Propolis particles incorporated in aqueous formulations with enhanced antibacterial performance

Chourmouziadi Laleni, Nelli; De Carvalho Gomes, Paulo; Gkatzionis, Kostas; Spyropoulos, Fotios

DOI:
10.1016/j.fhfh.2021.100040

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Chourmouziadi Laleni, N, De Carvalho Gomes, P, Gkatzionis, K & Spyropoulos, F 2021, 'Propolis particles incorporated in aqueous formulations with enhanced antibacterial performance', *Food Hydrocolloids for Health*, vol. 1, 100040. https://doi.org/10.1016/j.fhfh.2021.100040

Food Hydrocolloids for Health 1 (2021) 100040

Contents lists available at ScienceDirect

Food Hydrocolloids for Health

journal homepage: www.elsevier.com/locate/fhfh

Propolis particles incorporated in aqueous formulations with enhanced antibacterial performance

Nelli Chourmouziadi Laleni[a,*], Paulo De Carvalho Gomes[a], Konstantinos Gkatzionis[b,*], Fotis Spyropoulos[a]

[a] Food Microstructure Group, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom
[b] Laboratory of Consumer Responses and Sensory Perception of Food and Drinks, Department of Food Science and Nutrition, University of the Aegean, Metropolite Ioakeim 2, Lemnos, Myrina 81400, Greece

ARTICLE INFO

Keywords:
Propolis
Sonication
Dispersion
Antibacterial activity
Flow cytometry

ABSTRACT

In recent years, the use of natural bioactives in food, pharmaceutical and cosmetic industries has emerged as a global formulation development trend. Although natural bioactives exhibit promising properties, they are also associated with chemical instability or poor aqueous solubility. One such bioactive with beneficial functionalities but limited industrial applicability within industry is propolis. The purpose of this study was to investigate means to enable enhancement to the antibacterial activity of propolis-based aqueous formulations. Dry propolis was firstly extracted from crude material and the effect of common carrier phases used for dissolution of propolis for antibacterial assays was investigated. Consequently, the extract was formulated into propolis sub-micron aqueous dispersions *via* direct ultrasonication. Processing time was varied, and all formed particles were characterised immediately after production in terms of size, polydispersity and zeta potential, and then again after a month-long storage period. When tested on *E. coli* cells, 15% propolis dispersions caused a bactericidal effect, which was sonication time and time of exposure dependent. Particles formed at the shortest sonication period (4 min) resulted in higher cell injury while those processed the longest (10 min) caused greater cell death and with AFM imaging, cell membrane alterations were confirmed. Chemically, for whole dispersions and carrier phases alone, free radical scavenging activity and total phenol content were slightly enhanced at longer sonication times. Overall, the present work suggests that formulating propolis extract sub-micron aqueous dispersions *via* sonication enhances their antibacterial performance *via* a synergistic effect involving both their carrier and dispersed phases.

Introduction

In recent years there has been a movement to reduce the use of synthetic actives in food, pharmaceuticals and cosmetics. This largely stems from the consumers' conceptualisation of the term "natural", which ultimately leads to preferences towards products containing natural bioactives, as they are perceived to possess positive attributes such as being healthier or more environmentally friendly than synthetic equivalents (Rozin, 2005; Newburger, 2009; Asioli et al., 2017). Studies demonstrate the public's apprehension on being exposed to synthetic chemicals, concerns that may stem from misunderstandings related to the significance of the quantities used, despite the fact that these are closely regulated (Dickson-Spillmann, Siegrist & Keller, 2011). Consequently, more and more research is being directed towards natural bioactives shown to possess a wide range of functional performances that could be utilised within the context of formulated products. Some examples include curcumin which has been reported to possess antioxidant and anti-inflammatory properties (Hatcher, Planalp, Cho, Torti & Torti, 2008), aloe vera which has been associated with antitumor/anticancer, anti-diabetic and anti-inflammatory properties (Boudreau & Beland, 2006), and is widely used among cosmetic products (Gallagher & Gray, 2003), and essential oils from oregano or lavender (Dadalioğlu & Evrendilek, 2004) that have been reported to exhibit antibacterial, antifungal and antiviral properties (Lang & Buchbauer, 2012).

Another versatile bioactive with multiple reported benefits is propolis or bee glue; a resinous mixture produced by *Apis mellifera* honeybees (Eom, Lee, Yoon & Yoo, 2010). It consists of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and other substances such as organic debris (Pietta, Gardana & Pietta, 2002). Propolis extracts have been reported to exhibit antibacterial activity against both gram-positive (*S. aureus* (Akca et al., 2016), *S epidermidis* (Uzel et al., 2005), *L. monocytogenes* (Chen, Ye, Ting & Yu, 2018), *S. typhimurium* (Righi et al., 2011)) and gram-negative (*E. coli*

* Corresponding authors.
E-mail addresses: nxc742@student.bham.ac.uk (N. Chourmouziadi Laleni), kgkatzionis@aegean.gr (K. Gkatzionis).

https://doi.org/10.1016/j.fhfh.2021.100040
Received 31 August 2021; Received in revised form 15 October 2021; Accepted 12 November 2021

(Netíková, Bogusch & Heneberg, 2013), *P. aeruginosa* (Regueira et al., 2017) and *K. pneumoniae* (Lopez et al., 2015)) bacteria; with a less pronounced antibacterial action on gram-negative (Silva-Carvalho, Baltazar & Almeida-Aguiar, 2015). In current literature, propolis extracts have also been associated with antifungal, antioxidant and anti-inflammatory properties (Kuropatnicki, Szliszka & Krol, 2013). In terms of applications, propolis has been used as a dietary supplement (Moret, Purcaro & Conte, 2010),in dental care (Vanni et al., 2015), in dermatological creams and lotions (Barros, Neto & Fonteles, 2019) and in food industries it has been effective in preserving meat, fish and juice products (Mohammadzadeh et al., 2007; Pobiega, Kraśniewska & Gniewosz, 2019b).

Although the consistency of propolis varies depending on the local flora, season and climate, it is widely accepted that phenols and flavonoids are the main compounds responsible for its antibacterial activity (Castaldo & Capasso, 2002; Bankova, 2005b). By being such a complex mixture, propolis has the advantage of providing a synergistic functional response that is stronger compared to that of its individual components alone (Amoros, Simõs, Girre, Sauvager & Cormier, 1992; Kharsany, Viljoen, Leonard & van Vuuren, 2019). However, it is the level of heterogeneity that can cause issues such as variability in chemical constitution and functional performance (e.g. antibacterial activity), and its plethora of organic compounds that greatly diminishes its water solubility (Bankova, 2005a; Permana et al., 2020). Moreover, crude propolis needs to be purified prior to use as it contains contaminants and debris that are not functional nor safe (Burdock, 1998). This usually involves an extraction process, which itself can be highly variable depending on the solvent and method used. While efforts have been made to extract propolis' bioactive compounds in water (Park & Ikegaki, 1998) or oil (Ramanauskienė & Inkėnienė, 2011), aqueous ethanol (a mixture of ethanol and water) tends to be the most effective solvent (Kubiliene et al., 2015; Sun, Wu, Wang & Zhang, 2015).

Propolis can remain diluted in the extraction solvent for further investigation, or the solvent can be evaporated to recover the extracts as dry matter. The solid propolis extracts can then be incorporated within a formulation medium that among others should easily allow the realisation of the desired propolis functionalities in the final application. Solvents that effectively dissolve propolis extracts and tend to be used in literature (such as ethanol, methanol or dimethyl sulfoxide (DMSO)) (Tosi, Ré, Ortega & Cazzoli, 2007; Regueira et al., 2017; Ristivojević et al., 2018), have limited industrial applications and can themselves give rise to an antibacterial response that is beyond the sole capacity of propolis; for instance some *E. coli* strains can be susceptible to DMSO (O'Neill & Chopra, 2004; Cao et al., 2017).

To avoid such issues, water can be used as the formulation medium instead. However, literature on aqueous-based propolis systems and their antibacterial activity is much more scarce, since, although ideal for application purposes, water is not an efficient extraction solvent nor carrier/formulation medium for propolis (Pobiega et al., 2019b; Ghavidel, Javadi, Anarjan & Jafarizadeh-Malmiri, 2021). Alternative approaches to enhance the bioavailability of poorly water-soluble functional compounds have been reported in literature. Basniwal, Khosla and Jain (2014) produced aqueous dispersions of curcumin nanoparticles that were shown to exhibit a similar or even stronger response against different cancer cell lines, when compared to curcumin dissolved in DMSO. Elsewhere, attempts to increase the bioavailability of quercetin in water have studied its inclusion within solid dispersions fabricated using a range of carrier compounds such as monoolein, polyvinylpyrrolidone and hydroxypropyl methyl cellulose, and utilising homogenization, solvent evaporation or freeze drying methods (Park, Song & Choi, 2016; Cortesi et al., 2017; Porcu et al., 2018). Finally, and with direct relevance to the presence study, Elbaz, Khalil, Abd-Rabou and El-Sherbiny (2016) developed chitosan-based nano-in-microparticles carriers to facilitate the oral delivery of propolis, enhance its aqueous solubility and bioavailability, enable its controlled release and enhance its anticancer activity.

The current work adopts the formulation engineering ethos of such studies and aims to investigate approaches that enable enhancement to the antibacterial activity of propolis-based aqueous systems. Initially, the study reveals that the use of ethanol or DMSO as carrier phases for the dissolution of propolis extracts, produces antibacterial responses beyond the capacity of the bioactive itself. Therefore, water was exclusively used as the carrier phase of crude propolis dry extracts which were formulated into sub-micron propolis aqueous dispersions *via* direct ultrasonication. Although sonication has been shown to offer advantages when employed as part of the propolis extraction process (Gokce, Cengiz, Yildiz, Calimli & Aktas, 2014; Jug, Končić & Kosalec, 2014) or to facilitate encapsulation (e.g. in β- cyclodextrin),(Kalogeropoulos et al., 2009b), its use to promote functionality of the sole active directly within the final formulation/carrier medium, to the best of our knowledge, has not been previously explored. Here, sonication time was varied and all formed propolis particles were characterised in terms of size, polydispersity and zeta potential both immediately after production as well as over a month-long storage period. Sonication time and time of exposure were considered for their impact on the antibacterial activity (tested on *E. coli* cells) of all propolis dispersions and AFM imaging was selectively utilised to confirm potential damage to the cell membrane. All dispersions were tested for their free radical scavenging activity and total phenol content as means to gain further insight into their antioxidant activity and the causes of their antibacterial performance. Finally, the contribution to the overall bactericidal functionality arising by the carrier phase of the propolis aqueous dispersions alone, was investigated. Overall, the present work demonstrates that formulating propolis extracts into sub-micron propolis aqueous dispersions can indeed enhance their antibacterial performance *via* a synergistic effect involving both their carrier and dispersed phases.

Materials and methods

Propolis samples and materials

Crude propolis was collected from Fthiotida region, Greece during spring 2019, purchased from ANEL (Thessaloniki, Greece) and was stored in the dark at -20 °C. Ethanol (Absolute, 99.8%, analytical reagent grade), Nutrient agar (NA), Dimethyl Sulfoxide (DMSO) and Phosphate Buffered Saline (PBS) were purchased from Fisher Scientific (Loughborough, UK). Luria Bertani broth (LB), Tween 80, Propidium Iodide (PI), Bis-(1,3- Dibutylbarbituric acid) trimethine Oxonol (BOX), Folin-Ciocalteu reagent and gallic acid were purchased from Sigma-Aldrich Ltd (Gillingham, UK). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Trolox were purchased from EMD Millipore Corp. (Darmstadt, Germany). Sodium Carbonate was purchased from LP Chemicals Ltd (Winsford, UK).

Microbial culture

E. coli K12 (MG1655) cells were maintained on Luria Bertani agar at 4 °C. In order to obtain cells in the stationary phase, a colony was transferred into 50 ml of Luria Broth, incubated at 37 °C for 18 h while shaking at 150 rpm. The concentration of overnight cultures was fixed at approximately 1.5×10^7 CFU/ml. Cells were centrifuged at 4600 *g* for 3 min (Eppendorf Centrifuge 5430, Germany) and washed thrice with PBS.

Preparation and characterisation of propolis samples

Propolis extraction

Frozen propolis samples were ground (using a commercial food grade grinder, MosaicAL) into submillimetre particles as shown in Fig. 1. Extraction was carried out by mixing 30 g of propolis with 300 ml of 70% ethanol in water (i.e. a mass to volume ratio of 1:10 propolis to

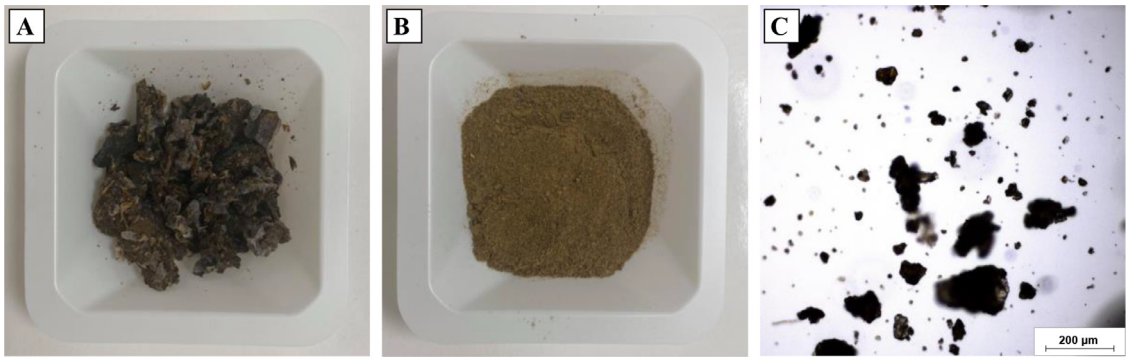

Fig. 1. (A) Frozen crude propolis, (B) grinded propolis and (C) light microscopy image (10 × magnification) of grinded propolis particles.

solvent) and stored at 25 °C in dark for one week with occasional shaking. The resulting tincture was centrifuged (Sigma 3K30, UK) at 8800 *g* and 4 °C, and filtered through no 4 Whatman filter paper, in order to remove waxes and other non-soluble compounds (Kalogeropoulos, Konteles, Troullidou, Mourtzinos & Karathanos, 2009a). This process was repeated for three consecutive days or until no visible precipitation was present, while the tincture was stored at 4 °C. The solvent was evaporated under reduced pressure (approximately -90 kPa gauge) at 40 °C (Buchi Rotavapor R-215, Switzerland) and the dry propolis extracts were obtained at approximately 20% yield. 25, 10, 5, 1 and 0.1 mg/ml of propolis dry extracts were diluted in 20% ethanol and 80% water for further testing.

Preparation of propolis aqueous dispersions

An aqueous dispersion was made of 1.88% propolis dry extract (approximately 1-2 g), 1% Tween 80 and water. Tween 80 was selected as a non-ionic, small weight surfactant, typically used in foods as an emulsifier or dispersing agent (Pourreza & Rastegarzadeh, 2004). The mixture was sonicated using a high intensity ultrasonic vibracell processor with a 13 mm probe (Sonics & Materials, Inc., CT, USA) for 2, 4, 6, 8 and 10 min at 20 kHz, 750 W and 95% amplitude. Pulse mode of 4 s on 2 s off was used to avoid heat buildup. A pre-dispersion was made of 7% propolis dry extract (approximately 4–6 g), 1% Tween 80 and water and processed as described above. Part of some water content of the dispersion was evaporated under reduced pressure (approximately -95 kPa gauge) at 40 °C, which resulted in dispersions with a higher propolis content of 15%. Dispersions of 4 and 10 min were diluted to 11.24%, 7.5%, 5.6% and 3.75% for further testing. A 15% propolis, non-sonicated, aqueous sample was also prepared for reference testing.

It should be stressed that the exact content of propolis ultimately retained within the formed aqueous dispersions (post sonication) used in this study differs from the amount of propolis extract initially added (prior to sonication). The discrepancy arises due to extract losses during the sonication stage where a fraction of the propolis was not possible to be dispersed; either precipitated at the bottom and/or adhered onto the walls of the vessel. A series of preliminary experiments have indicated this loss to be more or less consistent across the different propolis contents (and sonication times) used; amounting to ~1/3 of the initially introduced propolis extract mass. The authors have elected to retain the initial propolis content in the discussion that follows only because this represents a more unambiguous means to distinguish between different samples.

Isolation of the aqueous carrier phase of the propolis dispersions

The aqueous carrier medium was isolated from the propolis dispersion by ultrafiltration (Vivaspin 100 kDa, Uppsala, Sweden) and was further tested separately. The filtration tubes were filled with 1.2 ml propolis dispersions and centrifuged for 30 min at 3900 *g* (Eppendorf Centrifuge 5810, Germany).

Size, polydispersity index and zeta potential measurements

A zetasizer Nano (Malvern Instruments Ltd., U.K.) instrument was used to measure the particle size distribution (intensity,%) and determine the mean particle size and polydispersity index (PdI) using dynamic light scattering (DLS). Zeta potential was measured by electrophoretic mobility at room temperature. Samples were diluted 10 fold prior to measurement using ultrapure water (Milli-Q).

Total phenol content

Total phenolic content was determined using the colorimetric Folin-Ciocalteu method. The method was adapted from Kubiliene et al. (2015). The propolis dispersions and carrier phases were diluted 1:10 with distilled water and 77 μl were mixed with 77 μl of Folin- Ciocalteu reagent, 385 μl 20% sodium carbonate and 960 μl distilled water. After 1 h incubation in the dark at room temperature absorbance was taken at 760 nm using the Orion AquaMate 8000 UV–vis spectrophotometer (Thermo-Scientific, UK). Total phenol content was calculated as gallic acid equivalents (GAE) in micrograms per ml using a calibration curve(y = 0.007x-0.045, R^2 = 0.9997), from a concentration range of 15.63–250 μg/ml.

Free radical scavenging activity

The method was adapted from Jansen-Alves et al. (2019) All dispersions and carrier phases were diluted 1:100 with distilled water and 100 μl were mixed with 100 μl 0.78 mg/ml DPPH reagent and 2.3 ml ethanol. Absorbance was taken after half an hour at room temperature in the dark at 517 nm using the Orion AquaMate 8000 UV–vis spectrophotometer (Thermo-Scientific, UK). Results were expressed as Trolox equivalents (TRE) in micrograms per ml using a calibration curve (y = -0.0041x+0.9624, R^2 = 0.9988), from a concentration range of 12.5–200 μg/ml.

Assessment of biological properties of samples

Antibacterial assay

The antibacterial activity was determined by using a dilution assay in a 96-well microplate format. Samples were mixed with the microbes in a 1:1 ratio, incubated at 37 °C while at constant shaking at 150 rpm and plated at appropriate time points from 0 up to 24 h.

Plate counting (CFU)

Viability of bacteria after treatment was assessed by serial 10-fold dilutions in PBS. 10μl were measured and plated in Nutrient Agar plates and incubated overnight at 37 °C. Colony Forming Unites (CFU) were counted at the appropriate dilution (3–30 colonies) to determine cell viability. The lower limit of detection was 30 CFU /ml.

Flow cytometry

The viability of the cells and the physiology of their membrane was analyzed using an Attune NxT, Acoustic Focusing Cytometer (ThermoScientific, Singapore). Samples were diluted with filtered PBS and

stained with Propidium Iodide (PI) and Bis-(1,3- Dibutylbarbituric acid) trimethine Oxonol (BOX) at final concentrations 5 μg/ml and 10 μg/ml, respectively. PI binds to the nucleic acid but cannot permeate an unharmed cytoplasmic membrane (Hewitt & Nebe-Von-Caron, 2004). BOX is a lipophilic and anionic dye which binds with depolarised (damaged) cytomplasmic membranes (Berney, Weilenmann & Egli, 2006). In order for a cell to be significantly damaged and be characterised as "dead", detection of both dyes are needed. If there is only BOX dye detection, that gives an indication of injury to the cell membrane. Samples were vortexed and incubated for five minutes in the dark. Cells were excited using a blue laser at 488 nm and the emitted fluorescence was detected through a 400 nm band- pass filter for both dyes. The trigger was set for the green fluorescence (550 nm) channel and data were acquired on forward versus side scatter. Untreated bacteria cells in PBS were tested as controls.

Atomic force microscopy

Atomic force microscopy (AFM) images of control and treated bacterial samples with the 10 min sonicated dispersions (15%), were acquired with a JPK NanoWizard II atomic force microscope (Oxford Instruments, UK) in dry condition on a 25 mm^2 p-type silicon wafer (Sigma-Aldrich, UK). The silicon wafer was initially cleaned with a CO_2 snow jet while being held on a hot surface at 300 °C, followed by placing a suspension of 5 μl of *E. coli* (approximately 10^5 CFU/ml) in filtered PBS. Samples were allowed to air dry for 3-4 h. Then they were rinsed with deionised water to avoid crystallisation of PBS. The images were acquired in tapping mode using NCHV-A cantilever (Bruker, UK) (T: 8 nm; L: 117 μm; W: 33 μm; f_0: 320 kHz; k: 40 Nm^{-1}).

Statistical analysis

Experiments were conducted at least in duplicate unless otherwise stated. Results are expressed as averages and error bars represented the standard deviation. Statistical analysis was performed using IBM SPSS Statistics software. Kaplan-Meier survival plots were constructed within different bacteria treatments. Results were compared with 1-way ANOVA (Tukey's test) and 2-way ANOVA (Sidak's multiple comparisons test). Differences were considered significant at $p < 0.05$.

Results and discussion

The impact of the carrier phase used to dissolve propolis extracts on the overall antibacterial performance of the systems

Different quantities of propolis extracts ranging from 0.1 to 25 mg were dissolved in 1 ml of a carrier phase consisted of 20% aqueous ethanol solution and tested against *E. coli* cells in their stationary phase. PBS was used as the continuous phase for *E. coli* cells as it does not provide any nutrients for growth and is an appropriate medium for bacteria preservation. (Liao & Shollenberger, 2003) Data were collected immediately after the active-containing formulations were added to the cells and following 24 h of incubation.

Results show that at immediate contact, the highest concentrations of propolis (10 and 25 mg/ml) eliminated the population completely, while a dose dependent bactericidal effect was prominent for the concentrations ranging from 0.1 to 5 mg/ml (Fig. 2). However, after 24 h of incubation, there was no population present for any of the samples, including the control (20% ethanol). Therefore, the antibacterial effect appeared to be dominated by the carrier phase.

Flow cytometry was utilised to investigate the effects of the propolis extracts and carrier phase (20% ethanol in water) on the *E. coli* membrane potential and cellular integrity. Measurements were taken after immediate exposure (Fig. 3A) and after 24 h (Fig. 3B). All propolis extracts concentrations caused depolarisation of the cell membrane (injury) and affected cell integrity (death), even after immediate exposure, further confirming the CFU measurements (Fig. 2). The 20% aqueous

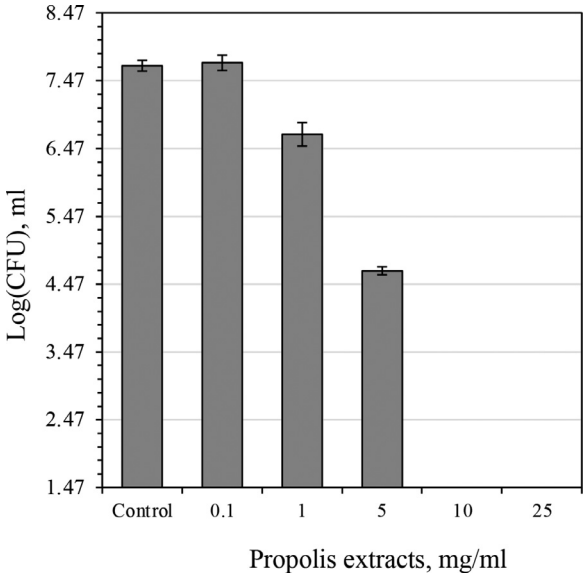

Fig. 2. Antibacterial activity of propolis extracts dissolved in 20% ethanol. Data for population of stationary phase *E. coli* MG1655 are presented as a function of concentration of propolis extracts added (0.1, 1, 5, 10 and 25 mg/ml) after immediate contact. The control is 20% ethanol. Data shown are averages ($n = 3$; biological replicates) and errors represent ± one standard deviation (s.d.).

ethanol mixture alone (control) caused a significant amount of injury to the cell membrane (43%), at immediate contact. As the concentration of propolis applied increases, the amount of injury is maintained, while the total amount of cell death is increasing, showing signs of propolis' bactericidal activity. After 24 h, the control and 0.1 mg/ml of propolis extracts exhibited similar antibacterial effects of low injured cells and a higher percentage of dead cells, whereas the rest of the samples mainly contained either alive or dead cells with low injury percentages. That could be due to the time effect which at high propolis extracts quantities, results in eventual death of the previously injured cells, whereas for the control and 0.1 mg/ml propolis concentration, the rate of action is slower.

It is apparent that ethanol, even at a relatively low percentage of 20%, provides an underlying antibacterial response. Ethanol, like other alcohols, is anticipated to cause damage to the cell membrane, denaturation of the proteins, followed by inhibition of the cell metabolism and eventually causing lysis (McDonnell & Russell, 1999). To further establish the antibacterial contribution arising from the carrier phase alone, a range of ethanol content (below the previously examined 20%) aqueous solutions were tested, while also investigating another popular carrier phase used in literature to dissolve propolis for antibacterial assays, DMSO (Fig. 4). The lowest percentage of ethanol tested (5%) achieved two logs of *E. coli* CFU /ml reduction, showing the sensitivity of this method to antibacterial agents. After 24 h of incubation, 100% DMSO completely eliminated the population and 20% DMSO achieved approximately one log CFU /ml reduction. DMSO has the ability to cross cell membranes ((Brito et al., 2017)), causing morphological changes in the cytoplasm (Ansel, Norred & Roth, 1969). However it should be noted that DMSO has been reported to protect *E. coli* cells by scavenging ROS (reactive oxygen species) caused by antibacterial agents ampicillin, kanamycin, oxolinic acid and ciprofloxacin (Mi et al., 2016).

Although both carrier phases are widely used in literature for dissolving propolis extracts for MIC assays (Uzel et al., 2005; Mohammadzadeh et al., 2007; Silva, Rodrigues, Feás & Estevinho, 2012; Freitas, Cunha, Cardoso, Oliveira & Almeida-Aguiar, 2019), they are usually added in overall more dilute concentrations (Moncla et al., 2012; Regueira et al., 2017; Belwal et al., 2018), to cells suspended in broth (Morais, Moreira, Feás & Estevinho, 2011; Ristivojević et al., 2018;

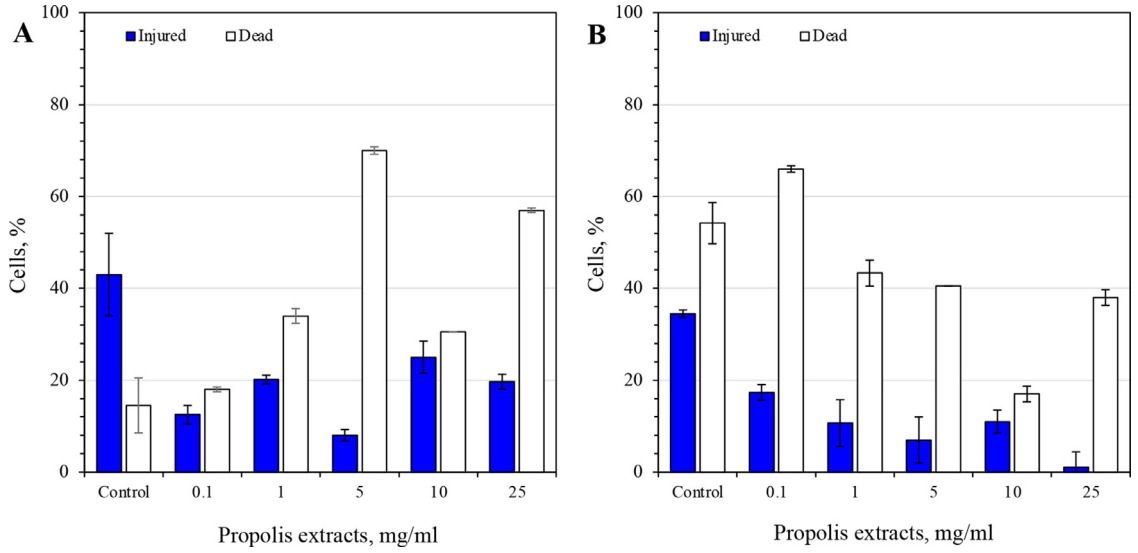

Fig. 3. Effect of membrane integrity of *E. coli* (MG1655) cells A) after immediate exposure and B) after 24 h with propolis extracts diluted in ethanol 20%. Data for percentages of injury and death are presented as a function of propolis extracts concentration (0.1, 1, 5, 10, and 25 mg/ ml). Data shown are averages (n = 3; biological replicates) and errors represent ± one standard deviation (s.d.).

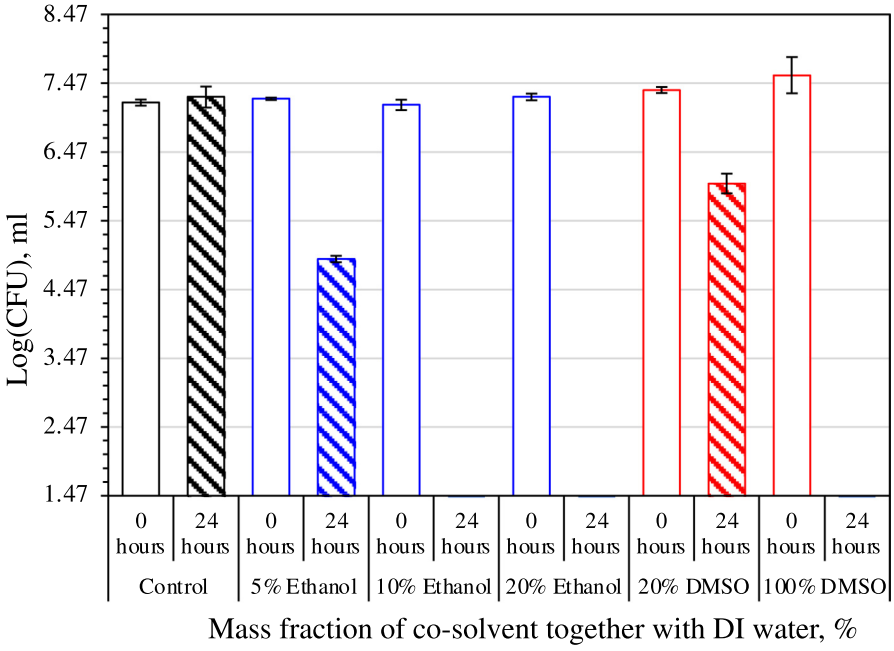

Fig. 4. Antibacterial activity of different fractions of ethanol and DMSO mixed with deionised water. Data for population of stationary phase *E. coli* MG1655 are presented as a function of different percentages of ethanol and DMSO in water after immediate exposure and 24 h. The control is deionised water. Data shown are averages (n = 3; biological replicates) and errors represent ± one standard deviation (s.d.).

Freitas et al., 2019) which provides nutrients for the bacteria population to grow (Silva et al., 2012; Al-Waili, 2018; Ristivojević et al., 2018) rather than at stationary phase in PBS. Furthermore, during a 37 °C incubation, part of the ethanol (particularly at such low concentrations) can evaporate, minimising/masking its antibacterial contribution. In the current method used, the actives to be tested are added in higher quantities (1:1) making their effect more prominent even at low ethanol concentrations (i.e. 5%). MIC assays show the minimum concentration needed to inhibit the growth of a microorganism (Wiegand, Hilpert & Hancock, 2008), without being able to distinguish between static and cidal status. The current method was chosen as there was greater interest in the rate of killing and how that can be influenced under different formulation conditions while understanding the effects on the cell membranes of fully grown stationary cells. In addition to choosing a carrier phase that is relevant to industrial applications, the results presented here clearly demonstrate the importance of such selection in terms of anticipated antibacterial performance by the bioactive of interest, to disentangle any effects arising from the bioactive alone, thus reducing environmental interferences/contributions.

The effect of sonication on the formation of propolis extract dispersions within an aqueous carrier phase

In order to create formulations with less toxic, environmental friendly and more industrially applicable solvents, as well as to study the propolis' antibacterial performance without external interferences, water was selected as the carrier phase for all further investigation. As propolis is insoluble in water, the hypothesis that its bioavailability and activity would be enhanced by formulating it as a colloidal aqueous dispersion, was tested.

Initially, aqueous dispersions of 1.88% of propolis extracts were formed in the presence of 1% Tween 80. The effect of the duration of the

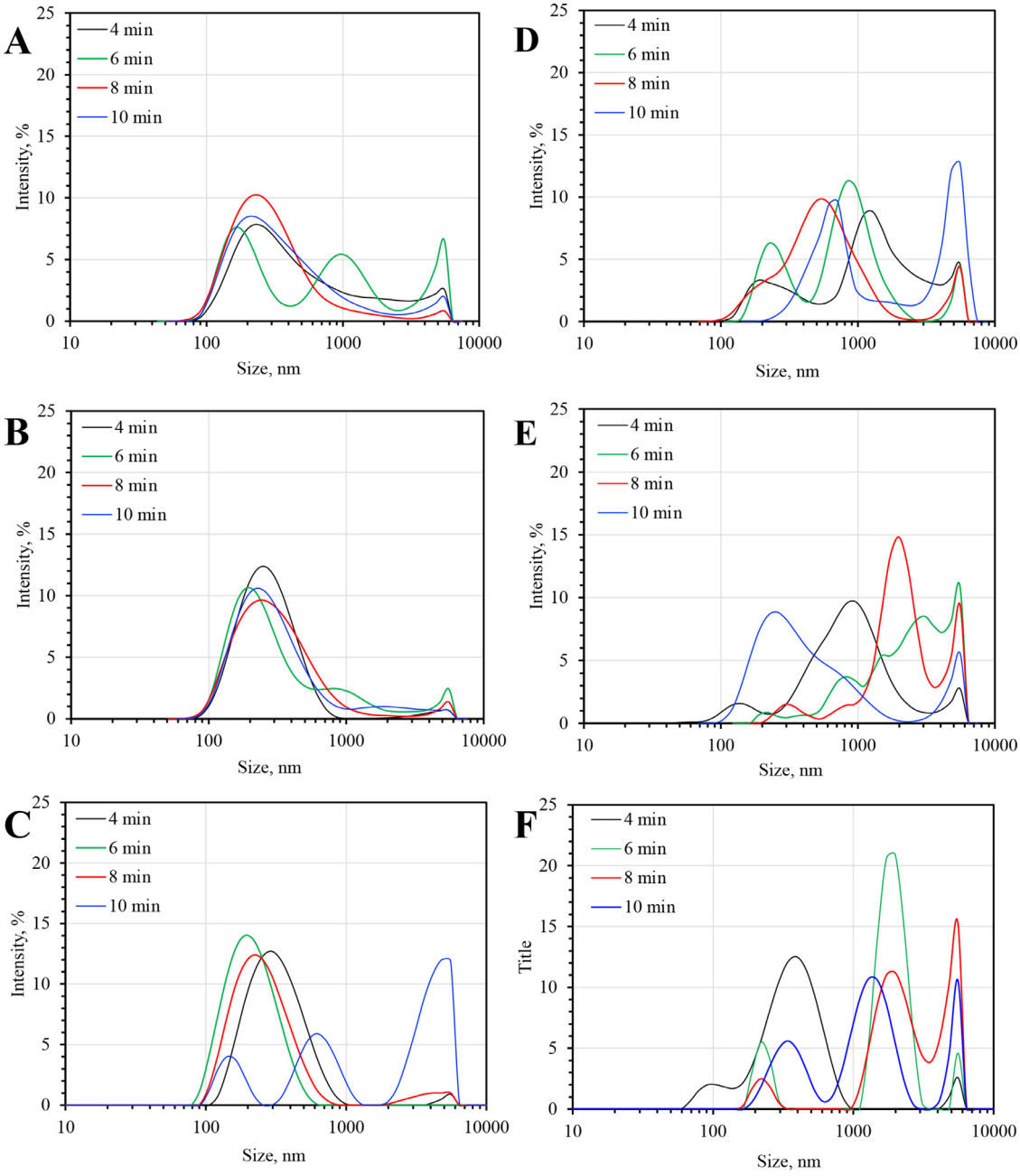

Fig. 5. Average particle size distributions of propolis particles dispersed in aqueous media. Data for dispersions of 7% (A, B and C) and 15% (D, E and F) (concentrated) propolis mass fractions are presented for different sonication times (4, 6, 8 and 10 min) and storage periods; at 0 (A and D), 7 (B and E) and 30 (C and F) days, at 25 °C in the dark. Data shown are averages (n = 3; repeats), except results shown for 30 days which represent averages n = 3; replicates due to deterioration of samples.

sonication step and the subsequent storage period on the particle size and polydispersity index (PdI) of the formed propolis particles were investigated (Table 1). The average particle size decreases with increasing sonication time, reaching a minimum at 6 min (206 ± 1 nm), with possible aggregation after; this was also reflected in the PdI values, as it can be seen in the supplementary material provided (Fig. S1A). What is more, all particle sizes remain constant with storage time.

Aqueous dispersions containing a higher propolis mass fraction of 7% were also prepared. These were further concentrated post sonication (by evaporating the appropriate amount of water) to form systems with 15% propolis content. The effect of the duration of sonication (> 2 min), water evaporation and period of storage on the formed propolis dispersions was investigated by measuring the average particle size, polydispersity index (PdI) and zeta potential; results are presented in Table 2. The average particle size between 1.88% and 7% propolis mass fraction was not statistically significant, but significantly increased with 15% propolis mass fraction ($p < 0.05$).

One-way ANOVA of the average size measurements of the initial 7% propolis dispersions revealed no statistically significant differences between the sonication times. However, considering the PdI data, there is a significant increase in polydispersity after 6 min of sonication ($p < 0.05$), compared with 4, 8 and 10 min sonication. This can be explained by observing the particle size distributions (Fig. 5A). The 6 min distribution is multimodal, justifying the rise in average size.

Time of sonication, along with other parameters including sonication power or the diameter of the probe can affect the particle size

Table 1
Average size and polydispersity index (PdI) of propolis particles dispersed in aqueous media. Data for aqueous dispersions containing a 1.88% propolis extract mass fraction are presented as a function of sonication time (2, 4, 6, 8 and 10 min) and storage period (7, 14, 21 and 30 days at 25 °C in the dark). Data shown are averages (n = 3; replicates) and errors represent ± one standard deviation (s.d.).

Sonication time (min)	Storage period (days)	Size (nm)	PdI
2	7	492 ± 9	0.63 ± 0.07
	14	565 ± 55	0.54 ± 0.01
	21	606 ± 20	0.60 ± 0.06
	30	727 ± 42	0.54 ± 0.03
4	7	333 ± 1	0.24 ± 0.01
	14	328 ± 6	0.18 ± 0.02
	21	393 ± 8	0.31 ± 0.01
	30	427 ± 17	0.34 ± 0.04
6	7	206 ± 1	0.25 ± 0.01
	14	209 ± 2	0.23 ± 0.01
	21	215 ± 3	0.24 ± 0.01
	30	218 ± 2	0.23 ± 0.01
8	7	239 ± 1	0.36 ± 0.01
	14	248 ± 1	0.22 ± 0.01
	21	251 ± 2	0.23 ± 0.01
	30	256 ± 1	0.24 ± 0.01
10	7	245 ± 7	0.42 ± 0.02
	14	230 ± 3	0.32 ± 0.02
	21	231 ± 2	0.29 ± 0.01
	30	227 ± 4	0.27 ± 0.01

(Smith & Dairiki, 1975). This could lead to a reduction of size and particle agglomeration (Pérez-Maqueda, Duran & Pérez-Rodríguez, 2005), which can be observed particularly in the 6 min distribution. It is challenging to compare these results with literature as sonication concerning propolis or other bioactives, appears to be used mostly as means of extraction (Anandan et al., 2017; Gargouri, Osés, Fernández-Muiño, Sancho & Kechaou, 2019; Pobiega, Kraśniewska, Derewiaka & Gniewosz, 2019a; Yusof, Munaim & Veloo Kutty, 2021) or encapsulation (Kalogeropoulos et al., 2009b; Sharma, Kaur & Khatkar, 2021), rather than creating dispersions. Kalogeropoulos et al. (2009b) formulated propolis extract suspensions in aqueous β- cyclodextrin solutions, but size measurements were not reported in this study.

Concentrating the propolis content in the dispersions *via* water evaporation (from 7% to 15%) reveals a consistent and statistically significant increase ($p < 0.05$ for all sonication times). Particles move in a Brownian motion and tend to collide and flocculate (Singer, Barakat, Mohapatra & Mohapatra, 2019), which can be further enhanced at smaller sizes due to the higher surface area created (Usune et al., 2019). Although solvent evaporation is used as means to concentrate formulations (Avgoustakis, 2004; Vauthier, Cabane & Labarre, 2008), aggregation can be a result (Vauthier et al., 2008) due to an increased likelihood of particles colliding. Therefore, the significant rise in particle size (Table 2) is expected and the same phenomena can equally explain the observed increase in polydispersity; also seen in Fig. 5.

Comparing the particle sizes between 0, 7 and 30 days, in some cases a significant increase is observed for both 7% and 15% propolis mass fractions. However, the standard deviations between measurements are too large indicating deterioration of samples.

Zeta potential was also measured as it is a good indicator of dispersion stability. Zeta potential values were all highly negative, in agreement with other propolis formulated systems (Kazemi, Divsalar & Saboury, 2018; Hegazi, Elhoussiny & Fouad, 2019), and remained mostly stable over 30 days of storage, with sample deterioration being accounted for some variability. In the 7% propolis fraction systems, the zeta potential was not statistically significant between 8 and 10 min, due to the possibility of the sonication time not causing any effect after that. In the 15% propolis fraction systems (post evaporation) there was no statistically significant difference between 6 and 10 min, although the 8 min sonication was close. This could be occurring due to flocculation of the particles.

2-way ANOVA showed a statistically significant difference of zeta potential values between 7% and 15% propolis fractions ($p < 0.05$), with increasing zeta potential (towards less negative values) as the fraction of propolis was raised from 7% to 15%. This could be a result of particle agglomeration (post water evaporation; see Table 2); the subsequent reduction to the particles' surface area would be associated with a decrease in the 'overall' repulsion. This increase in zeta potential with increasing propolis amount is a subject of some disagreement in literature. Ramli, Ali, Hamzah & Yatim (2021) studied the zeta potential with an increasing propolis (encapsulated in liposomes) concentration, reporting the same trend as here, although the authors claim this was due to interactions between the phospholipids and propolis. However, other studies (Elbaz et al., 2016; Soleimanifard, Feizy & Maestrelli, 2021) have reported the opposite effect.

The effect of sonication on the content of 'relevant' chemical species in propolis extract dispersions within an aqueous carrier phase

Although the duration of sonication did not have a significant effect on the average particle size, further investigation was performed on its effect on the chemical composition of the formed propolis dispersions. The dispersions were characterised in terms of propolis extracts' functional compounds; total phenol content (reducing capacity), which can indicate if there are phenolic compounds present that could contribute to propolis' antibacterial activity (Fig. 6A) and free radical scavenging

Table 2
Average size, polydispersity index (PdI), and zeta potential of propolis particles dispersed in aqueous media. Data for aqueous dispersions containing a 7% and 15% (concentrated) propolis mass fraction are presented as a function of sonication time (4, 6, 8 and 10 min) and storage period (0, 7 and 30 days at 25 °C in the dark). Data shown are averages (n = 3; repeats) and errors represent ± one standard deviation (s.d.).

Sonication time (min)	Storage Period (days)	7% propolis dispersions			15% propolis dispersions		
		Size (nm)	PdI	Zeta Potential (mV)	Size (nm)	PdI	Zeta Potential (mV)
4	0	357 ± 51	0.51 ± 0.07	−26.85 ± 2.62	928 ± 162	0.71 ± 0.01	−20.27 ± 2.93
	7	250 ± 26	0.19 ± 0.03	−22.0 ± 1.13	456 ± 68	0.41 ± 0.03	−28.76 ± 7.44
	30	2799 ± 2494	0.30 ± 0.06	−24.99 ± 4.96	462 ± 3	0.39 ± 0.05	−21.76 ± 2.89
6	0	466 ± 84	0.78 ± 0.08	−41.24 ± 4.22	1031 ± 187	0.86 ± 0.12	−30.12 ± 2.32
	7	321 ± 48	0.37 ± 0.08	−38.08 ± 4.13	3531 ± 312	0.40 ± 0.09	−29.11 ± 3.46
	30	350 ± 156	0.38 ± 0.23	−38.84 ± 1.13	3134 ± 102	0.49 ± 0.05	−31.07 ± 1.07
8	0	330 ± 47	0.39 ± 0.03	−36.59 ± 3.29	694 ± 76	0.63 ± 0.11	−33.09 ± 2.69
	7	289 ± 24	0.36 ± 0.05	−36.11 ± 1.35	2810 ± 177	0.47 ± 0.05	−30.28 ± 1.49
	30	368 ± 131	0.42 ± 0.21	−38.11 ± 0.69	2965 ± 43	0.62 ± 0.13	−30.26 ± 4.44
10	0	347 ± 24	0.48 ± 0.07	−35.80 ± 3.32	666 ± 87	0.72 ± 0.12	−26.84 ± 3.51
	7	249 ± 19	0.27 ± 0.03	−37.87 ± 2.20	455 ± 50	0.61 ± 0.05	−32.35 ± 1.76
	30	3224 ± 2279	0.60 ± 0.23	−29.22 ± 7.19	1116 ± 745	0.62 ± 0.21	−22.45 ± 6.77

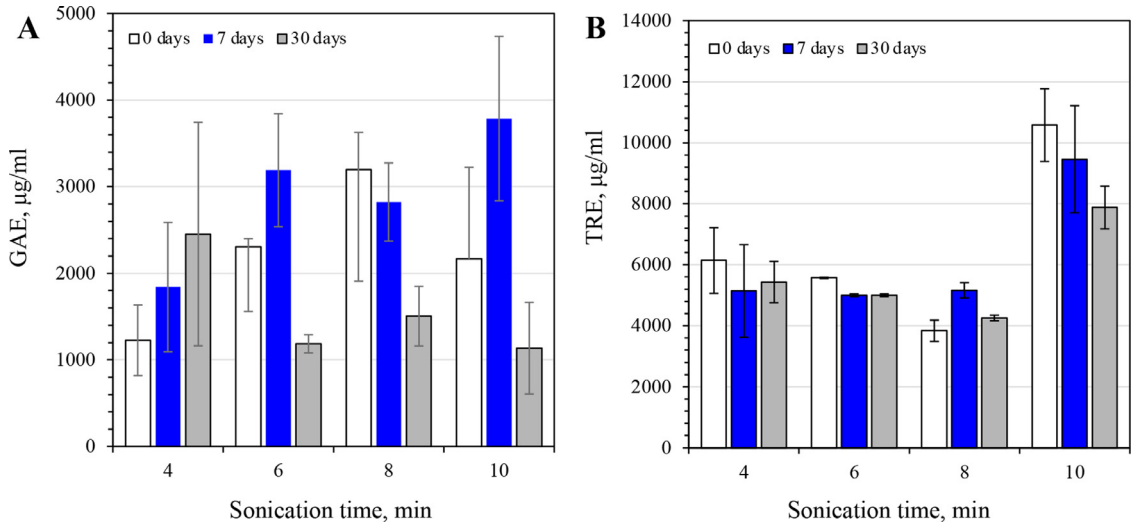

Fig. 6. (A) Total phenol content and (B) Free radical scavenging activity of propolis particles dispersed in aqueous media. Data for 15% (concentrated) propolis mass fractions are presented as a function of sonication time (4, 6, 8 and 10 min) and storage period (0, 7 and 30 days at 25 °C in the dark).

activity, which is associated with propolis' antioxidant activity (Fig. 6B) (Bittencourt et al., 2015).

The data suggest that there were no statistically significant differences between different sonication times and the amount of phenolic compounds present. However, there is an increasing trend in the total phenol content of dispersions and sonication time from 4 min to 8 min (from ~1226 to ~3199 μg/ml GAE) or above (Fig. 6A). This could be due to the increased forced dissolution of phenolic compounds in the aqueous carrier phase.

The total phenol content values although in some cases comparable (Bittencourt et al., 2015), (Christina et al., 2018), were generally smaller than reported literature values (Gargouri et al., 2019; Pratami, Sahlan, Mun'im & Sundowo, 2018; Sulaiman et al., 2011), probably due to the fact that less quantity was used in the formulation from the total amount extracted or less was available for testing since the highest abundance when sampling, was not in solution. Other systems have shown that especially phenolic compounds are bound to the biological cell walls and sonication has assisted their release into solution resulting in the detection of larger phenolic amounts. (Cheng, Soh, Liew & Teh, 2007; Abid et al., 2014) However, in terms of propolis, the variability of the results is very frequent making it challenging to compare between different samples, as the amount detected is not only related with the local flora or time of collection, but also with the degree of digestion by β-glycosidase from bees' saliva, and the percent of beeswax mixed with propolis. (Ristivojević et al., 2018)

The free radical scavenging activity method can provide an indication of the antioxidant capacity using the stable free DPPH radical by the ability of an active to donate a hydrogen atom to the nitrogen radical. In terms of free radical scavenging activity, there was a statistically significant difference ($p < 0.05$) between the 10 min sonicated dispersions and all the others tested (Fig. 6B), indicating that the time of sonication influences the antioxidant capacity of the samples. The stability was adequate after 30 days, revealing that the storage period of samples did not affect their antioxidant activity.

Similarly with the total phenol content, the free radical scavenging activity was comparable with the literature (Kalogeropoulos et al., 2009b; Ristivojević et al., 2018), although still lower probably due to less compounds being available in solution or due to the lower amount of propolis being used in the formulation.

To distinguish between chemical compounds associated with the propolis particles and those in the carrier phase, the aqueous phase of the formed dispersions was isolated and examined further for its total phenol content and free radical scavenging activity (Fig. 7).

The total phenol content of the carrier phase (Fig. 7A) showed a similar pattern to that of the full systems (Fig. 6A); i.e. total phenol content increased for systems sonicated for 6 min and over, although once more the trend was not statistically significant. The phenol content detected in the aqueous carrier phase is lower than that in the full systems (i.e., ~2164 and ~850 μg/ml GAE for 10 min of sonication, respectively), an average ratio of 0.34 ± 0.13 across all systems. This is expected as plenty of phenolic compounds, including flavonoids, found in propolis show a higher affinity for the solid phase (Ahn et al., 2007), were probably detected early on without further assistance of ultrasound energy, which could explain the non statistical significance of the results. However, there is evidence of sonication-assisted dissolution of functional compounds to the carrier phase. Campos, Assis and Bernardes-Filho (2020), compared the amount of phenolic compounds extracted in different fractions of water and ethanol, reporting that the percentage concentration reached a maximum when 1:1 ratio of ethanol and water was used, clarifying that some phenolic compounds can be partially water soluble.

When examining the full systems (Fig. 6), there was higher variability of the results than their respective carrier phases. This high variability could be associated with the alteration of the physical properties of the dispersions that were evident from the size measurements of the propolis particles (Table 2), rather than from chemical degradation of functional compounds.

Sonication time although not statistically significant, in terms of free radical scavenging activity of the carrier phase (Fig. 7B), presented a clear trend of increasing antioxidant activity with increasing sonication time (~880 and ~1587 μg/ml TRE after 4 and 10 min of sonication, respectively). The average ratio of compounds exhibiting free radical scavenging activity in the carrier phase over those in the whole dispersion was 0.19 ± 0.06, showing that similarly with the total phenol content, free radical scavenging activity was primarily associated with the solid phase. There has been evidence in the literature that antioxidant activity could be correlated with the total phenol content which also agrees with our results (Ristivojević et al., 2018).

Sonication has shown to encourage dissolution, especially as part of an extraction set-up. Oroian, Ursachi and Dranca (2020) investigated sonication as means of propolis extraction by altering parameters such as, ethanol and water concentration in the extraction media, temperature, amplitude and time, revealing that time did not have a significant effect on propolis extraction. That could be because the minimum amount of time tested (15 min) was already sufficient enough for complete extraction. In this study the maximum amount of sonication investigated was 10 min to avoid aggregation of particles or chemical deteri-

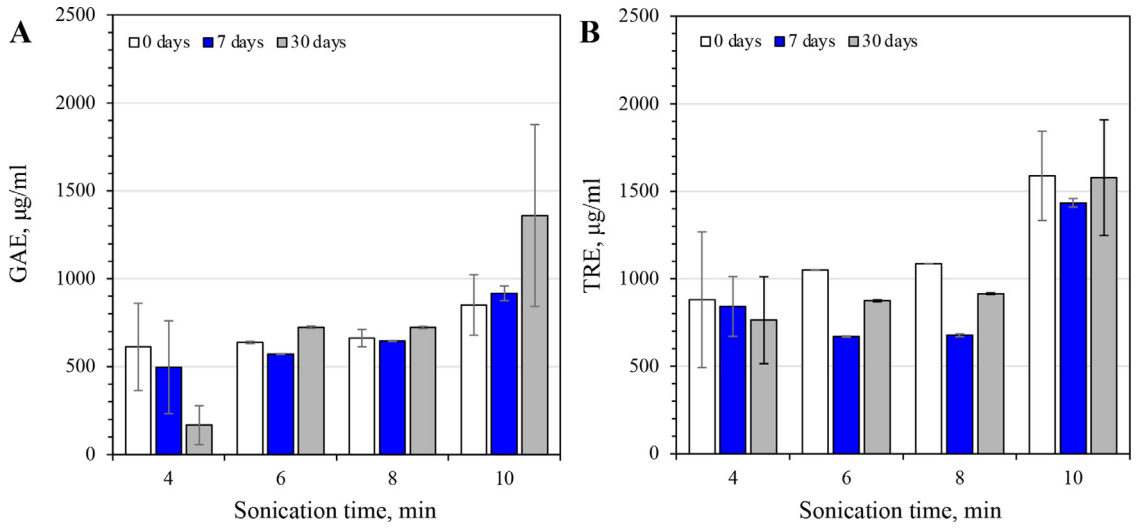

Fig. 7. (A) Total phenol content and (B) Free radical scavenging activity of propolis aqueous carrier phases post isolation from dispersions. Data for propolis carrier phases are presented as a function of sonication time (4, 6, 8 and 10 min) and storage time (0, 7 and 30 days at 25 °C in the dark).

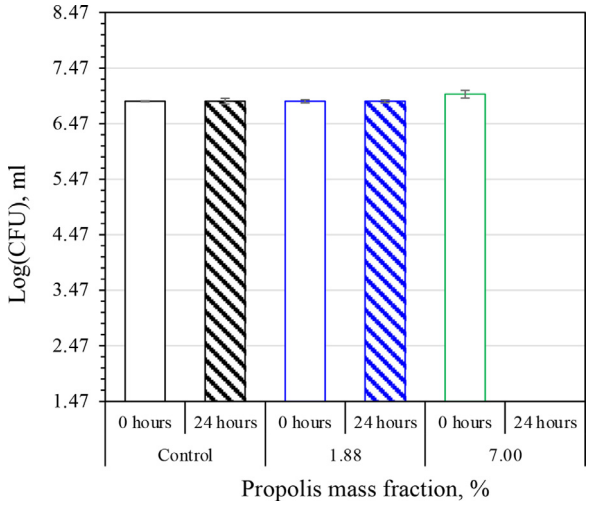

Fig. 8. Antibacterial activity of propolis particles dispersed in aqueous media. Data for population of stationary phase *E. coli* MG1655 are presented as a function of propolis mass fraction (1.88% and 7%) and time (0 and 24 h). The control is deionised water. Data shown are averages (n = 3; biological replicates) and errors represent ± one standard deviation (s.d.).

oration of the sample. In different systems the duration of sonication has played a significant role in the antioxidant capacity and phenolic compounds dissociated in the carrier phase. Bhat and Goh (2017) showed that a sonication time of 30 min increased significantly the total phenol compounds and free radical scavenging activity of strawberry juice. The present work suggests that sonication times between 8 and 10 min are enough to maximise the chemical species in the propolis extract aqueous dispersions, both in terms of total phenol content as well as free radical scavenging activity.

The effect of sonication on the antibacterial performance of propolis extracts within an aqueous carrier phase

The antibacterial response 1.88% and 7% propolis extract aqueous dispersions were tested against *E. coli* cells (Fig. 8; showing the antibacterial response from the 10 min sonicated 1.88% and 7% propolis aqueous dispersion). The 1.88% propolis mass fraction did not reveal any meaningful effect. There have been reports in literature where propolis extracts were not effective against *E. coli* (Eslami, Ariamanesh & Ariamanesh, 2016; Freitas et al., 2019; Grecka et al., 2019). This is typically ascribed to an inadequate (low) propolis concentration or differences in chemical consistency depending on the location and the time of collection (Katekhaye, Fearnley, Fearnley & Paradkar, 2019). The 7% propolis mass fraction eliminated the population after 24 h.

The antibacterial effect (over a 24 h period) of the aqueous formulations with a higher propolis content (15%) was subsequently also determined by exposing *E. coli* cells in their stationary phase to propolis dispersions formed at varying sonication times (Fig. 9A). The non-sonicated sample exhibited a similar behaviour with the control, highlighting the major enhancement of antibacterial activity of the samples post sonication. The 10 min sonicated dispersions acted rapidly, eliminating the bacterial population within one hour. Both the 6 min and 8 min sonicated systems responded similarly, eliminating *E. coli* population after 2 h of exposure, while finally the 4 min sonicated dispersions managed the same after 8 h of exposure

In addition, the data were fitted to a Kaplan-Meier survival plot model to estimate the percentage of survival of *E. coli* cells post exposure at each time point within the 24 h testing period (Fig. 9B). Unlike the particle size or chemical characterisation data, 2-way ANOVA revealed a statistically significant ($p < 0.05$) effect of sonication time on the antibacterial performance of the propolis extract aqueous dispersions.

There was no statistical difference between fresh samples and those stored for 30 days, with both data sets showing a practically equivalent antibacterial response. As such, this is hypothesised to predominantly arise by the preservation of compounds possessing antibacterial activity in the propolis aqueous systems and not to be affected by the deterioration of their physical characteristics (reported earlier in terms of particle size).

Propolis particles have previously shown to exhibit antibacterial activity. Dobrowolski et al. (1991) suspended propolis granules and tablets in water with 1% acacia and tested them against *E. coli* exhibiting an inhibition zone of 13.8–15 cm after 30 min at a concentration of 10 mg/ml. Similarly, Abdullah et al. (2019) suspended 20 mg/ml propolis particles in water which also exhibited antibacterial activity against *E. coli, B. subtilis, S aureus and P. aeruginosa* after 24 h, even though the concentrations used in this case were greater in comparison. Pobiega et al. (2019b) investigated the antibacterial effect of propolis extracts, extracted via different sonication times in 70% ethanol in water, showing that higher sonication times were related with stronger inhibition effects of the extracts against the organisms tested, agreeing with our results.

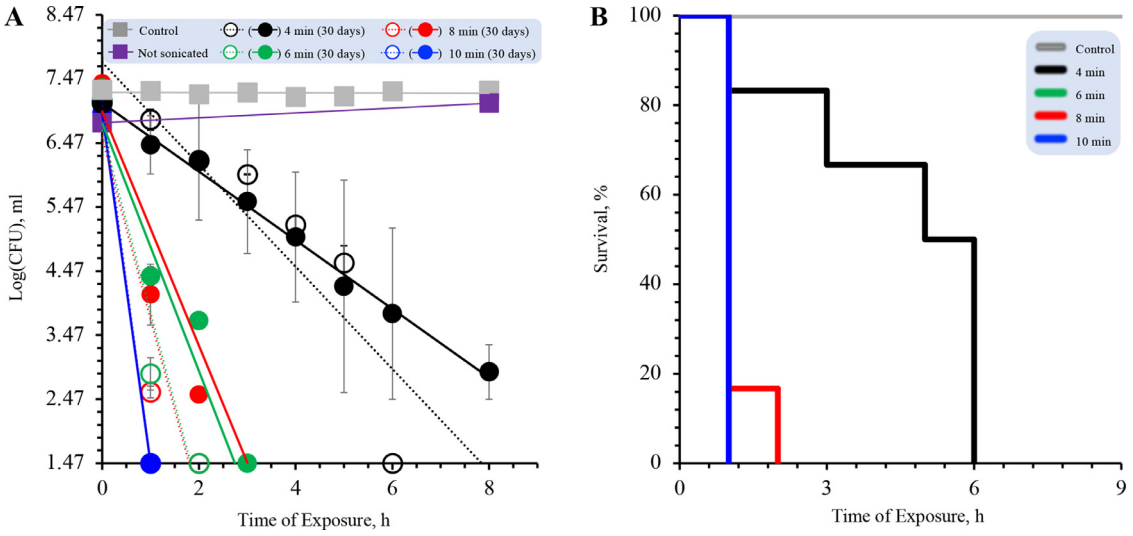

Fig. 9. A. Antibacterial activity of propolis particles dispersed in aqueous media. Data for the population of stationary phase *E. coli* MG1655 are presented as a function of 15% (concentrated) propolis mass fractions' exposure time, sonication time (4, 6, 8 and 10 min and not sonicated) and storage time (0 and 30 days at 25 °C in the dark). The control is deionised water. Data shown are averages (n = 3; repeats) and errors represent ± one standard deviation (s.d). B. Kaplan-Meier survival plot of stationary phase *E. coli* MG1655 cells are presented as a function of 15% (concentrated) propolis mass fractions' exposure time and sonication time (4, 6, 8 and 10 min).

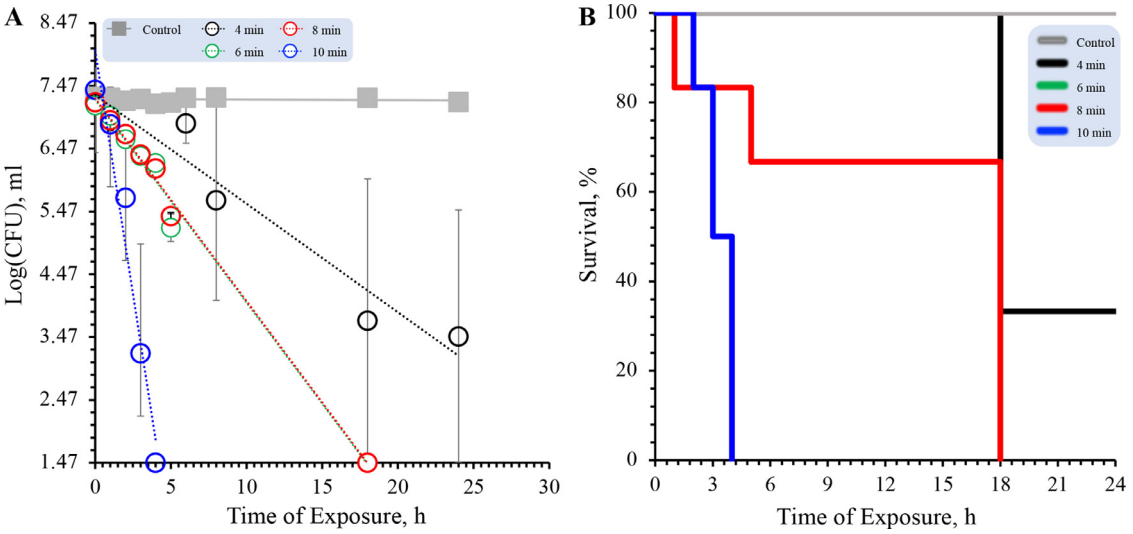

Fig. 10. A. Antibacterial activity of aqueous carrier phases post isolation from dispersions. Data for the population of stationary phase *E. coli* MG1655 are presented as a function of 15% (concentrated) propolis carrier phases' exposure time, sonication time (4, 6, 8 and 10 min) and storage time (0 days at 25 °C in the dark). The control is deionised water. Data shown are averages (n = 3; repeats) and errors represent ± one standard deviation (s.d). B. Kaplan-Meier survival plot of stationary phase *E. coli* MG1655 cells are presented as a function of 15% (concentrated) propolis carrier phases' exposure time and sonication time (4, 6, 8 and 10 min).

The antibacterial activity of the dispersions' carrier phases alone was also tested (Fig. 10). However, the resulting antibacterial response was weaker compared to the full systems, but a similar pattern of action was revealed (Fig. 10A); the obtained data were also fitted to the Kaplan-Meier survival plot model (Fig. 10B). 2-way ANOVA showed once more that the differences in the antibacterial activities of the carrier phases of propolis dispersions sonicated for varying times were statistically significant ($p < 0.05$). The carrier phases of propolis dispersions that had undergone 10 min sonication had the highest bactericidal effect, followed by those processed for 8 and 6 min (which had a similar effect), and lastly by 4 min where the effect was bacteriostatic.

Both whole systems and carrier phases showed statistically significant difference ($p < 0.05$) in antibacterial activity with increasing sonication time. It is hypothesized that this enhancement is probably not anticipated from the solid phase but rather from an increased forced dissolution of functional compounds to the aqueous phase. In terms of the resulting antibacterial activity, both the colloidal solid propolis component and the aqueous carrier phase contribute, exhibiting a synergistic antibacterial action. Propolis particles need foremostly to come in contact with the cells in order to act (Ge et al., 2014), whereas functional compounds in the carrier phase are more available to exhibit their antibacterial performance.

Effect of concentration of propolis extracts within an aqueous carrier phase on the membrane integrity of E. coli *cells*

Propolis dispersions were sonicated for 4 and 10 min and further diluted in water to different propolis mass fractions; these were taken to represent the two extremes in terms of processing intensity, and were tested against *E. coli* cells in their stationary phase (Fig. 11). The 4 min

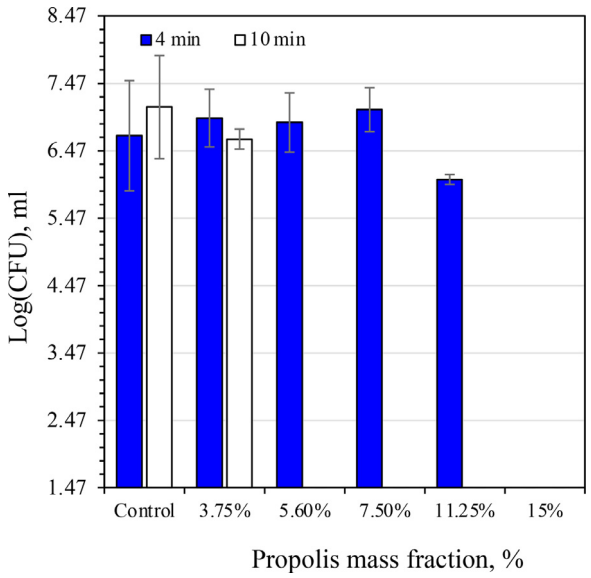

Fig. 11. Antibacterial activity of propolis particles dispersed in aqueous media. Data for population of stationary phase *E. coli* MG1655 are presented as a function of 15% propolis mass fraction sonicated for 4 and 10 min and further diluted to 11.25%, 7.5%, 5.60% and 3.75% after 24 h of exposure. The control is deionised water. Data shown are averages (n = 3; biological replicates) and errors represent ± one standard deviation (s.d.).

sonicated dispersions eliminated the bacteria population at the highest propolis mass fraction of 15%, and caused approximately one log CFU reduction at 11.25%. The 10 min sonicated dispersions eliminated the E. coli population at most propolis mass fractions apart from the lowest (3.75%) tested. This additionally confirms that the 10 min sonicated dispersions had a stronger antibacterial activity than the 4 min sonicated dispersions. In order to further understand the difference in the biological activity of the samples, flow cytometry was utilised to assess the level of injury and death of the cells, at immediate contact with the dispersions and at the same propolis mass fractions.

According to the flow cytometry data (Fig. 12), the propolis extract aqueous dispersions that had undergone 4 min sonication predominantly caused injury (rather than death) to the *E. coli* cells. In this case there is a gradual (almost linear) rise to the injured cell population that follows the increase in the propolis extract mass fraction in the systems (Fig. 12A). Cell death was both minimal and relatively independent of the propolis extract content (Fig. 12B). However, the mode by which the propolis extract aqueous dispersions subjected to 10 min impacted cell integrity was much more severe. Here, although at the lowest propolis content, injury and death is brought upon more or less equally across the cell population (Fig. 12A), further increases to the propolis mass fraction resulted in an almost linear enhancement to cell fatality. What is more, for the higher propolis fractions tested, cell injury upon immediate exposure is significantly reduced while cell mortality dominates (Fig. 12B). The fact that even the 15% propolis extract aqueous dispersions sonicated for 4 min did not significantly affect cell membrane permeability, provides further evidence that the propolis particles themselves are not the sole culprits in terms of antibacterial activity and that chemical species present in their aqueous carrier phases (dissolution of which is much more pronounced at higher sonication times) also play an important role. To the best of the authors' knowledge, this is the first time flow cytometry has been utilised to study the physiology of *E. coli* cells after exposure to propolis extracts either dissolved in ethanol (Fig. 3) or formulated in an aqueous dispersion (Fig. 12). Flow cytometry associated with propolis is commonly used to examine eukaryotic cells. (Chiao et al., 1995; Park et al., 2004; Nor et al., 2021)

AFM imaging and topography were employed to detect the *E. coli* cell membrane disruption caused by the 15% propolis extract aqueous dispersions sonicated for 10 min (Fig. 13). Cells A and B are examples of untreated bacteria with dimensions of around 2 μm length and approximately 1 μm height; these are similar to the previously reported dimensions for the same *E. coli* strain using AFM (Masoura, Passaretti, Overton, Lund & Gkatzionis, 2020). Following 1 h of exposure to the propolis extract aqueous dispersion there was evidence of membrane alteration, with a shift in cell dimensions to around 3 μm in height and approximately 150 nm in width; evidence of membrane disruption can also be seen. This results in a higher cell volume and therefore indicating swelling possibly due to water absorbance by the compromised membrane. This way of propolis antibacterial action has been observed in literature with different strains. Kim and Chung (2011) and Campos et al. (2020) imaged *E. coli* and *S. aureus* cells after being treated with propolis extracts diluted in different concentrations of ethanol in water. The cells had ruptures in the cell membrane which caused leakage of intracellular contents along with swelling due to possible water absorption, agreeing with our results. The concentration of propolis extracts used at 137.5 mg/ml was higher compared to ours. Similarly, when *S. aureus* was exposed to magnetite propolis nanoparticles

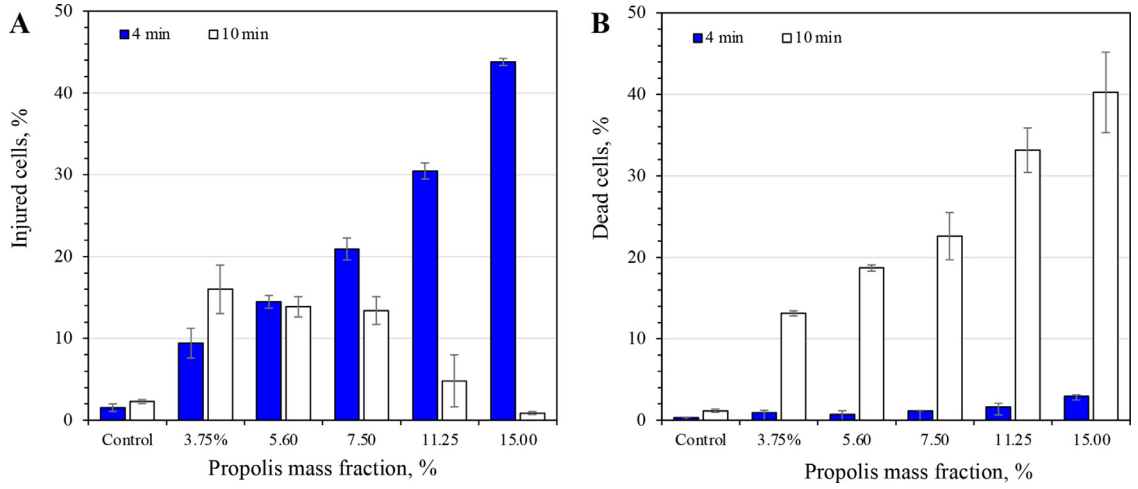

Fig. 12. Effect of membrane integrity of *E. coli* MG1655 cells after immediate exposure with propolis particles dispersed in aqueous media. Data for percentages of (A) injury and (B) death are presented as a function of propolis mass fraction added (15%, 11.25%, 7.5%, 5,6% and 3.75%) and sonication time (4 and 10 min). Data shown are averages (n = 3; biological replicates) and errors represent ± one standard deviation (s.d.).

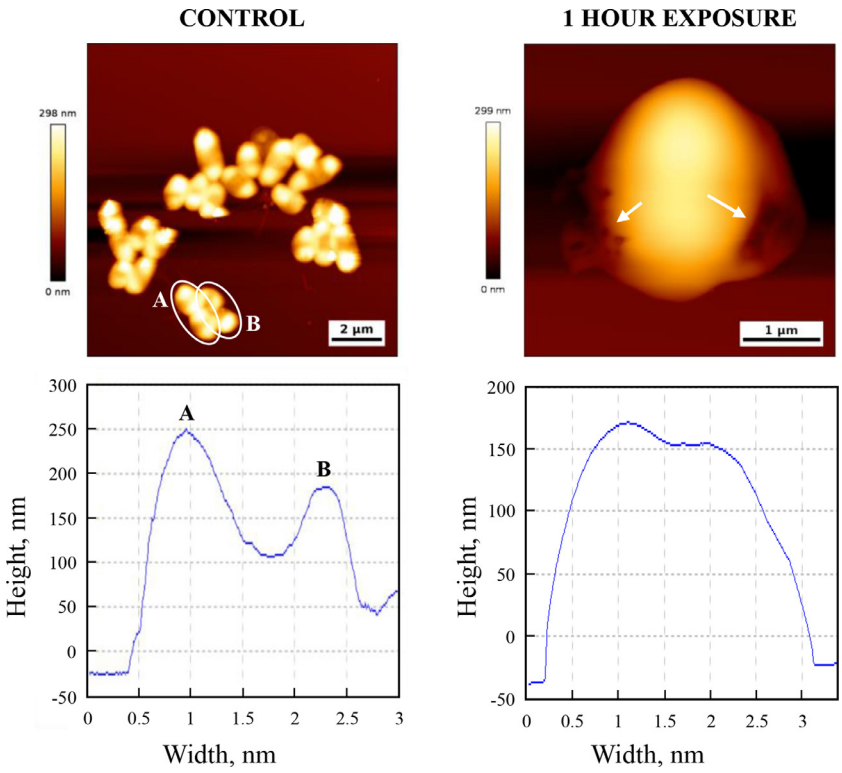

Fig. 13. Topography of *E. coli* MG1655 cells. Data for control (deionised water) and treated (15% propolis mass fraction sonicated for 10 min) cells are presented after 1 h exposure. Cells within the white circles were used for measurement while the white arrows indicate potential membrane damage.

in Brain Heart Infusion broth, disruptions on the cell wall were observed, at a lower concentration of propolis extracts compared to ours (El-Guendouz et al., 2019). To the best of our knowledge, is the first time cell swelling has been observed post propolis particles exposure, in an aqueous carrier phase. Therefore, there are similar mechanisms of action for propolis extracts both in a solid form (particles) and diluted in a solvent. The antibacterial performance of propolis has been mainly ascribed to phenolic and flavonoid compounds. Molecules such as galangin, pinocembrin and chrysin, often found in propolis extracts, have been shown to act synergistically and compromise the outer membrane of cells (Boisard et al., 2015). Terpenoids, species that are more specific to Greek propolis samples, have also been associated with targeting the cell membrane (Melliou & Chinou, 2004; Popova, Chinou, Marekov & Bankova, 2009; Guimarães et al., 2019).

Conclusions

In conclusion, the use of sonication to formulate aqueous dispersions of propolis extracts has shown promise. DMSO and ethanol were inadequate carrier solvents for investigation of antibacterial performance *via* the antibacterial assay used. Water based propolis formulations sustained propolis' antibacterial and antioxidant activity, diminishing any underlying effects. This study has demonstrated that utilising direct ultrasonication to formulate propolis aqueous dispersions, enables taking advantage of the ability to reduce the particle size and force dissociation of compounds in the aqueous carrier phase, resulting an enhanced bioactivity of propolis in an aqueous environment. Dispersions exhibited adequate physical and chemical stability and excellent antibacterial activity over the course of a month. Concentrating the dispersions resulted in agglomeration of particles increasing the polydispersity. Assays gave an indication of the activity and antioxidant capacity of the formulations exhibiting similar results among different sonication times, with a trend of increasing total phenol content and free radical scavenging activity with increasing sonication time. Investigating the dissociation ability of direct ultrasonication *via* separating the aqueous carrier phase showed a high affinity of phenolic compounds and antioxidant capacity to the solid phase. Exploration into the antibacterial activity of the two phases showed synergism, revealing a stronger effect than when the isolated carrier phase was tested separately. The bactericidal effect was sonication and exposure time dependant. Combination of flow cytometry and AFM gave an insight into the formulations dose dependant antibacterial activity and its mechanism of action targeting the cell membrane.

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.

Acknowledgments

This research has been funded by BBSRC, Midlands Integrative Biosciences Training Partnership (MIBTP) Doctoral Training Partnership (grant number BB/M01116X/1).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fhfh.2021.100040.

References

Abdullah, N. A., Ja'afar, F., Yasin, H. M., Taha, H., Petalcorin, M. I. R., Mamit, M. H., et al. (2019). Physicochemical analyses, antioxidant, antibacterial, and toxicity of propolis particles produced by stingless bee Heterotrigona itama found in Brunei Darussalam. *Heliyon, 5*(9), e02476 e02476.

Abid, M., Jabbar, S., Wu, T., Hashim, M. M., Hu, B., Lei, S., et al. (2014). Sonication enhances polyphenolic compounds, sugars, carotenoids and mineral elements of apple juice. *Ultrasonics Sonochemistry, 21*(1), 93–97.

Ahn, M. R., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., et al. (2007). Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chemistry, 101*(4), 1383–1392.

Akca, A. E., Akca, G., Topçu, F. T., Macit, E., Pikdöken, L., & Özgen, I. Ş. (2016). The comparative evaluation of the antimicrobial effect of propolis with chlorhexidine against oral pathogens: An *in vitro* study. *BioMed Research International, 2016*, Article 3627463 3627463.

Al-Waili, N. (2018). Mixing two different propolis samples potentiates their antimicrobial activity and wound healing property: A novel approach in wound healing and infection. *Veterinary World, 11*(8), 1188–1195.

Amoros, M., Simõs, C. M. O., Girre, L., Sauvager, F., & Cormier, M. (1992). Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis. *Journal of Natural Products, 55*(12), 1732–1740.

Anandan, S., Keerthiga, M., Vijaya, S., Asiri, A. M., Bogush, V., & Krasulyaa, O. (2017). Physicochemical characterization of black seed oil-milk emulsions through ultrasonication. *Ultrasonics Sonochemistry, 38*, 766–771.

Ansel, H. C., Norred, W. P., & Roth, I. L. (1969). Antimicrobial activity of dimethyl sulfoxide against Escherichia coli, Pseudomonas aeruginosa, and Bacillus megaterium. *Journal of Pharmaceutical Sciences, 58*(7), 836–839.

Asioli, D., Aschemann-Witzel, J., Caputo, V., Vecchio, R., Annunziata, A., Næs, T., et al. (2017). Making sense of the "clean label" trends: A review of consumer food choice behavior and discussion of industry implications. *Food Research International, 99*, 58–71.

Avgoustakis, K. (2004). Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: Preparation, properties and possible applications in drug delivery. *Current Drug Delivery, 1*(4), 321–333.

Bankova, V. (2005a). Chemical diversity of propolis and the problem of standardization. *Journal of Ethnopharmacology, 100*(1), 114–117.

Bankova, V. (2005b). Recent trends and important developments in propolis research. *Evidence-based Complementary and Alternative Medicine: ECAM, 2*(1), 29–32.

Barros, K. B. N. T., Neto, E. M. R., & Fonteles, M. M. F. (2019). Propolis and its cosmetic applications: A technological prospection. *Journal of Young Pharmacists, 11*(4), 350–352.

Basniwal, R. K., Khosla, R., & Jain, N. (2014). Improving the anticancer activity of curcumin using nanocurcumin dispersion in water. *Nutrition and Cancer, 66*(6), 1015–1022.

Belwal, T., Ezzat, S. M., Rastrelli, L., Bhatt, I. D., Daglia, M., Baldi, A., et al. (2018). A critical analysis of extraction techniques used for botanicals: Trends, priorities, industrial uses and optimization strategies. *TrAC Trends in Analytical Chemistry, 100*, 82–102.

Berney, M., Weilenmann, H. U., & Egli, T. (2006). Flow-cytometric study of vital cellular functions in Escherichia coli during solar disinfection (SODIS). *Microbiology, 152*(6), 1719–1729.

Bhat, R., & Goh, K. M. (2017). Sonication treatment convalesce the overall quality of hand-pressed strawberry juice. *Food Chemistry, 215*, 470–476.

Bittencourt, M. L. F., Ribeiro, P. R., Franco, R. L. P., Hilhorst, H. W. M., de Castro, R. D., & Fernandez, L. G. (2015). Metabolite profiling, antioxidant and antibacterial activities of Brazilian propolis: Use of correlation and multivariate analyses to identify potential bioactive compounds. *Food Research International, 76*, 449–457.

Boisard, S., Le Ray, A. M., Landreau, A., Kempf, M., Cassisa, V., Flurin, C., et al. (2015). Antifungal and antibacterial metabolites from a french poplar type propolis. *Evidence-Based Complementary and Alternative Medicine, 2015*, Article 319240.

Boudreau, M., & Beland, F. (2006). An evaluation of the biological and toxicological properties of aloe barbadensis (Miller), aloe vera. *Journal of Environmental Science and Health. Part C, Environmental Carcinogenesis & Ecotoxicology Reviews, 24*, 103–154.

Brito, R., Silva, G., Farias, T., Ferreira, P., & Ferreira, S. (2017). Standardization of the safety level of the use of DMSO in viability assays in bacterial cells.

Burdock, G. A. (1998). Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology, 36*(4), 347–363.

Campos, J. V. D., Assis, O. B. G., & Bernardes-Filho, R. (2020). Atomic force microscopy evidences of bacterial cell damage caused by propolis extracts on E. coli and S. aureus. *Food Science and Technology, 40*, 55–61.

Cao, H., Wei, D., Yang, Y., Shang, Y., Li, G., Zhou, Y., et al. (2017). Systems-level understanding of ethanol-induced stresses and adaptation in E. coli. *Scientific Reports, 7*(1), 44150.

Castaldo, S., & Capasso, F. (2002). Propolis, an old remedy used in modern medicine. *Fitoterapia, 73*, S1–S6.

Chen, Y. W., Ye, S. R., Ting, C., & Yu, Y. H. (2018). Antibacterial activity of propolins from Taiwanese green propolis. *Journal of Food and Drug Analysis, 26*(2), 761–768.

Cheng, L. H., Soh, C. Y., Liew, S. C., & Teh, F. F. (2007). Effects of sonication and carbonation on guava juice quality. *Food Chemistry, 104*(4), 1396–1401.

Chiao, C., Carothers, A. M., Grunberger, D., Solomon, G., Preston, G. A., & Barrett, J. C. (1995). Apoptosis and altered redox state induced by caffeic acid phenethyl ester (CAPE) in transformed rat fibroblast cells. *Cancer Research, 55*(16), 3576.

Christina, D., Hermansyah, H., Wijanarko, A., Rohmatin, E., Sahlan, M., Pratami, D. K., et al. (2018). Selection of propolis Tetragonula sp. extract solvent with flavonoids and polyphenols concentration and antioxidant activity parameters. *AIP Conference Proceedings, 1933*(1), Article 030020.

Cortesi, R., Cappellozza, E., Drechsler, M., Contado, C., Baldisserotto, A., Mariani, P., et al. (2017). Monoolein aqueous dispersions as a delivery system for quercetin. *Biomedical Microdevices, 19*(2), 41.

Dadalioğlu, I., & Evrendilek, G. A. (2004). Chemical compositions and antibacterial effects of essential oils of turkish oregano (Origanum minutiflorum), bay laurel (Laurus nobilis), Spanish lavender (Lavandula stoechas L.), and fennel (Foeniculum vulgare) on common foodborne pathogens. *Journal of Agricultural and Food Chemistry, 52*(26), 8255–8260.

Dickson-Spillmann, M., Siegrist, M., & Keller, C. (2011). Attitudes toward chemicals are associated with preference for natural food. *Food Quality and Preference, 22*(1), 149–156.

Dobrowolski, J. W., Vohora, S. B., Sharma, K., Shah, S. A., Naqvi, S. A. H., & Dandiya, P. C. (1991). Antibacterial, antifungal, antiamoebic, antiinflammatory and antipyretic studies on propolis bee products. *Journal of Ethnopharmacology, 35*(1), 77–82.

Elbaz, N. M., Khalil, I. A., Abd-Rabou, A. A., & El-Sherbiny, I. M. (2016). Chitosan-based nano-in-microparticle carriers for enhanced oral delivery and anticancer activity of propolis. *International Journal of Biological Macromolecules, 92*, 254–269.

El-Guendouz, S., Lyoussi, B., Lourenço, J. P., Rosa da Costa, A. M., Miguel, M. G., Barrocas Dias, C., et al. (2019). Magnetite nanoparticles functionalized with propolis against methicillin resistant strains of Staphylococcus aureus. *Journal of the Taiwan Institute of Chemical Engineers, 102*, 25–33.

Eom, H. S., Lee, E. J., Yoon, B. S., & Yoo, B. S. (2010). Propolis inhibits the proliferation of human leukaemia HL-60 cells by inducing apoptosis through the mitochondrial pathway. *Natural Product Research, 24*(4), 375–386.

Eslami, H., Ariamanesh, N., & Ariamanesh, A. (2016). Synergistic effect of honey and azarian propolis on oral microorganisms: An *in vitro* study. *Journal of Advanced Oral Research, 7*(3), 31–36.

Freitas, A. S., Cunha, A., Cardoso, S. M., Oliveira, R., & Almeida-Aguiar, C. (2019). Constancy of the bioactivities of propolis samples collected on the same apiary over four years. *Food Research International, 119*, 622–633.

Gallagher, J., & Gray, M. (2003). Is aloe vera effective for healing chronic wounds? *Journal of Wound Ostomy & Continence Nursing, 30*(2), 68–71.

Gargouri, W., Osés, S. M., Fernández-Muiño, M. A., Sancho, M. T., & Kechaou, N. (2019). Evaluation of bioactive compounds and biological activities of Tunisian propolis. *LWT, 111*, 328–336.

Ge, L., Li, Q., Wang, M., Ouyang, J., Li, X., & Xing, M. M. Q. (2014). Nanosilver particles in medical applications: Synthesis, performance, and toxicity. *International Journal of Nanomedicine, 9*, 2399–2407.

Ghavidel, F., Javadi, A., Anarjan, N., & Jafarizadeh-Malmiri, H. (2021). New approach in process intensification based on subcritical water, as green solvent, in propolis oil in water nanoemulsion preparation. *Green Processing and Synthesis, 10*(1), 208–218.

Gokce, Y., Cengiz, B., Yildiz, N., Calimli, A., & Aktas, Z. (2014). Ultrasonication of chitosan nanoparticle suspension: Influence on particle size. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 462*, 75–81.

Grecka, K., Kuś, P. M., Okińczyc, P., Worobo, R. W., Walkusz, J., & Szweda, P. (2019). The anti-staphylococcal potential of ethanolic polish propolis extracts. *Molecules (Basel, Switzerland), 24*(9), 1732.

Guimarães, A. C., Meireles, L. M., Lemos, M. F., Guimarães, M. C. C., Endringer, D. C., Fronza, M., et al. (2019). Antibacterial activity of terpenes and terpenoids present in essential oils. *Molecules (Basel, Switzerland), 24*(13), 2471.

Hatcher, H., Planalp, R., Cho, J., Torti, F. M., & Torti, S. V. (2008). Curcumin: From ancient medicine to current clinical trials. *Cellular and Molecular Life Sciences, 65*(11), 1631–1652.

Hegazi, A., Elhoussiny, A., & Fouad, E. (2019). Egyptian propolis 14: Potential antibacterial activity of propolis-encapsulated alginate nanoparticles against different pathogenic bacteria strains. *Advances in Natural Sciences: Nanoscience and Nanotechnology, 10*, Article 045019.

Hewitt, C. J., & Nebe-Von-Caron, G. (2004). The Application of Multi-Parameter Flow Cytometry to Monitor Individual Microbial Cell Physiological State. In *Physiological stress responses in bioprocesses* (pp. 197–223). Berlin, Heidelberg: Springer Berlin Heidelberg. -/-.

Jansen-Alves, C., Krumreich, F. D., Zandoná, G. P., Gularte, M. A., Borges, C. D., & Zambiazi, R. C. (2019). Production of propolis extract microparticles with concentrated pea protein for application in food. *Food and Bioprocess Technology, 12*(5), 729–740.

Jug, M., Končić, M. Z., & Kosalec, I. (2014). Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures. *LWT - Food Science and Technology, 57*(2), 530–537.

Kalogeropoulos, N., Konteles, S. J., Troullidou, E., Mourtzinos, I., & Karathanos, V. T. (2009a). Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. *Food Chemistry, 116*(2), 452–461.

Kalogeropoulos, N., Konteles, S., Mourtzinos, I., Troullidou, E., Chiou, A., & Karathanos, V. T. (2009b). Encapsulation of complex extracts in beta-cyclodextrin: An application to propolis ethanolic extract. *Journal of Microencapsulation, 26*(7), 603–613.

Katekhaye, S., Fearnley, H., Fearnley, J., & Paradkar, A. (2019). Gaps in propolis research: Challenges posed to commercialization and the need for an holistic approach. *Journal of Apicultural Research, 58*(4), 604–616.

Kazemi, F., Divsalar, A., & Saboury, A. A. (2018). Structural analysis of the interaction between free, glycated and fructated hemoglobin with propolis nanoparticles: A spectroscopic study. *International Journal of Biological Macromolecules, 109*, 1329–1337.

Kharsany, K., Viljoen, A., Leonard, C., & van Vuuren, S. (2019). The new buzz: Investigating the antimicrobial interactions between bioactive compounds found in South African propolis. *Journal of Ethnopharmacology, 238*, Article 111867.

Kim, Y. H., & Chung, H. J. (2011). The effects of Korean propolis against foodborne pathogens and transmission electron microscopic examination. *New Biotechnology, 28*(6), 713–718.

Kubiliene, L., Laugaliene, V., Pavilonis, A., Maruska, A., Majiene, D., Barcauskaite, K., et al. (2015). Alternative preparation of propolis extracts: Comparison of their composition and biological activities. *BMC Complementary and Alternative Medicine, 15*(1), 156.

Kuropatnicki, A. K., Szliszka, E., & Krol, W. (2013). Historical aspects of propolis research in modern times. *Evidence-based Complementary and Alternative Medicine: ECAM, 2013*, Article 964149 964149.

Lang, G., & Buchbauer, G. (2012). A review on recent research results (2008–2010) on essential oils as antimicrobials and antifungals. A review. *Flavour and Fragrance Journal, 27*(1), 13–39.

Liao, C. H., & Shollenberger, L. M. (2003). Survivability and long-term preservation of bacteria in water and in phosphate-buffered saline*. *Letters in Applied Microbiology, 37*(1), 45–50.

Lopez, B. G. C., de Lourenço, C. C., Alves, D. A., Machado, D., Lancellotti, M., & Sawaya, A. C. H. F. (2015). Antimicrobial and cytotoxic activity of red propolis: An alert for its safe use. *Journal of Applied Microbiology, 119*(3), 677–687.

Masoura, M., Passaretti, P., Overton, T. W., Lund, P. A., & Gkatzionis, K. (2020). Use of a model to understand the synergies underlying the antibacterial mechanism of H2O2-producing honeys. *Scientific Reports, 10*(1), 17692.

McDonnell, G., & Russell, A. D. (1999). Antiseptics and disinfectants: Activity, action, and resistance. *Clinical Microbiology Reviews, 12*(1), 147–179.

Melliou, E., & Chinou, I. (2004). Chemical analysis and antimicrobial activity of Greek propolis. *Planta Medica, 70*(6), 515–519.

Mi, H., Wang, D., Xue, Y., Zhang, Z., Niu, J., Hong, Y., et al. (2016). Dimethyl sulfoxide protects escherichia coli from rapid antimicrobial-mediated killing. *Antimicrobial Agents and Chemotherapy, 60*(8), 5054.

Mohammadzadeh, S., Shariatpanahi, M., Hamedi, M., Ahmadkhaniha, R., Samadi, N., & Ostad, S. N. (2007). Chemical composition, oral toxicity and antimicrobial activity of Iranian propolis. *Food Chemistry, 103*(4), 1097–1103.

Moncla, B. J., Guevara, P. W., Wallace, J. A., Marcucci, M. C., Nor, J. E., & Bretz, W. A. (2012). The inhibitory activity of typified propolis against Enterococcus species. *Zeitschrift fur Naturforschung. C, Journal of biosciences, 67*(5–6), 249–256.

Morais, M., Moreira, L., Feás, X., & Estevinho, L. M. (2011). Honeybee-collected pollen from five Portuguese natural parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology, 49*(5), 1096–1101.

Moret, S., Purcaro, G., & Conte, L. S. (2010). Polycyclic aromatic hydrocarbons (PAHs) levels in propolis and propolis-based dietary supplements from the Italian market. *Food Chemistry, 122*(1), 333–338.

Netíková, L., Bogusch, P., & Heneberg, P. (2013). Czech ethanol-free propolis extract displays inhibitory activity against a broad spectrum of bacterial and fungal pathogens. *Journal of Food Science, 78*(9), M1421–M1429.

Newburger, A. E. (2009). Cosmeceuticals: Myths and misconceptions. *Clinics in Dermatology, 27*(5), 446–452.

Nor, F., Nor, C., Bento, L. W., Zhang, Z. C., Bretz, W. A., & Nor, J. E. (2021). Propolis reduces the stemness of head and neck squamous cell carcinoma. *Archives of Oral Biology*, 125.

O'Neill, A. J., & Chopra, I. (2004). Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. *Expert Opinion on Investigational Drugs, 13*(8), 1045–1063.

Oroian, M., Ursachi, F., & Dranca, F. (2020). Influence of ultrasonic amplitude, temperature, time and solvent concentration on bioactive compounds extraction from propolis. *Ultrasonics Sonochemistry, 64*, Article 105021.

Park, J. H., Lee, J. K., Kim, H. S., Chung, S. T., Eom, J. H., Kim, K. A., et al. (2004). Immunomodulatory effect of caffeic acid phenethyl ester in Balb/c mice. *International Immunopharmacology, 4*(3), 429–436.

Park, S., Song, I. S., & Choi, M. K. (2016). Preparation and characterization of quercetin-loaded solid dispersion by solvent evaporation and freeze-drying method. *Mass Spectrometry Letters, 7*, 79–83.

Park, Y. K., & Ikegaki, M. (1998). Preparation of water and ethanolic extracts of propolis and evaluation of the preparations. *Bioscience, Biotechnology, and Biochemistry, 62*(11), 2230–2232.

Pérez-Maqueda, L. A., Duran, A., & Pérez-Rodríguez, J. L. (2005). Preparation of submicron talc particles by sonication. *Applied Clay Science, 28*(1), 245–255.

Permana, A. D., Utami, R. N., Courtenay, A. J., Manggau, M. A., Donnelly, R. F., & Rahman, L. (2020). Phytosomal nanocarriers as platforms for improved delivery of natural antioxidant and photoprotective compounds in propolis: An approach for enhanced both dissolution behaviour in biorelevant media and skin retention profiles. *Journal of Photochemistry and Photobiology B: Biology, 205*, Article 111846.

Pietta, P. G., Gardana, C., & Pietta, A. M. (2002). Analytical methods for quality control of propolis. *Fitoterapia, 73*, S7–S20.

Pobiega, K., Kraśniewska, K., Derewiaka, D., & Gniewosz, M. (2019a). Comparison of the antimicrobial activity of propolis extracts obtained by means of various extraction methods. *Journal of Food Science and Technology, 56*(12), 5386–5395.

Pobiega, K., Kraśniewska, K., & Gniewosz, M. (2019b). Application of propolis in antimicrobial and antioxidative protection of food quality – A review. *Trends in Food Science & Technology, 83*, 53–62.

Popova, M. P., Chinou, I. B., Marekov, I. N., & Bankova, V. S. (2009). Terpenes with antimicrobial activity from Cretan propolis. *Phytochemistry, 70*(10), 1262–1271.

Porcu, E. P., Cossu, M., Rassu, G., Giunchedi, P., Cerri, G., Pourová, J., et al. (2018). Aqueous injection of quercetin: An approach for confirmation of its direct *in vivo* cardiovascular effects. *International Journal of Pharmaceutics, 541*(1), 224–233.

Pourreza, N., & Rastegarzadeh, S. (2004). Kinetic spectrophotometric determination of Tween 80. *Talanta, 62*(1), 87–90.

Pratami, D., Sahlan, M., Mun'im, A., & Sundowo, A. (2018). Phytochemical profile and antioxidant activity of propolis ethanolic extract from tetragonula bee. *Pharmacognosy Journal, 10*, 128–135.

Ramanauskienė, K., & Inkėnienė, A. M. (2011). Propolis oil extract: Quality analysis and evaluation of its antimicrobial activity. *Natural Product Research, 25*(15), 1463–1468.

Ramli, N. A., Ali, N., Hamzah, S., & Yatim, N. I. (2021). Physicochemical characteristics of liposome encapsulation of stingless bees' propolis. *Heliyon, 7*(4), e06649.

Regueira, M. S., Tintino, S. R., Silva da, A. R. P., Costa, M. D. S., Boligon, A. A., et al. (2017). Seasonal variation of Brazilian red propolis: Antibacterial activity, synergistic effect and phytochemical screening. *Food and Chemical Toxicology, 107*, 572–580.

Righi, A. A., Alves, T. R., Negri, G., Marques, L. M., Breyer, H., & Salatino, A. (2011). Brazilian red propolis: Unreported substances, antioxidant and antimicrobial activities. *Journal of the Science of Food and Agriculture, 91*(13), 2363–2370.

Ristivojević, P., Dimkić, I., Guzelmeric, E., Trifković, J., Knežević, M., Berić, T., et al. (2018). Profiling of Turkish propolis subtypes: Comparative evaluation of their phytochemical compositions, antioxidant and antimicrobial activities. *Lebensmittel-Wissenschaft & Technologie, 95*, 367–379.

Rozin, P. (2005). The meaning of "natural": Process more important than content. *Psychological Science, 16*(8), 652–658.

Sharma, N., Kaur, G., & Khatkar, S. K. (2021). Optimization of emulsification conditions for designing ultrasound assisted curcumin loaded nanoemulsion: Characterization, antioxidant assay and release kinetics. *Lebensmittel-Wissenschaft & Technologie, 141*, Article 110962.

Silva, J. C., Rodrigues, S., Feás, X., & Estevinho, L. M. (2012). Antimicrobial activity, phenolic profile and role in the inflammation of propolis. *Food and Chemical Toxicology, 50*(5), 1790–1795.

Silva-Carvalho, R., Baltazar, F., & Almeida-Aguiar, C. (2015). Propolis: A complex natural product with a plethora of biological activities that can be explored for drug development. *Evidence-based Complementary and Alternative Medicine: ECAM, 2015*, Article 206439 206439.

Singer, A., Barakat, Z., Mohapatra, S., & Mohapatra, S. S. (2019). Chapter 13- nanoscale drug-delivery systems: *In vitro* and *in vivo* characterization. In S. S. Mohapatra, S. Ranjan, N. Dasgupta, R. K. Mishra, & S. Thomas (Eds.), *Nanocarriers for drug delivery* (pp. 395–419). Elsevier.

Smith, L. M., & Dairiki, T. (1975). Stability of milk fat emulsions. I. Preparation of model oil-in-water emulsions and evaluation of their stability. *Journal of Dairy Science, 58*(9), 1249–1253.

Soleimanifard, M., Feizy, J., & Maestrelli, F. (2021). Nanoencapsulation of propolis extract by sodium caseinate-maltodextrin complexes. *Food and Bioproducts Processing, 128*, 177–185.

Sulaiman, G. M., Sammarrae, K. W. A., Ad'hiah, A. H., Zucchetti, M., Frapolli, R., Bello, E., et al. (2011). Chemical characterization of Iraqi propolis samples and assessing their antioxidant potentials. *Food and Chemical Toxicology, 49*(9), 2415–2421.

Sun, C., Wu, Z., Wang, Z., & Zhang, H. (2015). Effect of ethanol/water solvents on phenolic profiles and antioxidant properties of Beijing propolis extracts. *Evidence-Based Complementary and Alternative Medicine, 2015*, Article 595393.

Tosi, E. A., Ré, E., Ortega, M. E., & Cazzoli, A. F. (2007). Food preservative based on propolis: Bacteriostatic activity of propolis polyphenols and flavonoids upon Escherichia coli. *Food Chemistry, 104*(3), 1025–1029.

Usune, S., Takahashi, T., Kubo, M., Shoji, E., Tsukada, T., Koike, O., et al. (2019). Numerical simulation of structure formation of surface-modified nanoparticles during solvent evaporation. *Journal of Chemical Engineering of Japan, 52*(8), 680–693.

Uzel, A., Sorkun, K. y., Önçağ, Ö., Çoğulu, D., Gençay, Ö., & Salïh, B. R. (2005). Chemical compositions and antimicrobial activities of four different Anatolian propolis samples. *Microbiological Research, 160*(2), 189–195.

Vanni, R., Waldner-Tomic, N. M., Belibasakis, G. N., Attin, T., Schmidlin, P. R., & Thurnheer, T. (2015). Antibacterial efficacy of a propolis toothpaste and mouthrinse against a supragingival multispecies biofilm. *Oral Health & Preventive Dentistry, 13*(6), 531–535.

Vauthier, C., Cabane, B., & Labarre, D. (2008). How to concentrate nanoparticles and avoid aggregation? *European Journal of Pharmaceutics and Biopharmaceutics, 69*(2), 466–475.

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols, 3*(2), 163–175.

Yusof, N., Munaim, M. S. A., & Veloo Kutty, R. (2021). Optimization of total phenolic compounds extracted from propolis by ultrasound- assisted extraction. *Chemical Engineering Communications, 208*(4), 564–572.