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Original Article

Characterization of bergamot essential oil: chemical, microbiological and colloidal aspects

Caracterização do óleo essencial de bergamota: aspectos químicos, microbiológicos e coloidais

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Abstract

Citrus bergamia is a citric species known as bergamot. The species is widely used due to its derivatives, such as juices, extracts, and essential oil. Specifically, the bergamot essential oil (BEO) is of great interest, with a chemical composition rich in terpenes and esters. Considering its chemical composition, bioactivity, and great economic potential, the characterization of BEO should be studied. However, this essential oil is almost unexplored in terms of a characterization associated with colloids. Chemical characterization was carried out by gas-chromatography coupled to a mass spectrometer and by gas-chromatography coupled to a flame ionization detector. Antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* was carried out to confirm the bioactivity of this important essential oil. Dynamic light scattering analysis was performed to create a pattern of droplet size distribution of BEO. Major compounds of BEO were linalyl acetate, limonene, and linalool. The BEO was active against *E. coli* and presented a MIC value of 2.000 µg/mL, while values of MIC and MBC higher than 2.000 µg/mL were observed for *S. aureus*. The dynamic light scattering analysis revealed a mean hydrodynamic diameter of 65.7 ± 2.2 nm. After a 1:10 dilution it was observed reduction of mean diameter and enhancement of the percentagem of low size droplets, resepctively 44.1 ± 1.2 nm and 14.5 ± 0.5 nm (28.8 ± 1.2%). Higher droplets and reduced polydispersity index were observed after 1:100 dilution. In the present study, the chemical characterization was in accordance with the species, as the characteristic chemical markers of the species were found. Moreover, it has presented antibacterial activity as expected for the BEO. The analysis of the colloid showed a pattern of droplet size distribution following the Ostwald ripening mechanism after dilution.

Keywords: *Citrus bergamia*, limonene, *Escherichia coli*, *Staphylococcus aureus*, nanoemulsion.

Resumo

Citrus bergamia é uma espécie cítrica conhecida como bergamota amplamente utilizada industrialmente devido aos seus derivados, como sucos, extratos e óleo essencial. Especificamente o óleo essencial de bergamota (OEB) é de grande interesse, com uma composição química rica em terpenos e seus ésteres. Devido a sua composição química, bioatividade e grande interesse econômico, a caracterização do OEB deve ser alvo de estudos. No entanto, esse óleo essencial é praticamente inexplorado em termos de uma caracterização associada a coloides. Sua caracterização foi feita por cromatografia em fase gasosa acoplada à espectrômetro de massas e cromatografia em fase gasosa acoplada a detector de ionização de chamas. Atividade antibacteriana frente a *Staphylococcus aureus* e *Escherchia coli* foi realizada para confirmar a bioatividade do óleo essencial. Análise por espalhamento dinâmico da luz foi feita para criar um padrão de distribuição de tamanho de gotícula. Os constituintes majoritários foram o acetato de linalila, limoneno e linalool. O OEB foi ativo frente à *E. coli*, apresentando um valor de CMI igual à 2.000 µg/mL, enquanto values de CMI e CMB maiores do que 2.000 µg/mL foram observados frente à *S. aureus*.. A análise por espalhamento dinâmico da luz revelou um diâmetro hidrodinâmico médio de 65.7 ± 2.2 nm. Após diluição de 1:10, houve redução do diâmetro médio acompanhado de aumento do percentual de gotículas de baixo tamanho (14.5 ± 0.5 nm, 28.8 ± 1.2%). Foi observado aumento do tamanho e redução do índice de polidispersividade após uma maior diluição (1:100). Discussão: No presente estudo, a caracterização química está em consonância com a espécie, sendo encontrados os marcadores químicos da mesma. Além disso, o OEB apresentou a atividade antibacteriana esperada. A análise do coloide mostrou um padrão de distribuição de tamanho de gotícula de acordo com o mecanismo de Maturação de Ostwald após diluição.

Palavras-chave: *Citrus bergamia*, limoneno, *Escherichia coli*, *Staphylococcus aureus*, nanoemulsão.

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1. Introduction

Essential oils (EOs) are complex mixtures of lipophilic and volatile substances of low molecular weight from the secondary metabolism of plants, biosynthesized in specific cells of different parts such as leaves, roots, fruits, flowers, and bark, or even produced as artifacts through extractive processes from chemical precursors present in the utilized plant material (Sadgrove et al., 2022; Silva et al., 2022; Mukurumbira et al., 2022).

Literature data indicate that several EOs have interesting biological activities, such as antioxidant, anti-inflammatory, larvicidal, antifungal, and antibacterial properties, among others. Therefore, they are of great interest to the food, pharmaceutical, and cosmetic industries (Ferreira et al., 2019; Silva et al., 2022; Hou et al., 2022). Data from Grand View Research (2023) point out that the global market for EOs was valued at USD 21.79 billion in 2022. It is expected to have an annual growth of 7.9% from 2023 to 2030, as essential oils increasingly gain more relevance due to the associated health benefits and reduced risk of side effects.

Citrus EOs are considered valuable, since they have a large spectrum of biological activities, especially antimicrobial, and are commonly used in the industry (Ceccato-Antonini et al., 2023). Bergamot essential oil (BEO) (*Citrus bergamia),* for example, is known as the fruit of health and is described as having an anti-inflammatory, anticancer, antioxidant, and anti-microbial activity (Marchese et al., 2020; Valussi et al., 2021; Adorisio et al., 2023). These biological activities are associated with the chemical composition of BEO, rich in monoterpenes and sesquiterpenes, such as limonene, linalyl acetate, α-pinene, β-pinene, and ϒ-terpinene (Valussi et al., 2021; Salvino et al., 2022).

Colloids are a large group of sub-micron systems that comprises nanoemulsions. They are colloidal dispersions constituted by an internal phase at the nanoscale. Most of the nanoemulsions are oil-in-water type, therefore being constituted by nanodroplets of the oil distributed in the water external phase (Solans and Solé, 2012). Most nanoemulsions need a surfactant in their composition and the properties may be intrinsically associated with these stabilizers. In this context, one would expect that pattern of dynamic light scattering (DLS) could be used for the quality control of essential oils (Fernandes et al., 2013). Changes in the hydrodynamic diameter of colloids constituted by surfactant-natural product are reported for flavonoids with detailed description of the size distribution profile (Singh et al., 2017). However, to the best of our knowledge, this important parameter as that can be userd as pattern for quality control (fingerprint) is almost unexplored for essential oils.

Therefore, considering the high relevance of BEO, the aim of the present study was to perform its characterization through different techniques, in term of chemical profile by gas-chromatograph analysis, biological profile of antimicrobial effect, and colloidal profile in term of dynamic light scattering analysis for the fingerprint of droplet size distribution.

2. Methodology

2.1. Materials

The BEO was purchased from Quinari (Ponta Grossa, Brazil). The polysorbate 20, polysorbate 80 and sorbitan monooleate were purchased by Sigma-Aldrich (São Paulo, Brazil). Bacterial strains were obtained from the Laboratory of Molecular Epidemiology and Biotechnology of Federal Fluminense University, Rio de Janeiro, Brazil. Bacterial strains *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were stored in Brain Heart Infusion (BHI) broth with 10% of glycerol at -80 °C.

2.2. Bergamot essential oil (BEO) characterization

The BEO was characterized by gas chromatography (GC) coupled to a mass spectrometer (MS) model GC-MS QP2010 (Shimadzu) and quantified by gas chromatography coupled to a flame ionization detector (FID) model GC-2014 (Shimadzu). One microliter of the essential oil was dissolved in CH_2CL_2 (1:100 mg/mL) and injected in a DB5 for MS column (id = 0.25 mm, length 30 m, thickness = 0.25 µm). The chromatographic conditions were: helium as carrier gas with a flow rate of 1 mL/min, injector temperature was 260 °C with a split injection of 1:40. The oven temperature was initially 60 °C and then increased to 3 °C/min until 290 °C. The MS analysis was realized at 70 eV and 1 scan/s of scan rate.

The arithmetic index (AI) was calculated by interpolating the retention time (RT) of the aliphatic hydrocarbon standard (C7-C40) analyzed under the same chromatographic conditions described above. The BEO chemical identification was realized by comparing the AI and/or mass spectra fragmentation pattern with those described in the literature (Adams, 2017, NIST). The relative abundance of the essential oil constituent was quantified by GC-DIC with the FID peak area normalization method.

2.3. Minimum Inhibitory Concentration (MIC)

Strains were streaked in Tryptic Soy Agar (TSA) plates, and after 24 hours, colonies were transferred to 0.85% NaCl solution and adjusted to a 0.5 McFarland standard scale. BEO previously solubilized in DMSO was applied in 96-well plates with Tryptic Soy Broth (TSB) followed by a serial dilution in ranging concentrations from 2 mg/mL to 0.015 mg/mL. The antimicrobial agents vancomycin and ciprofloxacin were used as positive controls, for the Gram-positive and the Gram-negative bacteria, respectively. Plates were incubated overnight for 24 h at 37 °C and after preparing the sample for further Minimum Bactericidal Concentration (MBC), 20 µL of resazurin dye was added to reveal MIC results. Tests were performed in triplicates based on the standard broth microdilution method set under laboratory conditions (CLSI, 2015).

2.4. Minimum Bactericidal Concentration (MBC)

After incubation of the plates for the MIC determination, aliquots of 10 µL from each well were applied in Petri plates with TSA divided in fields corresponding to each concentration of the microdilution, including positive controls,

followed by incubation for 24 h at 37 °C. MBC endpoint is defined according to the last quadrant without bacterial growth indicating the elimination of 99.9% of bacteria.

2.5. Preparation of BEO nanoemulsions

The nanoemulsions were prepared by a low-energy method by phase inversion. The oil phase consisted of BEO and a mixture of surfactants composed of non-ionic surfactants (polysorbate 20 or polysorbate 80 and sorbitan monooleate). The oil phase was homogenized by using a vortex tube homogenizer model AP-56 (PHOENIX), then the aqueous phase (deionized water) was slowly added dropwise into the oil phase under constant homogenization.

2.5.1. Influence of surfactant (s)

Non-ionic surfactants were used in pairs, aiming to reach a wide range of hydrophile-lipophile balance (HLB) values (Table 1). The compositions were as follows: 95% (w/w) of deionized water, 2.5% (w/w) of *C. bergamia*, and 2.5% (w/w) surfactant mixture. The masses of surfactants defined in the mixtures were made based in the Equation 1:

$$\frac{HLB = (HLBa\ x\ Ma) + (HLBb\ x\ Mb)}{Ma + Mb} \quad (1)$$

where: HLB = HLB resulting from the mixture of surfactants; HLBa = HLB of the surfactant of the more hydrophilic pair; HLBb = HLB of the surfactant of the more lipophilic pair; Ma = mass of the most hydrophilic surfactant; Mb = mass of the most lipophilic surfactant.

2.5.2. Influence of surfactant to BEO ratio (SBEOR)

The composition of the nanoemulsions was changed in terms of surfactant to BEO ratio at a constant water content (95%, w/w). The SBEOR values of 1, 2, 3, 4, and 5 were made based in the Equation 2:

$$SBEOR = m_{surfactant} / m_{BEO} \quad (2)$$

Table 1. Different surfactants used as mixtures and HLB ranges used in the study.

Surfactants	HLB range
Polysorbate 20 + Sorbitan monooleate	9-16.7
Polysorbate 80 + Sorbitan monooleate	9-15

2.5.3. Characterization of BEO nanoemulsions

A macroscopical evaluation was carried out to identify which formulations did not show signs of instability, such as creaming and phase separation, adherence, opacity, fluidity, transparency, and presence of the Tyndall effect (bluish reflection).

In the physicochemical evaluation, the average particle size (Z-average), polydispersity index (PdI), and zeta potential (ZP) of the *C. bergamia* nanoemulsions were obtained using the Dynamic Light Scattering (DLS) technique in a Zetasizer Advance Lab Blue (Malvern, UK), applying non-invasive back-scatter (diffusion angle of 90º) at room temperature (25 °C). All analyses were diluted in ultrapure water to minimize the multiple scattering effects.

3. Results and Discussion

The BEO presented 27.0% of linalyl acetate and 25.9% of monoterpene limonene, the characteristic chemical markers of the species (Cebi and Erarslan, 2023). Other relevant constituents found in the oil were β-pinene (2.9%), terpinene (3.0%), and linalool (13.4%) (Table 2). Variations in the chemical profile of essential oils may be associated with variations in plant secondary metabolism biosynthesis or accumulation due to extrinsic factors, such as temperature, circadian cycle, soil nutrients, collection time, extraction type, and others (Zhang et al., 2023). Regarding BEO, Dosoky and Setzer (2018) described the terpenoids *d*-limonene (27.4-52%), linalool (1.7-20.6%), linalyl acetate (17.1-40.4%), β-pinene (4.4-11.0%) and γ-terpinene (5.0-11.4%) as usual components of BEO obtained by pressing, therefore corroborating with the chemical profile found in the herein presented study. Regarding the main compounds that totalized more than 65% of the BEO, the U.S. Food and Drug Administration considers the linalyl acetate, limonene and linalool as substances generally recognysed as safe (GRAS) (FDA, 2023).

Table 2. Chemical characterization of *Citrus bergamia* peel essential oil by GC-MS and GC-FID.

	RT	AI_{calc}	AI_{lit}	Substances	%
1	6.685	978	974	β-pinene	2.9
3	8.272	1029	1024	Limonene	25.9
4	9.308	1058	1054	γ-Terpinene	3.0
5	10.856	1102	1095	Linalool	13.4
6	17.179	1255	1254-	Linalool acetate	27.0
		TOTAL			72.2
		Monoterpene hydrocarbons			34.7
		Oxygenated monoterpene			40.4

RT: retention time; AI_{lit}: arithmetic index from literature; AI_{calc}: arithmetic index calculated.

The results of the antibacterial assay of the BEO against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) strains by microdilution in the 96-well plates method are shown in Table 3. It was not possible to verify growth inhibition against *S. aureus* strain, considering that MIC and MBC values in the present study were greater than 2 mg/mL, which was the highest concentration of the sample used in this study. However, concerning the *E. coli*, it was possible to observe MIC = 2 mg/mL and MBC > 2 mg/mL, indicating the bioactivity of the BEO.

The BEO bacteriostatic activity against *E. coli* strain may be associated with the presence of its terpenes. Regarding the monoterpene limonene, one of BEO chemical markers, it is well described in the literature as an antibacterial molecule, especially against *E. coli* strains. It has MIC of 2000 µL/L with pharmacological mechanisms related to oxidative DNA damage and cellular membrane damage. The Gram-negative outermost cellular membrane is constituted of lipopolysaccharides, structures with hydrophilic characteristics, that prevent the permeability of lipophilic substances. In that way, it is suggested that hydrocarbon monoterpenes, such as limonene, may induce enhanced membrane permeability (Gupta et al., 2021).

Regarding the preparation of BEO nanoemulsion, the systems prepared with polysorbate 20 and sorbitan monooleate at HLBs 9 - 11 and 15 presented creaming, while those corresponding to HLBs 16 and 16.7 were opaque. The pair consisting of polysorbate 80 and sorbitan monooleate induced the formation of creaming for the nanoemulsions in all HLBs, except for the system formed solely with polysorbate 80 (HLB = 15).

Macroscopical appearance gives an important interpretation of colloidal systems and should not be neglected, since opaque systems are often associated with larger droplets when compared to transparent/translucent systems. Moreover, creaming is a destabilization indicative that relies on the gravitational force-induced formation of an upper layer of droplets when the internal phase has a lower density than the external phase (McClements and Jafari, 2018).

BEO-nanoemulsions previously prepared through a high-energy nanoemulsification method showed no major differences in the size diameter and PdI of BEO-droplets population generated with polysorbate 20 and polysorbate 80 (Severino et al., 2014, 2015; Ribes et al., 2017). However, it is worth mentioning that high-energy methods may induce small narrow populations of colloids due to high disruptive forces. Low-energy methods rely on the chemical energy of the system and therefore, composition may have a major influence. One would expect that because polysorbate 20 is more hydrophilic than polysorbate 80, it would also facilitate destabilization if the release of more hydrophilic compounds of the EO migrates from small to larger droplets through the aqueous phase (Solans and Solé, 2012).

Although the HLBs 12 - 14 prepared with polysorbate 20/sorbitan monooleate also showed a bluish reflection, the surfactant polysorbate 80 used individually for nanoemulsification was chosen due to its good compatibility and possible cost reduction for nanoemulsified prototypes. Then, a prototype of formulation constituted by 95.0% (w/w) of water and 5% oily phase (polysorbate 80 and BEO) was chosen to investigate the influence of different ratios between the components of the oily phase.

The macroscopical appearance indicated that a tendency for more translucent/transparent systems was observed as the SBEOR increased (1:1 → 5:1) (Figure 1). At SBEOR 1 and 2, the system presented a fine appearance, however, a main white aspect was observed, while the system at SBEOR 3 presented a tendency for translucence instead of an opaque appearance. When SBEOR 4 and 5 were reached, a more transparent aspect with remarkable bluish reflection associated with the Tyndall effect was observed. Even though one would expect that at higher SBEOR, a minimum droplet size would be reached, no main differences in the macroscopical appearance were observed between nanoemulsions at SBEOR 4 and 5 immediately after preparation. In fact, after a few minutes of preparation, it was possible to observe a tendency for a slight increase in turbidity of nanoemulsion at SBEOR 5. This would also be in accordance with a tendency for Ostwald ripening, since despite higher SBEOR often allowing size reduction, it may also facilitate the release of hydrophilic compounds.

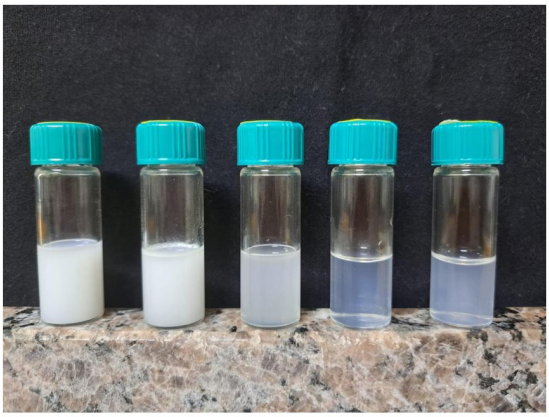

Figure 1. Macroscopical appearance of BEO-nanoemulsions prepared with 95% (w/w) of water and oily phase constituted by mixtures of polysorbate 80/BEO. From the left to the right the system were prepared at respective SBEOR: 1, 2, 3, 4 and 5.

Table 3. Minimum inhibitory and bactericidal concentration (µg/mL) of BEO against *S. aureus* and *E. coli* strains.

Strains	Essential oil (µg/mL)	
	MIC	MBC
Staphylococcus aureus (ATCC 25923)	>2,000	>2,000
E. coli (ATCC 25922)	2,000	> 2,000

Therefore, considering not only the ease to generate but also stabilizing the system, the nanoemulsion prepared at SBEOR 4 was chosen for a dynamic light scattering analysis to confirm the colloidal system formed with BEO. Figure 2 shows the droplet size distribution of BEO nanoemulsion and it can be observed that the concentrated colloid has a mean droplet diameter of 65.7 ± 2.2 nm with a predominance of droplets around 190 nm (~70%) followed by droplets around 15 nm (~20%). After dilution at a 1:10 ratio, an increase of lower droplets abundance to around 30% was accompanied by reduction of mean droplet diameter, reaching 44.1 ± 1.2 nm. This is in accordance with a slight disintegration of larger droplets without a main breakdown of the system, probably due to the incorporation of EO compounds in the micelles, the probable structures associated with a low-size population (Rao and McClements, 2012). Then, at higher dilution (1:100), a great droplet size increase is observed reaching mean size diameter of 469 ± 13.2 nm, with around 90% of high droplets (~600 nm) that corroborates with the Ostwald ripening mechanism of destabilization (Solans and Solé, 2012).

Utilization of dynamic light scattering is promising for spectral information of natural products. The self-aggregation of linoleic acid was studied and no major alteration of the pattern of size distribution was observed in a range of concentrations (Degrand et al., 2023). Investigations of the profile of size distribution of some flavonoids, such quercetin and kaempferol, with surfactants revealed enhancement of size diameter after solubilization in the micelles. Since different size of colloidal structures result in different intensity of light scattering, distinct profiles of size distribution can be reached (Singh et al., 2017).

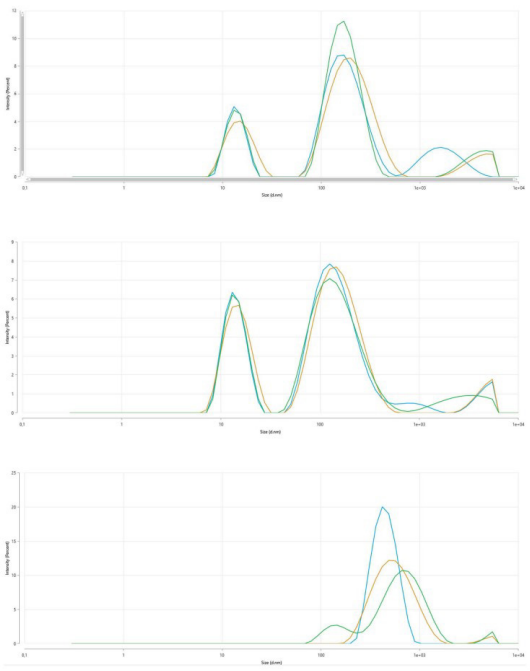

Figure 2. Droplet size distribution graphs of BEO nanoemulsion obtained by dynamic light scattering. From upper figure to down figure are respective the graphs of concentrated colloid, 1:10 diluted colloid and 1:100 diluted colloid.

4. Conclusion

Most of studies aiming to characterize herbal derivatives focus in single phytochemical markers. In the present we provide chemical characterization that confirmed the presence of main compounds of BEO and also the presence of an antimicrobial activity, corroborating the authenticity of this valuable natural raw material. Moreover, we use the dynamic light scattering technique to provide a pattern of droplet size distribution of the BEO. Since chemical pattern, biological potential and size distribution are intrinsically associated and due to high potential of the BEO, our data contributes significantly to chemistry of phytocolloids and provides a suitable combination of techniques that can be further used for quality control of this herbal derivative.

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