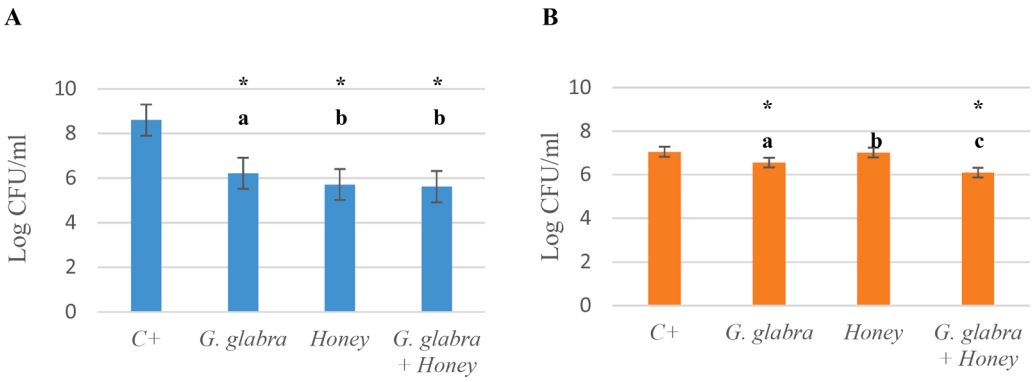

Fig. 4. Antibiofilm effect of *Glycyrrhiza glabra* hydroethanolic extract (1.5 mg/ml) and honey (37.5 %) alone and in combination on 24 h- preformed single biofilms of *Staphylococcus aureus* ATCC 25,923 (A) and *Candida tropicalis* ATCC 750 (B), at 24 h. C+ positive growth control. Data represent the mean ± Standard deviation. * $p < 0.0001$ indicates statistical significance as compared to control. Different letters mean significant differences from the different treatments ($p < 0.0001$).

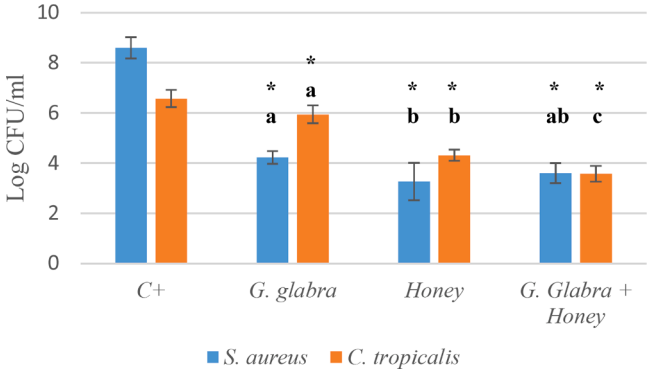

Fig. 5. Antibiofilm effect of *Glycyrrhiza glabra* hydroethanolic extract (1.5 mg/ml) and honey (37.5 %) alone and in combination on biofilm formation of mixed biofilms of *Staphylococcus aureus* ATCC 25,923 and *Candida tropicalis* ATCC 750, at 24 h. C+ positive growth control. Data represent the mean ± Standard deviation. * $p < 0.0001$ indicates statistical significance as compared to control. Different letters mean significant differences from the different treatments ($p < 0.0001$).

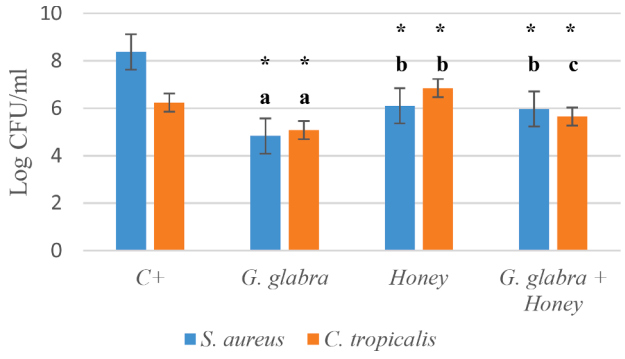

Fig. 6. Antibiofilm effect of *Glycyrrhiza glabra* hydroethanolic extract (1.5 mg/ml) and honey (37.5 %) alone and in combination on 24 h- preformed mixed biofilms of *Staphylococcus aureus* ATCC 25,923 and *Candida tropicalis* ATCC 750, at 24 h. C+ positive growth control. Data represent the mean ± Standard deviation. * $p < 0.0001$ indicates statistical significance as compared to control. Different letters mean significant differences from the different treatments ($p < 0.0001$.

seventeen were flavonoids and liquiritigenin was the most abundant and consistently reported as one of the major flavonoids in licorice. Antimicrobial activities have been reported as strongly correlated with phenolic composition, with flavonoids having a preponderant role (Araújo et al., 2012).

Comparing the effect of both agents alone and in combination on the formation of mixed biofilm, an additive effect was observed on the *C. tropicalis* population and a similar effect of each agent alone and the combination for *S. aureus*, demonstrating good antimicrobial activity. In preformed mixed biofilms, *G. glabra* exhibited a superior antibiofilm activity on both populations. As reported by Fernandes et al. (2020) this is a very rare situation since in the majority of cases antimicrobials are only able to reduce one of the species. *C. tropicalis* suffered an increase of their population density after honey exposure. In preformed single biofilms, honey had no antimicrobial effect on *C. tropicalis*, while in mixed biofilms it was shown to stimulate the fungal growth. This can be explained by the fact that *C. tropicalis* takes advantage of the antimicrobial effect exerted on the *S. aureus* population, and of the lower sugar concentration present in honey, to promote their own growth since on mixed biofilms, a common interaction is the competition for nutrient acquisition and space occupation (Rao et al., 2020). No synergy was observed when combining both antimicrobials. In general, a better antibiofilm effect was obtained on biofilm formation than on preformed biofilms.

Although in some conditions synergy has not been observed when combining both agents, combined treatment seems to be the most promising therapeutic strategy, presenting good results for *S. aureus* and *C. tropicalis* either when growing alone or in co-habitation. Manuka honey, already used in the medical setting and approved for some clinical applications, has been widely reported as a safe therapeutic alternative. In turn, Martins (2017) showed that hydromethanolic extract of *G. glabra* has no toxic effects for concentrations up to 1.5 mg/ml (Martins, 2017).

Conclusion

The increasing evidence of polymicrobial infections highlighted the need for new antimicrobial therapies targeted at this type of infection. The results obtained highlighted the antimicrobial potential of both hydrothenolic extract of *G. glabra* and Manuka honey. The combination of Manuka honey and *G. glabra* has demonstrated a general good antibiofilm activity namely against single and mixed biofilms of *S. aureus* and *C. tropicalis* being active in both in the prevention of biofilm formation and the eradication of preformed biofilms. Thus, the combination tested can act as a good biofilm reducer for both pathogens and be a potential therapeutic strategy either in cases of single-species infections or in infections of polymicrobial origin.

CRediT authorship contribution statement

Fernanda Gomes: Writing – original draft, Validation,

Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Inês Dias:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Maria Elisa Rodrigues:** Data curation, Formal analysis, Investigation. **Lillian Barros:** Data curation, Formal analysis, Investigation, Supervision. **Mariana Henriques:** Investigation, Supervision, Validation, Writing – original draft.

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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