Research Article

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Nanoemulsions of essential oils stabilized with saponins exhibiting antibacterial and antioxidative properties

https://doi.org/10.1515/rams-2022-0337
received February 20, 2023; accepted June 20, 2023

Abstract: Functional foods, drug delivery systems, and cosmetics are the main areas of application for multiphase systems, where the use of naturally derived compounds is preferred. Hence, this study aimed to assess the possibility of using natural surfactants and saponin-rich extracts to produce emulsions containing antibacterial and antioxidant cinnamon and clove essential oils (EOs). The analyses of nanoparticles using dynamic light scattering showed that the addition of plant extracts to solutions allows one to obtain stable emulsions and decreased zeta potential (< -40 mV) and droplet size (<200 nm). In all investigated emulsions, the increase of antioxidative properties was observed when both EOs and plant extracts were used. The emulsion with clove oil stabilized with *Quillaja saponaria* bark saponins has the highest combined antioxidative properties (3.55 ± 0.01 µg gallic acid equivalent per g). Additionally, a stronger antibacterial action against *Pseudomonas* bacteria was observed for clove oil with *Quillaja saponaria* and cinnamon oil with *Glycyrrhiza glabra*. In addition, plant extracts did not affect significantly the other properties of the oil emulsions, *e.g.* wettability, colour, and refractive index. All results show that the proposed emulsions can be helpful in the preparation of multifunctional emulsions, where the co-action of saponins and EOs is especially beneficial.

Keywords: emulsions, cinnamon oil, clove oil, natural surfactants

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1 Introduction

Essential oils (EOs) are complex mixtures of terpenes, terpenoids, phenylpropanoids, fatty acids, oxides, and sulphur compounds. Many EOs possess antimicrobial and antioxidant properties. The most effective EOs acting against pathogenic microorganisms are, among others, clove oil and cinnamon oil [1,2]. The constituents of clove EO can disrupt the wall and cellular membrane of microorganisms, penetrating *via* cytoplasmic membranes or entering cells, and subsequently, inhibit the correct synthesis of DNA and proteins [3,4]. In another investigation, it was indicated that eugenol, the prime clove EO component, can block the production of α-amylase, protease, and subtilisin by *Bacillus subtilis*, leading to the destruction of the cell wall and resulting in the lysis of cells [5].

Another group of antibacterial and antioxidative compounds of plant origin is natural surfactants – saponins [6]. Therefore, saponins are gaining popularity as possible therapeutic research and development targets. In addition, the amphiphilic properties of saponins may be very helpful in the interruption of pathogen cells allowing for EOs to penetrate microbial cells more effectively. Additionally, emulsifying properties of saponins were also proved [7]. It should also be emphasized that saponins, compared to biosurfactants of bacterial origin, are a cheaper source of natural surfactants. In addition, compared to other plant-based surfactants, such as soy lecithin, saponins show better emulsifying properties [8,9].

Nowadays, a rapidly growing threat of drug-resistant microorganisms is observed; therefore, EO formulations enriched with saponins exhibiting antimicrobial properties

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may be an effective solution [10]. Additionally, saponin's antioxidant properties make them additionally beneficial [11,12]. The question arises, however, whether the interaction of several natural antibacterial and antioxidant substances would not increase effectiveness. Such a beneficial co-action effect may be particularly evident for substances of different natures, *i.e.* lipophilic EOs and water-soluble surfactants [13,14]. At the same time, saponins, as surfactants, can provide a tool for better dispersion of EOs and thus enhance their effects.

Hence, the objective of this study was to extensively investigate the properties of emulsions based on EOs stabilized by plant extracts rich in saponins. The research hypothesis posed is that the proposed multiphase systems can constitute novelty and effective systems acting against pathogenic microbes and provide high antioxidative properties. This work reports on the antimicrobial properties of emulsions of EOs stabilized with saponins from *Glycyrrhiza glabra* and *Quillaja saponaria.* Moreover, the physicochemical properties of the emulsions are the subject of the study. It should be highlighted that here the potential synergistic effect from the nanosized emulsion system (enhanced stability), the surface active compound (plant extract), and the EO composition is presented.

2 Materials and methods

2.1 Chemicals

In this study, three plant extracts were used. The first one was the commercially available *Quillaja saponaria* bark extract provided by Sigma-Aldrich (Germany). The other two were extracted from *Glycyrrhiza glabra* L. roots (Flos, Poland) or *Sapindus mukorossi* L. fruits (Mohani, Poland). The extraction of plants was performed analogically as described by Smulek *et al.* [15]. The crushed roots of *G. glabra* Land fruits of *S. mukorossi* L. were subjected to extraction in a Soxhlet apparatus for 8 h at 65°C using 10 mL methanol per gram of the plant material. After solvent evaporation, the solid extract was freeze-dried (Alpha 1–2 LD Plus, Christ, Germany).

The EOs from cloves (from *Syzygium aromaticum* flower buds), cinnamon (from *Cinnamomum verum* bark), lemon (from *Citrus lemon* peel), rosemary (from *Salvia rosmarinus* leaves), eucalyptus (from *Eucalyptus globulus* leaves), and their mix (in equal volume ratio, called "five thieves oil") were purchased from Avicenna-Oil (Wrocław, Poland). All other chemicals used in the experiments were purchased from Merck (Germany).

2.2 Emulsion preparation

Based on the analysis of the antioxidative properties, described above, and the antibacterial properties from our previous study [16], extracts from *Q. saponaria* and *G. glabra* as well as EOs from clove and cinnamon have been chosen for further experiments with emulsion systems (Table 1). After our preliminary research described by Siejak *et al.* [16], the concentration of the components was selected as 0.5% oils and 0.5 $g\cdot L^{-1}$ extract within the sample (20 mL). The emulsion systems were prepared in a one-step method, where an ultrasound homogenizer (sonicator Sonoplus, Bandelin, Berlin, Germany) was used. The homogenization process was adjusted with an energy equal to 12,500 kJ for 5 min, in cycles action/break 5 s/5 s.

Table 1: Plant extracts and EO systems chosen for further investigations

Sample no.	EO		Plant extract	
	Type	Concentration ($g\cdot L^{-1}$)	Type	Concentration ($g\cdot L^{-1}$)
S01	None	NA	*Q. saponaria*	0.5
S02	None	NA	*G. glabra*	0.5
S03	Cinnamon	0.5	None	NA
S04	Cinnamon	0.5	*Q. saponaria*	0.5
S05	Cinnamon	0.5	*G. glabra*	0.5
S06	Clove	0.5	None	NA
S07	Clove	0.5	*Q. saponaria*	0.5
S08	Clove	0.5	*G. glabra*	0.5

NA – not applicable.

2.3 Antioxidative properties

The first part of the study was focused on the analysis of antioxidative properties of *Q. saponaria*, *S. mukorossi*, and *G. glabra* extracts as well as EOs of clove, cinnamon, eucalyptus, lemon, rosemary, and the mixture of all oils (so-called "thieves oil"). The extract was analysed in water solutions at concentrations of 0.5 $g\cdot L^{-1}$. The oils were tested directly as liquids. Then, the emulsions obtained containing selected oils and extracts were analysed for their antioxidative properties.

There is no single antioxidant assay due to the lack of standard quantification of methods. The methods differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, and expression of results. Therefore, to properly measure the antioxidant capacity of a given sample, multidirectional analysis was done using several methods [17].

The antioxidant activity of the samples was determined using the ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical method described by Jeszka-Skowron *et al.* [18]. The results were expressed as μM of Trolox equivalent (TE) to a gram of the oil or a litre of emulsion ($TE\cdot L^{-1}$).

The ability of plant extracts, oils, and emulsions to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was studied according to the method previously described [19]. The results were expressed as μM of TE to a gram of the extract/oil or a litre of emulsion.

The total phenolic content of plant extracts, oils, and emulsions was determined by using Folin–Ciocalteu's reagent according to the method described in previous study [18]. The results were expressed as mg of gallic acid equivalent (GAE) to a gram of the extract/oil or a litre of emulsion. The antioxidant properties of the samples determined by all the above methods were performed under dark conditions.

2.4 Determination of emulsion properties

The hydrodynamic diameters (d_H) of the emulsion droplets and the zeta potential (ζ) of the emulsions were measured using a Zetasizer Nano-ZS (Malvern Instruments Ltd., United Kingdom), which worked based on non-invasive back-scattering method. In addition, the contact angle, the colour of samples, and the refractive index (RI) were also determined. The contact angle of the emulsions was calculated based on the measurements performed on the surfaces of glass microscopic slides (Levenhuk, Inc., USA) using a Drop Shape Analyzer DSA100E (KRÜSS GmbH, Hamburg, Germany). Before measurements, the glass slides were washed with 96% ethanol and dried. Finally, the emulsions' stability was determined based on the measurement of the ratio between the volume of the emulsified layer and the total volume of the sample. The experiments were conducted after 24 and 72 h according to the emulsification index (EI) measurement method [20] at 25°C.

An NH310 portable spectrophotometer (Shenzhen ThreeNH Technology Co., Ltd., Shiyan, China) equipped with internal software was used for colour evaluation. Before the examination, 2 mL of the sample was introduced into the transparent plastic cuvette. The colour tests were repeated ten times, and average values with SD were recorded. The RIs were determined using an analogous optical Abbe Refractometer (RL, Polskie Zakłady Optyczne S.A.the, Warsaw, Poland).

2.5 Microscopic imaging of the emulsion

Microscopic imaging was performed using an inverted microscope ZEISS Axio Verta.A1 (Zeiss, China) equipped with a colour camera Axiocam 208 (Zeiss, China). The studies were performed with the objective magnifications LD A-Planx40/0,55 ph1 (air), A-Planx100/1,25 Oil Ph2 oil. For microscopic observations, the emulsions were introduced into 1 μm-Slide VI0,1 cuvette (Ibidi GmbH, Germany). For the better visualisation of the emulsions 2.5D images were prepared using ZEN 3.1 software (Zeiss, Germany).

2.6 Emulsion bioactivity measurements

To assess the bioactive properties of the prepared emulsions, three strains of bacteria of the genus *Pseudomonas* were used: *Pseudomonas plecoglossicida* IsA (NCBI GenBank accession no. KY561350), *Pseudomonas* sp. OS4 (NCBI GenBank accession no. KP096512), and *Pseudomonas fluorescens* ATCC17400. *Pseudomonas* bacteria are Gram-negative, rod-shaped, mostly motile, aerobic, and endospore negative. They are ubiquitous in soil, plants, freshwater, and saltwater. Many of the *Pseudomonas* strains are opportunistic pathogens [21].

Microbial cell viability tests were performed using a resazurin-based dye alamarBlue, according to the method described in a previous study [22]. Briefly, the cultures were prepared in 50 mL sterile laboratory tubes that contained 20 mL of nutrient broth inoculated with a loopful of bacterial biomass from agar plates. The cultures were incubated at 30°C with shaking (120 rpm) for 24 h. Then, the OD_{600} was adjusted to 0.1 and transferred to a 96-well spectrophotometric plate and mixed with emulsions in a 1:1 v/v ratio. Afterward, the indicator was added and incubated at 30°C for 24 h. The spectrophotometric measurements were then carried out. As blank samples, the samples without bacteria but with emulsions or plant surfactants or EOs were used. As control samples, the samples with bacteria suspension were only used.

2.7 Statistical analysis

In all analyses, at least three independent experiments were performed, unless otherwise indicated. The mean values were considered as the final results. The statistical significance of differences between the mean values was determined by ANOVA (two-way analysis of variance) with Tukey's range test applied as *post hoc* analysis. Differences at $p < 0.05$ were considered statistically significant. The calculations were performed using GraphPadPrism (GraphPad Prism version 8.4.3 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

3 Results and discussion

3.1 Antioxidative properties of emulsion components

First, antioxidant properties and total phenolic compounds of plant extracts of emulsion components were analysed (Table 2). The extracts of *Quillaja saponaria* showed the highest level of phenolic compounds: 2-fold higher compared to *Glycyrrhiza glabra* and 23-fold higher compared to the *Sapindus mukorossi* extract ($p < 0.05$). A similar observation was also found in the DPPH method between *Quillaja saponaria* and *Glycyrrhiza glabra* extracts. The *Sapindus mukorossi* fruit extract possessed the lowest antioxidant activity in both assays.

The saponin-rich soapbark tree *Quillaja saponaria* aqueous extract contains phenolic compounds such as (+)-piscidic acid (a major constituent), glucosyringic acid, and vanillic acid derivatives, which are responsible for the hypoglycemic, hypocholesterolemic, and antioxidant effects in streptozotocin-induced diabetic rats [23]. On the other hand, glycyrrhizic acid, glabridin, and other flavonoids were found in the roots and leaves of *Glycyrrhiza glabra* [24]. The *Sapindus mukorossi* extract exhibited over ten times lower antioxidative properties than *Quillaja*

Table 2: Antioxidative properties of the tested saponin-rich plant extracts, EOs, and emulsions

	Folin–Ciocalteu reagent method (μg GAE·g^{-1})	ABTS method (μM TE·g^{-1})	DPPH method (μM TE·g^{-1})
Plant extracts*			
Quillaja saponaria bark	1354.9 ± 0.9$^{(c)}$	ND	5,566 ± 122$^{(c)}$
Sapindus mukorossi fruits	58.1 ± 2.8$^{(b)}$	ND	862 ± 17$^{(a)}$
Glycyrrhiza glabra roots	797.5 ± 1.4$^{(a)}$	ND	2,764 ± 28$^{(b)}$
EOs**			
Clove oil	635 ± 4$^{(c)}$	124 ± 3$^{(f)}$	2,517 ± 36$^{(e)}$
Rosemary oil	<LOD	0.06 ± 0.01$^{(a)}$	10.7 ± 0.06$^{(a)}$
Cinnamon oil	34 ± 1$^{(a)}$	1.92 ± 0.25$^{(c)}$	3,216 ± 20$^{(f)}$
Lemon oil	<LOD	6.41 ± 0.22$^{(d)}$	288 ± 2$^{(c)}$
Eucalyptus oil	<LOD	0.95 ± 0.05$^{(b)}$	195 ± 12$^{(b)}$
Mix of 5 oils	165 ± 7$^{(b)}$	44.8 ± 0.3$^{(e)}$	407 ± 10$^{(d)}$
Emulsions			
S04 cinnamon oil + *Quillaja saponaria* bark	0.33 ± 0.01$^{(b)}$	34.0 ± 0.9$^{(a)}$	7.98 ± 0.18$^{(a)}$
S05 cinnamon oil + *Glycyrrhiza glabra* roots	0.30 ± 0.01$^{(a)}$	34.9 ± 0.3$^{(a)}$	7.75 ± 0.10$^{(a)}$
S07 clove oil + *Quillaja saponaria* bark	3.55 ± 0.01$^{(c)}$	854 ± 16$^{(a)}$	114 ± 1$^{(b)}$
S08 clove oil + *Glycyrrhiza glabra* roots	3.33 ± 0.07$^{(c)}$	675 ± 20$^{(b)}$	112 ± 2$^{(b)}$

*Results calculated per gram of the dried mass extract; ** Results calculated per gram of oil; TE – trolox equivalent; GAE – gallic acid equivalent; ND – not determined; <LOD – below the limit of detection; and groups labelled with different letters in columns (superscript) are significantly different at $p < 0.05$.

saponaria and *Glycyrrhiza glabra* extracts, although fruits from *Sapindus mukorossi* contain relatively the most saponins of all the raw materials analysed Smułek *et al.*, [25]. This indicates that the antioxidant properties should not be attributed to the presence of saponins but rather to compounds associated with them such as polyphenols, flavonoids, or phenolic acids. The higher the level of phenolic content in plant extracts, the higher the antioxidant activity.

In the next stage of experiments, the antioxidant properties of EOs were determined using the bioassays with the Folin–Ciocalteu reagent, ABTS, and DPPH methods *in vitro* radical scavengers. The highest level of total phenolic compounds was found for clove oil followed by a mix of five oils and cinnamon oil ($p < 0.05$) (Table 2). The results for rosemary, lemon, and eucalyptus oils were below the limit of detection (LOD < 0.03 mg TE·L^{-1}).

In the case of the ABTS method, the same two oils, clove oil and a mix of five oils, showed the highest antioxidant activity. In the DPPH method, cinnamon oil showed the highest antioxidant activity followed by clove oil and a mix of five oils ($p < 0.05$). The lowest antioxidant activity of rosemary oil was found in both radical assays. The differences between the ABTS method (water used as a solution in the method) and DPPH (methanol used as a solution in the method) were due to the different polarities of antioxidants that were measured in the oils.

Eugenol is the major compound (approx. 88%) belonging to phenol derivatives, which was determined in clove oil [26]. The total phenolic content determined in the clove oil was higher than that previously reported [26]. The main compound of cinnamon oil is cinnamaldehyde and minor compounds are terpenes, aromatic compounds, and esters. The possible explanation for the high antioxidant activity of clove and cinnamon oils is that they possessed high levels of phenolic and other reducing compounds in comparison to the other oils.

In terms of antioxidative co-action of emulsion components, the combination of five oils (clove, rosemary, cinnamon, lemon, and eucalyptus oil), their properties appear to be the result of the shares of individual oils. However, the antioxidative properties of a mix of five oils were 3.8-fold lower in the Folin–Ciocalteu method as compared to single clove oil and 2.8-fold lower in the ABTS method, respectively (Table 2). The antioxidant activity measured by the DPPH method of a mix of five oils showed 7.9-fold lower values in comparison to clove oil and 6.2-fold lower values for cinnamon oil; therefore, clove and cinnamon oils were chosen to prepare the emulsions for the next analyses. An additional argument to select these oils was a previously published study [16], which has shown that clove and cinnamon oils exhibit the highest antibacterial properties among the five tested oils.

Based on the above conclusions, the final composition of the emulsion and the reference samples were proposed (see detailed description in Table 1) and used in further experiments to determine if the co-action of plant extracts and EOs can be observed (*i.e.* if oils have stronger antioxidative properties when combined with plant extracts).

3.2 Emulsion properties

3.2.1 Antioxidative properties

The highest antioxidant activity in the DPPH method, as well as total phenolic compounds for emulsions prepared with clove oil and *Quillaja saponaria* bark, and clove oil and Glycyrrhiza glabra roots, were determined (Table 2). The results were 10-fold or higher than the results obtained for the emulsions prepared with cinnamon oil ($p < 0.05$). The highest activity was observed for the emulsion prepared with clove oil and *Quillaja saponaria* bark in the ABTS method. The differences between the results in both methods, with the Folin–Ciocalteu reagent or DPPH, for emulsions with clove oil, were not statistically different ($p > 0.05$).

There were no significant differences between cinnamon oil and *Quillaja saponaria* bark emulsion, and cinnamon oil and *Glycyrrhiza glabra* roots emulsion in both ABTS and DPPH methods (Table 2). Emulsions prepared with cinnamon oil were not statistically different in both radical assays ($p > 0.05$). It is more important to note that all results for emulsions were expressed in units per litre instead of per gram because of the different physical properties of the emulsions in contrast to pure emulsion components, *i.e.* the concentration of 0.5 g·L^{-1} both for plant extracts and EOs. When the values were re-calculated to activity per dry mass of emulsions, the values were 1,000× higher, which shows a strong antioxidative effect. The mechanism for enhancing this antioxidant effect is difficult to explain clearly. The reason may be antioxidant chemical regeneration, as was observed in the case of vitamin E and vitamin C or vitamin E and flavonoids [27]. Moreover, as observed by Deng *et al.* [28], in the case of thymol and Tween 80, the separation of unsaturated terpenes by surfactant molecules increases the oxygen availability and thereby the antioxidative properties of thymol. We can assume that both the mentioned effects can be responsible for the observed increased antioxidative activity of the prepared emulsions when plant surfactants and EOs were used together.

Moreover, it can be expected that oils added to the emulsions affected their antioxidant activities rather than plant extracts. It could be due to the higher antioxidant activity of analysed EOs and extracts as compared to plant extracts. Clove oil is a potential source of antioxidants and has been recently used in nanoemulsions and capsulated EOs [29].

3.2.2 Stability and droplets dispersion

The visual observations of the emulsions proved that all plant extracts and EO emulsions remain stable after 24 and 72 h at 25°C when no phase separation was observed (samples S04, S05, S07, and S08). In the case of EOs dispersed in only water (S03 and S06), phase separation was noticed after 72 h. An important factor that impacts the stability of the colloidal system is the droplet size distribution. The results of the representative droplet size distribution of the prepared samples are presented in Figure 1 and Table 2. First, the aqueous solutions of plant extracts were evaluated. The non-invasive backscattering measurements showed high PDI, which was confirmed by the droplet size (expressed by the % number) and the % intensity results. Here, the *Z*-axis

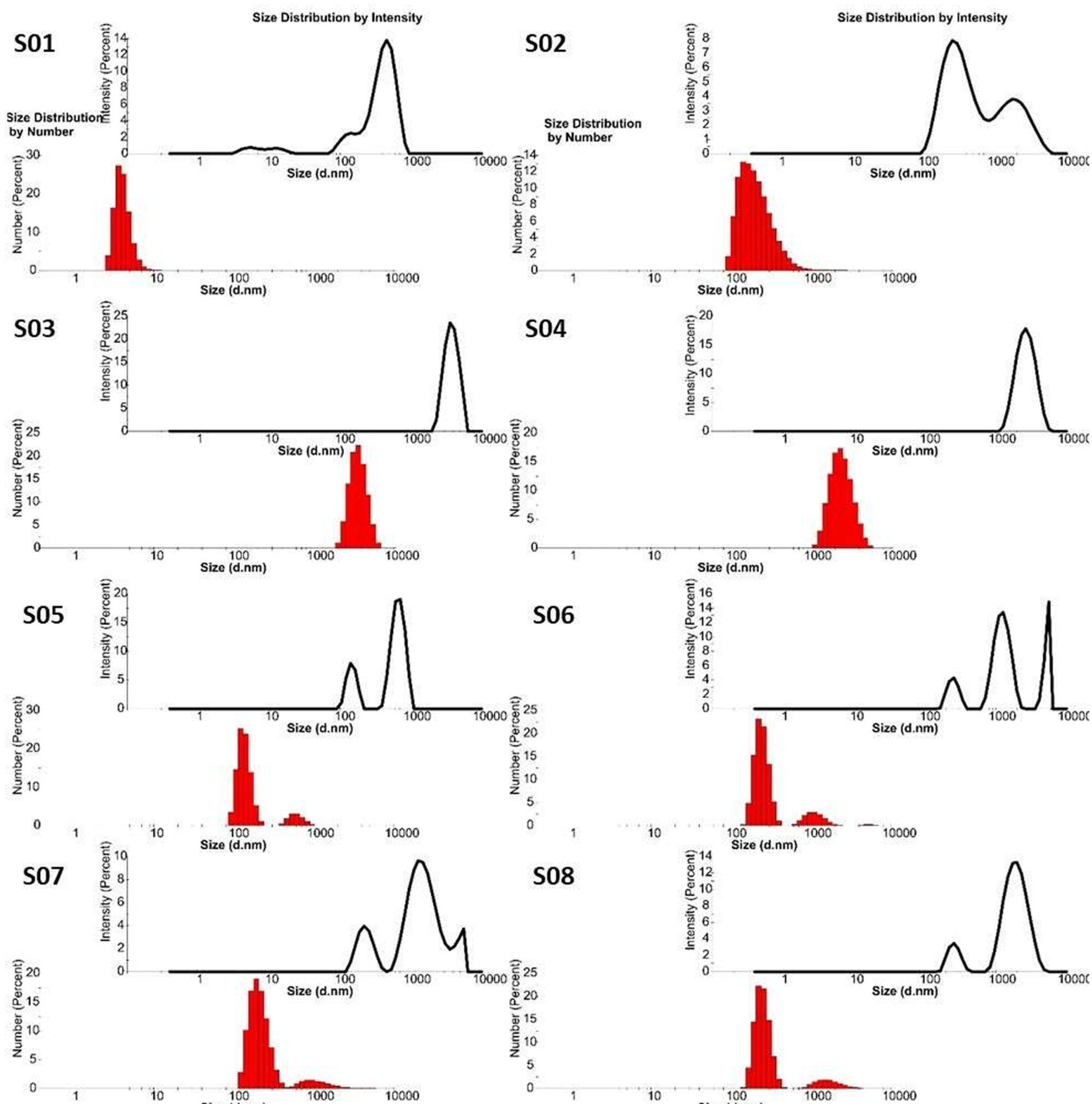

Figure 1: Droplet size distribution by % number and % intensity of the tested systems; sample labels are presented in Table 1.

(Z_{av}) parameter, recorded during the measurements, is not meaningful due to the very high polydispersity index (PDI). As was presented in the previously published studies [30], especially in the case of high polydispersed samples, evaluation of the droplet size *vs* the % number and % intensity should be considered. It was proved that even a small number of large particles (droplets) may affect the PDI value. Here, once again it was justified. Samples without plant extracts (S03 and S06) exhibited the presence of micron-sized droplets with quite high uniformity. The lowest PDI was observed for those two samples. Nevertheless, typically higher stability of the emulsion was recorded for the systems with smaller droplet sizes [31]. The results in Figure 1 suggest that the addition of saponin-rich plant extracts decreases the droplet size. Two, or a maximum of three, main fractions of the emulsion droplets were noticed.

Therefore, it was decided to perform additional light microscopy tests (Figure 2). The observations using a polarized light suggested that the samples are oil-in-water emulsions. Moreover, the analyses indicated that in the emulsions stabilized with plant extracts, such as *Quillaja saponaria* and *Glycyrrhiza glabra,* the droplet sizes are not so homogenous. Microscopic results showed that samples S05 and S08 represent better homogeneity and uniformity, even when larger droplets were observed. As mentioned, the effect of the saponin content on the emulsion structure and droplet size distribution is significant.

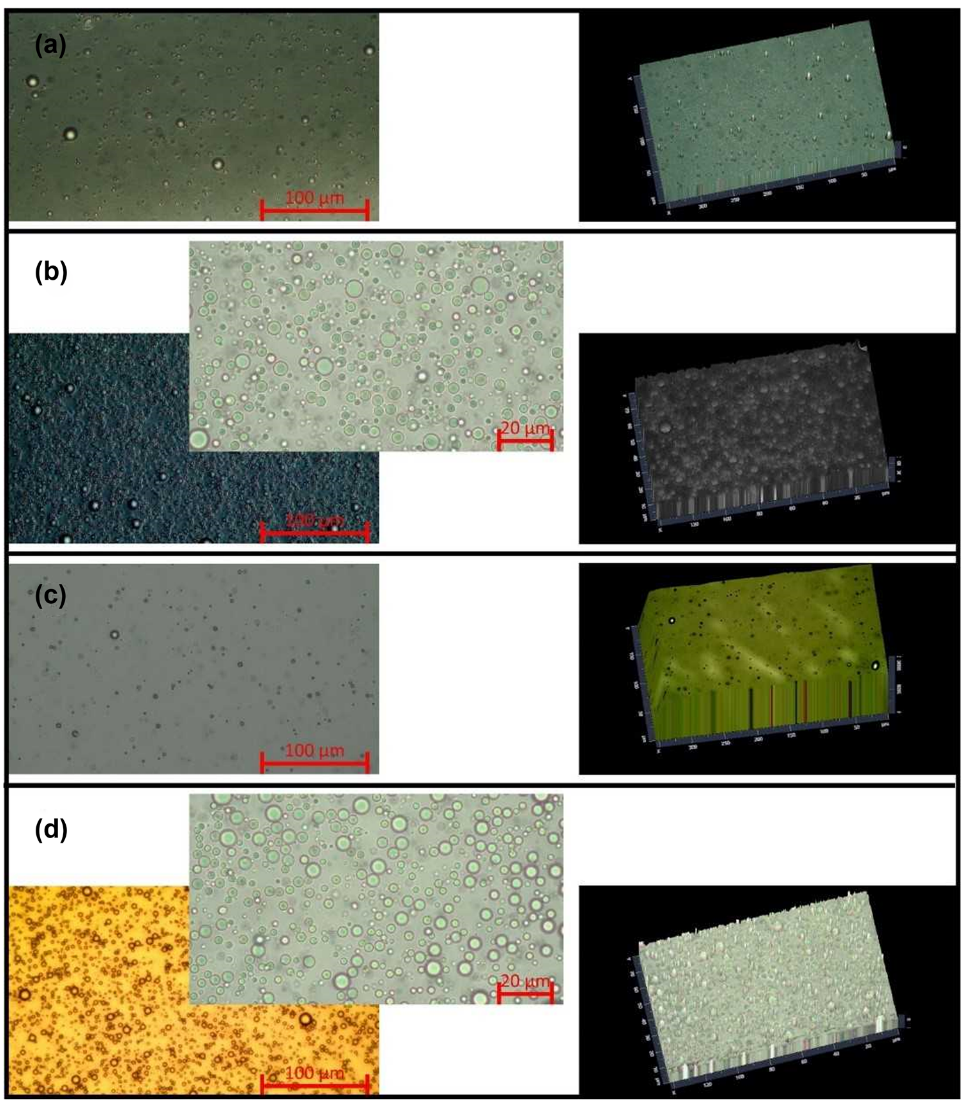

Figure 2: Microscopic images of the tested colloidal systems: (a) Sample S04 (from left magnification ×40 and 2.5D image), (b) S05 (from left magnification ×40, ×100 and 2.5D image), (c) Sample S07 (from left magnification ×40 and 2.5D image), and (d) S08 (from left magnification ×40, ×100 and 2.5D image); sample labels are according to Table 2.

The emulsifying properties of saponin-rich plant extracts were widely studied so far; mainly the general properties of emulsions (like the EI) [32]. The information on their impact on the droplet size is relatively less frequent. Jarzębski *et al.* [33] used the *Aesculum hippocastanum* L. extract containing saponins for preparing hempseed oil. They observed a decrease in the PDI along with the mean particle size after the addition of the extract, however, only in water systems. In the presence of hyaluronic acid, the effectiveness of *A. hippocastanum* L. extracts was no longer observed. Interestingly, the results presented by Taarji *et al.* [34] indicated that the mixture of different compounds present in plant extracts can be more effective in decreasing the droplet size in emulsions than in pure saponins. This suggests that during the application of extracts, there is no single mechanism of interaction responsible for the stabilization of smaller droplets. Perhaps, the resins, polysaccharides, and small particles promote emulsification together with saponin's surfactants.

3.2.3 Zeta potential

The measured zeta potential values of the emulsions varied significantly between the samples (Table 3). When EOs were dispersed in water, their zeta potential values were equal to −37.26 and −31.3 mV for cinnamon oil and clove oil, respectively. When cinnamon oil or clove oil was present along with the *Quillaja saponaria* extract, the zeta potential values dropped to below −40 mV, reaching −48.7 mV for the clove oil emulsion, which was also the lowest value noted for all analysed samples. The addition of EOs to both *Quillaja saponaria* and *Glycyrrhiza glabra* resulted in similar final zeta potential values, with only cinnamon oil emulsion significantly different. This might indicate the very good ability of both EOs to stabilize the *Quillaja saponaria* and *Glycyrrhiza glabra* emulsions. It is assumed that stable emulsions are solutions with zeta potential values higher than 30 mV (regardless of the positive or negative charge), and the stability of these emulsions is secured mainly by the electrostatic repulsions in the colloid [35]. Furthermore, the stability of the emulsion might also be increased by the steric effects. Pirozzi *et al.* [36] suggested that the average zeta potential of oregano EO emulsion was −23.7 mV, presuming also that the negative value of the zeta potential might be due to the adsorption of free fatty acids and polar constituents at the emulsion droplet interface.

In the presented study, the zeta potential values of EOs in water solutions were even lower, but the addition of amphiphilic non-ionic stabilizers such as *Quillaja saponaria* or *Glycyrrhiza glabra* further improved the emulsion stability. This is due to the dual mechanism of the

Table 3: Physical properties of the tested emulsion systems

Sample		Z_{av} (nm)	PDI (−)	Main peak maximum (nm)	Zeta potential (mV)	Contact angle on glass (°)
Emulsions						
S03	Cinnamon oil	3,746 ± 438$^{(e)}$	0.113 ± 0.099$^{(c)}$	3,756 ± 217$^{(d)}$	−37.3 ± 0.9$^{(e)}$	NA
S04	Cinnamon oil + *Quillaja saponaria*	786 ± 177$^{(c)}$	0.747 ± 0.217$^{(a)}$	847 ± 606$^{(b)}$	−42.7 ± 1.8$^{(c)}$	23.1 ± 0.1$^{(c)}$
S05	Cinnamon oil + *Glycyrrhiza glabra*	999 ± 77$^{(d)}$	0.545 ± 0.215$^{(ae)}$	1,526 ± 333$^{(a)}$	−47.6 ± 1.0$^{(a)}$	24.7 ± 0.1$^{(d)}$
S06	Clove oil	2,448 ± 135$^{(a)}$	0.186 ± 0.097$^{(c)}$	2,648 ± 344$^{(c)}$	−31.3 ± 1.1$^{(d)}$	NA
S07	Clove oil + *Quillaja saponaria*	1,861 ± 419$^{(a)}$	0.677 ± 0.036$^{(a)}$	1,673 ± 890$^{(a)}$	−48.7 ± 1.2$^{(a)}$	21.3 ± 0.2$^{(a)}$
S08	Clove oil + *Glycyrrhiza glabra*	1,209 ± 38$^{(b)}$	0.434 ± 0.063$^{(b)}$	1,909 ± 393$^{(a)}$	−45.8 ± 1.6$^{(b)}$	26.5 ± 0.1$^{(b)}$
Reference solutions						
	Water	NA	NA	NA	NA	24.4 ± 0.4$^{(d)}$
S01	*Quillaja saponaria* in water	592 ± 74$^{(f)}$	0.603 ± 0.053$^{(a)}$	446 ± 18$^{(e)}$	−8.8 ± 0.9$^{(f)}$	18.1 ± 0.1$^{(e)}$
S02	*Glycyrrhiza glabra* in water	362 ± 2$^{(g)}$	0.426 ± 0.019$^{(e)}$	331 ± 65$^{(f)}$	−40.3 ± 1.0$^{(c)}$	18.3 ± 0.2$^{(e)}$

Groups labelled with different letters in columns (superscript) are significantly different at $p < 0.05$; NA – not applicable.

surfactant behaviour. The hydrophilic head of the surfactant interacts with the solvent and reduces the interfacial tension due to its deposition on the O/W interface, while the hydrophobic tail interacts with the dispersed EO particles. This protects the oil droplets from aggregation [37].

3.2.4 Wettability

An important parameter describing emulsions' properties is their wettability. The potential of adhesion to different surfaces may be one of the limiting factors influencing the emulsions' bioavailability and bioactivity. The plant extracts, containing surface-active saponins, decreased the contact angle from 24.4° (value for water) to nearly 18° (Table 3). The clove oil emulsion with the addition of the *Quillaja saponaria* extract exhibits a decreased contact angle down to 21.3°. However, the addition of the *Glycyrrhiza glabra* extract led to an increase in the contact angle (up to 26.5°). The cinnamon oil-based emulsion had a little higher contact angle, which tends to decrease in plant extract-containing systems.

3.2.5 Emulsions' colour and RI

Taking into account the importance of product appearance in the food industry, we have carried out tests on colour and the RI changes depending on emulsions' compositions. Figure 3a shows the RI values of emulsions. The collected results indicated no statistically significant changes in these RI values caused by the addition of the plant extracts. The slight differences were caused by the presence of EOs, which led to a small increase in the RI compared to the water samples.

The results of changes in the colour of the emulsions and their components are presented in Figure 3b–d. All the emulsions exhibited slightly negative a^* values, evidence of yellowish-greenish tones in the systems. Considering the b^* parameter, it was positive only for solutions containing *Quillaja saponaria* and *Glycyrrhiza glabra.* The emulsions prepared with EOs had lower b^* values with the lowest for cinnamon oil/*Glycyrrhiza glabra* emulsion ($b^* = -2.6$). The brightness of the tested systems (L^* parameter) was similar for all systems and varied between 43 and 47%. In that case, the cinnamon oil emulsion with *Glycyrrhiza glabra* was an exception as well with L^* reaching nearly 57%. The obtained values are significantly lower when they are compared with L^*, a^*, and b for initial substances. For example, $a^* = -10$ and $b^* = +30$ for cinnamon oil, and $a^* = -5$ and $b^* = +85$ for the clove oil [16]. However, the dispersion of emulsions did not lead to an increase in brightness because the L^* parameter remains at a similar level in emulsions and pure oils.

3.3 Bioactive properties of emulsions

The final part of the research was to check whether the obtained emulsions show better antibacterial activity than solutions of the initial compounds. Three strains of the genus *Pseudomonas*, which belong to the Gram-negative bacteria and are ubiquitous microorganisms in the environment, were selected for testing. Some of these pathogens are also commonly responsible for infections of wounds and skin diseases [38]. The obtained results, presented in Figure 4, indicate that the addition of clove and cinnamon EOs to the formulations with *Quillaja saponaria* and *Glycyrrhiza glabra,* respectively, strongly affected the metabolic activity of the *Pseudomonas* sp. OS4 strain in comparison to the strain's metabolic activity in the emulsion formulation without EOs.

The *Pseudomonas fluorescens* ATCC17400 strain showed the highest resistance to the assumed antimicrobial activity of the EOs. However, this bacterial strain exhibited inhibition of its metabolic activity in the emulsions containing saponins (*Quillaja saponaria* and *Glycyrrhiza glabra*) alone as well as in the emulsions containing cinnamon EO with saponins. A similar behaviour was noticed for the *Pseudomonas plecoglossicida* IsA strain. Nevertheless, the *Pseudomonas plecoglossicida* IsA strain showed increased inhibition of metabolic processes within the emulsions containing saponins combined with EOs as compared to the emulsions with saponins alone. Then, a strong antibacterial activity was observed in cases of clove oil with *Quillaja saponaria* (S07) and cinnamon oil with *Glycyrrhiza glabra* (S05), especially against the strain *Pseudomonas* sp. OS4.

As noted earlier, the highest antioxidant properties were found for all emulsions (S04, S05, S08, and S09) and beneficial antibacterial activity for the following two systems: clove oil with *Quillaja saponaria* (S07) and cinnamon oil with *Glycyrrhiza glabra* (S05). These two emulsions did not distinguish themselves (from the other two emulsions) with some physicochemical properties like zeta potential or emulsion droplet samples. It is an interesting observation, suggesting the complexity of the mechanism of emulsions' components co-action, which cannot be simply explained by increased solubilization of EO components with surfactants and increased bioavailability of oil components. It can be rather a result of the co-action of different extract/oil components, leading to their increased antimicrobial activity.

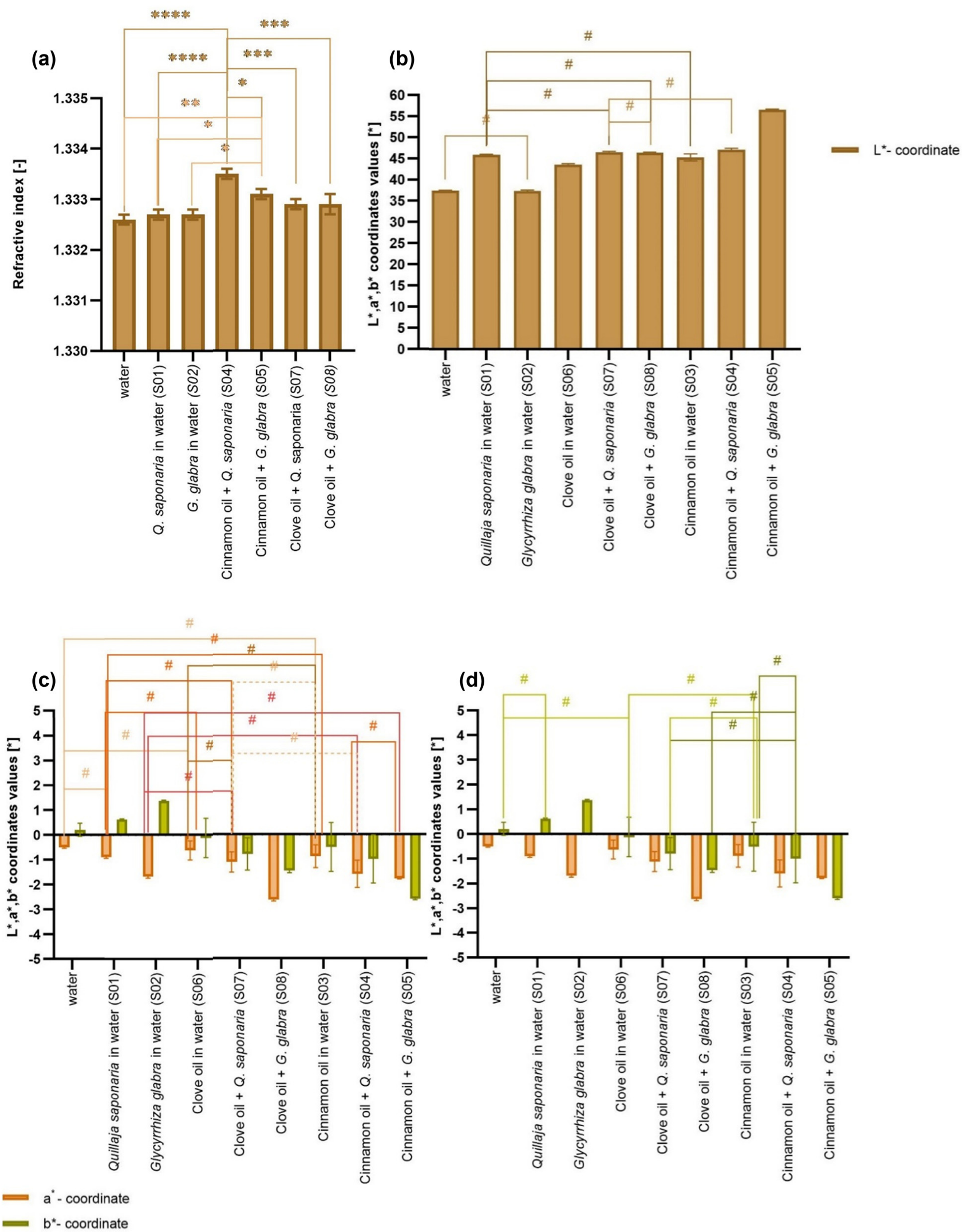

Figure 3: The optical properties of the tested colloidal systems: (a) RI; results labelled with an asterisk (*) in the data series are significantly different at $p < 0.05$. (b–d) Values of colour coordinates *L*, *a*, and *b*, respectively; results labelled with # in the data series are not significantly different at $p < 0.05$. Standard deviations are marked with error bars.

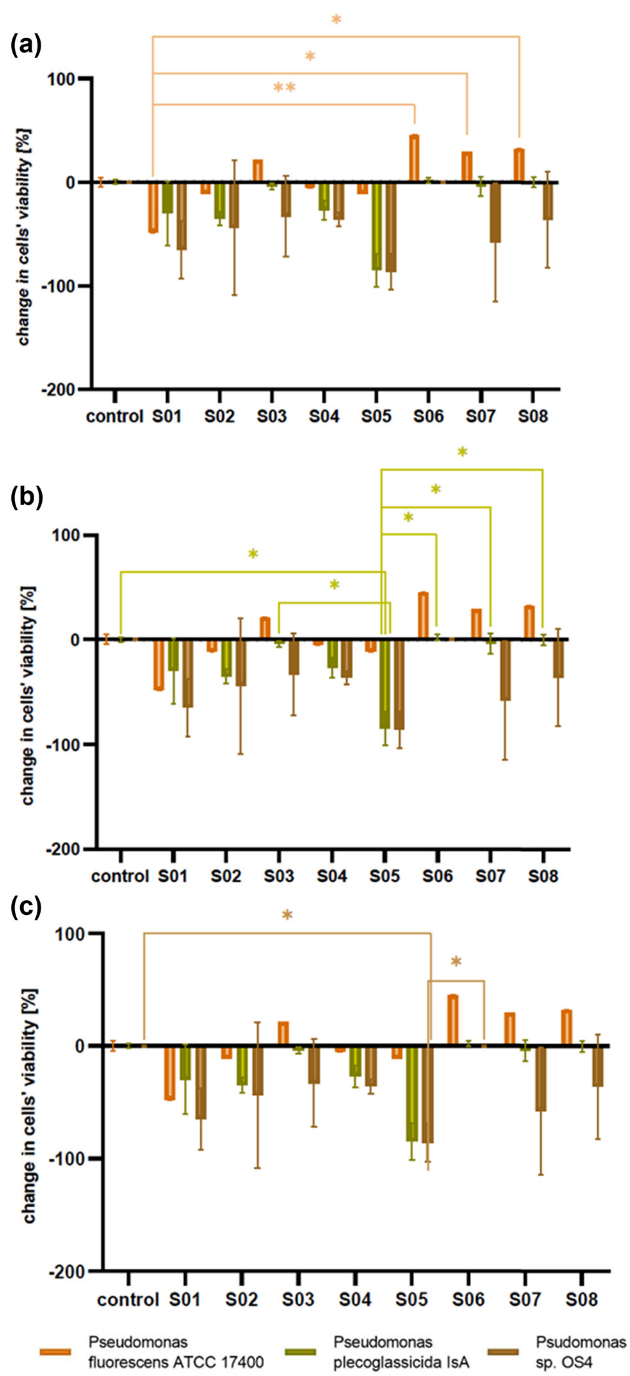

Figure 4: Impact of emulsions and plant extract solutions and their emulsions on bacteria cell viability; results labelled with an asterisk (*) in the data series (for each bacteria strain) are significantly different at $p <$ 0.05 for (a) *Pseudomonas fluorescens* ATCC 17400; (b) *Pseudomonas plecoglossicida* IsA; and (c) for *Pseudomonas* sp. OS4. Standard deviations are marked with error bars.

Many studies have been performed to evaluate the antibacterial activity of EOs. Siejak *et al.* [16] have studied the antibacterial properties of pure oils, which are components of the “five thieves” oil mixture and they found out that the most active was clove and cinnamon oil. A study by Shan *et al.* [39] determined that among the tested EO extracts, the highest antibacterial activity was shown by the clove extract. It was observed that cinnamon and clove oils had the best activity against all of the tested microorganisms. On the other hand, studies performed by Rosarior *et al.* [40] demonstrated the clove EO inhibitory behaviour against five urinary tract infections caused by both Gram-positive and Gram-negative strains. The examined opportunistic pathogens *Proteus mirabilis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* proved to be sensitive towards clove oil. However, no inhibition was observed with clove oil and the *Pseudomonas aeruginosa* strain.

3.4 Interrelationships between emulsion properties

In light of the conducted research, it can be noted that the use of extracts containing saponins as emulsifiers allows for modifying the emulsion properties of cinnamon and clove EOs. In the presence of plant extracts, the physicochemical properties changed, however, the direction and degree of change depended on the type of EO and the type of extract. The same was true for the antioxidant and antibacterial properties of the emulsions although it is possible to identify systems (clove oil with *Quillaja saponaria* and cinnamon oil with *Glycyrrhiza glabra)* where a significant increase in these properties was observed after the addition of plant extracts.

To get a better perspective of the correlations between the measured parameters describing the emulsions, an ANOVA was performed. The correlation matrix is presented in the Supplementary Materials (Table S1). The influence of the emulsion composition was slightly but statistically significantly influenced the wettability properties but not the RI of the investigated colloidal systems. Moreover, the ANOVA showed that the correlation between the analysed physical parameters of emulsions was relatively small and the most noticeable was the negative correlation between the average droplet size and the PDI of the samples.

4 Conclusions

A significant antibacterial action was observed in cases of clove oil with *Quillaja saponaria* and cinnamon oil with

Glycyrrhiza glabra. Moreover, the addition of plant extracts decreased the droplet size of the emulsions, which was different in samples without any plant extract. Enhanced emulsion stability, in the presence of selected extracts, was also confirmed by zeta potential measurements, where an increase in absolute values was observed. Importantly, the *Quillaja saponaria* extract did not have a significant impact on emulsion colour, RI, and wettability, which can be an advantage in using them in the food industry.

To conclude, the plant extracts containing saponins from *Quillaja saponaria* and *Glycyrrhiza glabra* appeared to be able to increase the stability and dispersion ratio of EO emulsions. They can be useful tools as food preservatives as well as systems useful in the pharmaceutical and cosmetic industry.

Funding information: This research was funded by the Ministry of Education and Science (Poland). Agata Zdarta was supported by the Foundation for Polish Science (FNP).

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Conflict of interest: Maciej Jarzębski, who is the co-author of this article, is a current Editorial Board member of *Reviews on Advanced Materials Science*. This fact did not affect the peer-review process. The authors declare no other conflict of interest.

Data availability statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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