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RESEARCH ARTICLE

Chemical Composition and Antibacterial Activity of Bergamot Peel Oil from Supercritical CO_2 and Compressed Propane Extraction

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Abstract:

Objective:

Essential oils are widely used as flavors and fragrances in the food, cosmetic and pharmaceutical industries, especially the bergamot peel oil due to the high polyphenols content, compared to other citrus species. Two types of polyphenols present in bergamot peel oil, brutieridin and melitidin, are directly related to cholesterol biosynthesis inhibition in a similar way as the statins. In this context, this work reports the extraction yields of bergamot peel oil obtained by supercritical carbon dioxide and compressed propane, together with the antimicrobial activity.

Methods:

The experiments were conducted at 55°C and 350 bar (density 0.881kg/m^3) for carbon dioxide and at 55 °C and 40 bar (density 0.441 kg/m^3) for propane.

Results:

Regarding the antimicrobial activity, the minimum inhibitory concentrations of bergamot oil were effective for the gram-positive bacteria growth inhibition, *Staphylococcus aureus* at 31.25 µg.mL^{-1} of bergamot oil, while 500 µg.mL^{-1} of oil extract was necessary to afford gram-negative bacterium (*Escherichia coli*) inhibition.

Keywords: Bergamot peel oil, Compressed solvents, Antibacterial activity, Citric fruits, S. aureus, E-coli.

1. INTRODUCTION

Research focused on the development of clean technologies, especially on the extraction of natural compounds, has been applied to several areas of science and largely devoted to food, cosmetics, and pharmaceutical industries [1, 2]. Recently, citrus essential oil has gained increasing attention due to the hypoglycemic and hypolipidemic activity as well as anti-inflammatory properties [3 - 6].

Among citric fruits, *Citrus deliciosa bergamia* (bergamot) has an associated peel essential oil with high commercial value due to the great variety of compounds of interest such as limonene, linalool and linalyl acetate [7, 8] that can be applied in different processing industry areas. Thus, bergamot peel obtained by cold extraction cannot be considered a residue of the citrus juice industry as it is a very valuable product, high price, used in perfumery and cosmetic industry.

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Concerning the biological activity of bergamot peel oil, some studies have reported the anti-inflammatory, antiproliferative, and analgesic effect, including effects on the cardiovascular and central nervous system [9 - 12].

Among several different techniques available for the extraction of essential peel oils from vegetable matrices, the use of pressurized solvents has demonstrated great potential, as long as physicochemical properties (density, diffusivity, viscosity, and dielectric constant) can be finely tuned by pressure and temperature [13, 14]. Besides, the use of pressurized fluids is considered an environmentally friendly, economic and safety technology demanded by industries and consumers, as it is associated with the use of non-toxic and relatively lower cost solvents [15 - 17]. When compared to the conventional methodologies that make use of organic liquid solvents, the use of compressed gases does not require an additional separation step for solvent removal, hence preventing possible degradation of thermo-sensitive compounds.

Although several research works have focused on the development of clean/green technologies for the natural compounds extraction, there is a lack of information in the literature regarding the bergamot peel oil extraction using supercritical carbon dioxide ($SCCO_2$) and compressed propane as solvents, considering the chemical profile and antimicrobial activity of the oils obtained. Though $SCCO_2$ has been the solvent selected for extraction of herbaceous matrices, propane may be advantageous for some applications due to its low dielectric constant, comparable to $SCCO_2$, the ability to extract lipophilic compounds, much reduced working pressure needed, which means lower Capex and Opex (Capex- capital expenditure costs; Opex- operational expenditure costs) [18]. Although the use of propane may cause doubts about safe operation, it might be taken into account that much lower pressures than $SCCO_2$ can be employed, typically in the order of 10 bar (as solvent density becomes almost invariant above this value). Additionally, good high precision detectors of light hydrocarbons are available in the market, hence preventing leakage problems - such points have recently drawn attention from the industrial sector [19, 20].

In this context, the main goal of this work was to evaluate the use of carbon dioxide and propane in the oil extraction of *Citrus deliciosa bergamia* peel (bergamot peel) and compare the results in terms of extraction yield and antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, as microorganism models. The target here is to use and environmental benign extraction technology combining with the use of a renewable, waste, raw material, as well as replacing the use of organic solvents without residue left in the final product.

2. EXPERIMENTAL

2.1. Materials

Bergamot was harvested in July 2014 from native plants in Descanso (Santa Catarina State, Brazil). After harvesting, the bergamot (*Delicious Citrus Bergamia*) was washed with abundant water and then peeled. Peels were cut in 1 cm^2 pieces and then stored at -18 °C in polypropylene bags. Neither in the peeling step nor in peel cutting, losses of bergamot peel oil were visually observed. Propane and carbon dioxide (CO_2) with minimum purity of 99.5% in the liquid phase was purchased from White Martins S.A. *Staphylococcus aureus* strains (ATCC 25923) and *Escherichia coli* strains (ATCC 25922) were used for the antimicrobial activity tests. Brain Heart Infusion Broth (BHI), 2,3,5-Triphenyl-tetrazolium chloride (TTC), and dimethylsulfoxide (DMSO, 99%) was purchased from Merck, Sigma-Aldrich and Vetec, respectively.

2.2. Bergamot Peel Oil Extraction With Pressurized Propane and Supercritical CO_2

Before extraction, the previously cut bergamot peels were crushed in a slicer and sieved to obtain particles of mean size ≤ 2 mm. Typically, around 30 g (±0.05 g) of raw material was used in all extraction experiments. The equipment consisted of four components: a solvent gas cylinder, a high-pressure pump, an extraction vessel, a micrometric valve connected to collection flask and a temperature and pressure control systems. A detailed discussion about experimental apparatus, methodology and extraction conditions can be found in the works of Capeletto *et al*. (2016) [21] and Scapinello *et al*. (2015) [22].

After preliminary tests, the extraction conditions for all assays were set as solvent flow rate of 3 mL min^{-1} and 2 h of extraction time for experimental runs. In fact, preliminary tests showed that extraction times greater than 2 h led to negligible gains of the extract obtained and therefore above this time resulted in practice in solvent losses with additional costs and unfruitful results. Also, preliminary tests and experience from previous works of our research group show that good extraction conditions may be 55 °C and 350 bar for $SCCO_2$ (density 0.881kg/m^3) and 55 °C and 40 bar

(density 0.441kg/m^3) for propane. The purpose for these extraction conditions was to increase the possibility of extraction a larger number of chemical compounds by raising vapor pressure of pure components while improving solvation trough adequate solvent extracting densities. For all extraction conditions using $SCCO_2$ and pressurized propane, bergamot peel oils obtained were collected in amber bottles and then stored under refrigeration (5 ºC ± 2 ºC). The overall extraction yield of bergamot peel oil was obtained by the relation between the total mass oil extracted by the mass of the sample. Based on triplicate experiments carried out for all the experimental conditions for both compressed solvents, the overall average standard deviation of the yields noticed was about 0.2 wt%.

2.3. Bergamot Peel Oil Analysis by Gas Chromatography (GC and GC-MS)

The chemical composition of bergamot peel oil was determined using a Shimadzu gas chromatograph model Varian 3800 equipped with a split-injection port, flame-ionization detector and a HP-Innowax column (25 m x 0.25 mm x 0.5 μm). The chromatograph operating conditions were: split = 1:150; column flux = 0.92 $mL.min^{-1}$, detector temperature: 250 °C, injector temperature: 250 °C, oven temperature: 60 °C (8 min), 180 °C (4 °C min^{-1}), 180 °C - 230 °C (20 °C min^{-1}), 230 °C (20 min) using helium as carrier gas: split ratio: 50:1, 34 kPa, and injected volume of 1.0 μL. The composition was obtained from electronic integration measurements using flame ionization detection. The GC-MS analysis was performed on a GC-MSD system model HP 5973-6890, operating in EI mode at 70 eV, equipped with a cross-linked column capillary (HP-5, 30 m x 0.25 mm). Helium was used as carrier gas (56 kPa, 1 $ml.min^{-1}$). The identification of compounds in the bergamot peel oil was based on the retention index, determined as a function of a homologous series of C_7-C_{30} n-alkanes, under identical experimental conditions, to the NBS mass Library (Massada, 1976) described by Felton *et al*. (2009) [24]. The relative amounts of the individual components were calculated based on the GC peak area (FID response).

2.4. Minimum Inhibitory Concentration Assays (MIC)

The antimicrobial analyses to determine Minimum Inhibitory Concentration (MIC) were done for the bergamot peel oil extracted with pressurized propane and supercritical CO_2 (Table **1**). The assays were carried out in triplicate on systems sterile microplates containing 96 cavities shaped bottom "U" containing 100 μL of Brain Heart Infusion Broth (BHI), following the adapted method described by Hentz and Santin (2007)] [25]. A 200 μL volume of the sample of bergamot peel oil extracted at the concentration from 27 $mg.mL^{-1}$ (diluted in 10% DMSO) was filtered (0.45 μm Millipore filter) and inoculated in BHI medium. Also, the blank test was done with 100 μL of BHI in 10% DMSO. The bacterial inoculums of *Staphylococcus aureus* and *Escherichia coli* with a concentration of 0.5 in the McFarland scale (10^8 UFC mL^{-1}) were diluted in 0.9% in a sterile saline solution and a volume of 5 μL (10^4 UFC mL^{-1}) was deposited in each cavity of the microplate utilizing a micropipette. In each line of the microplates containing different concentrations of the bergamot peel oil extract, adjusted with DMSO 10%. The 96-well microplates were incubated in a bacteriological oven at 35 °C for 18 hours. Afterward, 20 μL de TTC 0.5% was added in each cavity of the microplate and again incubated for another three hours. The MIC was defined as the lowest concentration, enough to inhibit the microbial growth (Mann and Markham, 1998).

Table 1. Chemical composition of bergamot peel oil obtained from supercritical CO_2 (55 °C and 350 bar) and compressed propane extraction (55 °C and 40 bar).

Components	RI[a]	RI[b]	Area (%)	
			CO_2	propane
α-Terpene	931	932	0.17	0.09
α-Pinene	939	938	0.83	0.54
Canfene	953	953	0.05	-
Sabinene	976	975	1.64	1.11
β-Pinene	980	982	6.27	4.08
Mircene	991	993	0.36	0.95
p-Cimene	1026	1026	0.18	0.46
Limonene	1031	1030	45.13	21.53
γ-Terpinene	1062	1062	3.25	6.08
Linalool	1098	1096	11.35	15.16
α-Terpineol	1189	1190	0.09	0.70
Neral	1228	1225	1.37	1.12

(Table 1) contd.....

Components	RI[a]	RI[b]	Area (%)	
			CO_2	propane
Linalyl acetate	1257	1255	23.67	19.74
Geranial	1270	1269	0.25	2.03
Citronelil acetate	1354	1351	-	0.18
Neryl acetate	1365	1362	1.34	0.57
β-Cariofilene	1418	1417	0.84	1.96
Neryl acetate	1434	1434	0.47	2.61
Trans-α-bergamotene	1436	1439	0.08	1.17
Germacrene D	1480	1480	0.15	0.90
β-Bisabolene	1509	1511	1.27	3.04
α-Humulene	1454	1457	-	0.10
γ-Muurolene	1477	1476	1.03	2.81
Carifilene oxide	1581	1580	0.06	-
Total identified (%)	–		**99.85**	**86.93**

[a]Retention indexes from literature (Adams, 1995).

[b]Experimental retention indexes (based on homologous series of *n*-alcanes C_7-C_{30}).

3. RESULTS AND DISCUSSION

3.1. Extraction Performance

The obtained extraction yields, defined as the weight percentage of the oil extracted with respect to the initial charge of the raw material in the extractor, were 0.48 wt% and 0.37 wt% for $SCCO_2$ and pressurized propane, respectively. Table **2** shows the bergamot peel oil composition obtained by $SCCO_2$ and pressurized propane extraction. It can be observed that more compounds were identified from the $SCCO_2$ (99.8%) extract compared to propane (~ 87%), which may be justified to the fact that in the experimental conditions used the propane has greater power of solvation and in this way extracted less volatile compounds, with higher molecular weight and consequently greater structural complexity that were not identifiable by GC-MS [26].

Table 2. Minimum inhibitory concentration (MIC) of bergamot peel oil extracts.

Target microorganism	Supercritical CO_2	Compressed propane
–	MIC (mg/ml)	MIC (mg/ml)
E. coli	500	125
S. aureus	31.2	31.2

Comparison of chemical profile obtained from the application of extraction solvents shows that the major constituent in both bergamot peel oils was limonene, an important monoterpene, precursor of many pharmaceuticals and chemicals [27], with 45% observed for $SCCO_2$ and 21.5% for propane. These results are in accordance with the studies reported by Sawamura *et al*. (2006) [28], in which the authors reported limonene as the major component in bergamot essential oil.

Other important components identified in bergamot peel oil were linalyl acetate, linalool and geraniol with 23.6%, 11.3%, and 0.25% of the total components extracted with CO_2 and 19.7%, 15.1%, and 2.03% for propane, respectively. As reported by Elisabetsky *et al*. (1995) [29], compounds such as linalyl acetate and linalool may be associated with anxiolytic activity. According to Camargo and Vasconcelos (2014) [30], linalool is a secondary metabolite, one of the most important substances in the pharmaceutical industry, used by popular medicine as anti-inflammatory, analgesic, hypotensive, and antimicrobial properties [10, 31, 32].

Results reported in this research work agree with those published by Dugo *et al*. (2000) [33] and Russo *et al*. (2013) [34], who reported that bergamot peel oil has a non-volatile fraction (4-7%), characterized by coumarins and furocoumarins, and a volatile fraction (93-96%) with hydrocarbons as monoterpenes and sesquiterpenes such as limonene, γ-terpinene, α-β-pinene, β-myrcene, sabinene, β-bisabolene, and oxygenated derivatives, such as linalool, linalyl acetate, neral, geraniol, neryl and geranyl acetate.

3.2. Antibacterial Assays

From the MIC results, it was noticed that the bergamot peel oils were efficient as bacterial growth-inhibitor. However, the antibacterial effect was higher against Gram-positive bacteria (*S. aureus*) with a determined MIC of 31.2 $\mu g.mL^{-1}$. On the other hand, Gram-negative bacteria (*E. coli*) was more resistant, requiring higher concentrations of bergamot peel oil, 500 $\mu g.mL^{-1}$ of oil obtained with $SCCO_2$ and 125 $\mu g.mL^{-1}$ of with pressurized propane. The resistance of Gram-negative bacteria such as *E. coli* may be associated with a complex cell wall compared to Gram-positive bacteria, thereby increasing their resistance to the antibacterial agents. Ashok Kumar *et al*. (2011) [35], using five different solvent extracts of *Citrus lemon* and *Citrus simensis*, analyzed the antimicrobial activity and reported a MIC of 12.5 $mg.mL^{-1}$ for *E. coli* and for 25 $mg.mL^{-1}$ for *S. aureus*, these results is similar to the strong antimicrobial activity of the bergamot oil obtained in this work.

The difference in the minimum inhibitory concentration for *E. coli* for the two bergamot peel oils obtained with CO_2 (500 $\mu g.mL^{-1}$) and propane (125 $\mu g.mL^{-1}$) may be related to differences regarding chemical composition of oils obtained, and might be associated with the compounds γ-terpinene (3.25% for CO_2 and 6.08% propane), geraniol (0.25% CO_2 and 2.03% propane), β-caryophyllene (0.84% CO_2 and 1.96% propane), neryl acetate (0.47% CO_2 and propane 2.61%), trans- α -bamamotene (0.08% CO_2 and 1.17% propane), β-bisabolene (1.27% CO_2 and 3.04% propane) and linalool (11.35% CO_2 and 15.16% propane).

According to Sokovié *et al*. (2010) [36], linalool has higher antimicrobial effects against Gram-positive bacteria compared to Gram-negative ones, with an effect on protein denaturation or dehydration of the cells. Limonene, a cyclic hydrocarbon, can accumulate in the biological membrane and change its structure and function [37]. In addition, limonene is reported as an antifungal agent [38], bacteriostatic [39, 40], and anti-bactericidal plasma membrane of the bacteria and causes the loss of the bacterial membrane integrity [41]. A study of *E. coli* inactivation by terpenes and terpenoids (such as carvacrol or citral) demonstrated the sublethal lesions in the cytoplasmic membrane [42] suggesting the bactericidal membrane rupture as the inactivation mechanism [43].

According to Prado *et al*. (2013) [44], Wang *et al*. (2008) [45], Sartoratto *et al*. (2004) [46] and Michielin *et al*. (2009) [47], it is possible to classify the antimicrobial agents based on MIC values. Michielin *et al*. (2009) [47] classified the extracts as strong inhibitors with MIC up to 500 $\mu g.mL^{-1}$, moderate inhibitor for MIC from 600 to 1500 $\mu g.mL^{-1}$, and low inhibitor for MIC over 1600 $\mu g.mL^{-1}$, allowing positioning the bergamot peel oils obtained in this work as a strong inhibitors agent against Gram-positive and Gram-negative bacteria.

It should be mentioned that many compounds present in the essential oil from vegetable matrices, *e.g.* limonene, becomes effective in combination with heat in the inactivation of some bacteria such as *E. coli* and *Listeria monocytogenes* [42] due to a synergistic effect on pathogens inactivation, making them able for use in food preservation, keeping the organoleptic properties of the fresh food products [43].

CONCLUSION

This work reports the application of a green technology for the extraction of bergamot peel oil, looking for the green chemistry approach in replacement of traditional organic liquid solvents, positively affecting the quality of the final product. It was possible to identify 99.85% and 86.93% of chemical compounds from bergamot peel oil extract using CO_2 and propane, respectively. In general, both extracts presented limonene as the major constituent - 45.13% for CO_2 and 21.53% for propane, followed by linalyl acetate - 23.67% for CO_2 and 19.74% for propane, and linalool - 11.35% for CO_2 and 15.16% for propane. Regarding the antibacterial activity, bergamot peel oil was effective on bacterial growth-inhibitory, especially for the Gram-positive (*S. aureus*), with MIC of 31.25 $\mu g.mL^{-1}$. On the other hand, for Gram-negative (*E. coli*), results showed a higher microbial resistance, with MIC of 500 $\mu g.mL^{-1}$ for bergamot peel oil extract by $SCCO_2$ and 125 $\mu g.mL^{-1}$ in the case of propane, allowing to classifying the bergamot peel oil obtained in this work as strong bacteria inhibitors.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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