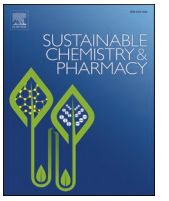
Sustainable Chemistry and Pharmacy 28 (2022) 100739

Contents lists available at ScienceDirect

Sustainable Chemistry and Pharmacy

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



Potential of supercritical fluid myrtle extracts as an active ingredient and co-preservative for cosmetic and topical pharmaceutical applications

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ARTICLE INFO

Keywords:
Myrtus communis L.
Supercritical Fluid Extraction (SFE)
Antimicrobial activity
Topical applications

ABSTRACT

This study investigated the applications of a myrtle extract obtained by supercritical fluid extraction (SFE), a technique considered environmentally friendly, as a possible antimicrobial ingredient in cosmetic and pharmaceutical formulations. The supercritical fluid extract was obtained at 230 bar and 45 °C, for 2 h, with a flow of CO_2 of 0.3 kg h^{-1}. A cosolvent (ethanol) was also used, with a flow 0.09 kg h^{-1}. The extracts thus obtained were tested against seven Gram-positive bacteria and one yeast using the well diffusion and the broth dilution techniques. The results showed that the myrtle extract exhibits good antibacterial activity against all the bacteria strains studied and is superior to most of those obtained by conventional extraction methods. Antifungal activity was also present but at a lesser extent. Cell viability studies were carried out by exposing HaCat cells to a range of extract concentrations, from 0.1 μg/mL up to 60 μg/mL for 24 h, using the MTT assay. The *Salmonella* mutagenicity assay was applied to evaluate the mutagenicity and antimutagenicity of the extract. The results obtained suggest that the myrtle extract obtained using a green solvent, supercritical CO_2, is safe and could reduce the genotoxic damage induced by reactive oxygen species (ROSs).

1. Introduction

Nowadays there is an increasing public demand for cosmetic and pharmaceutical formulations containing natural ingredients instead of synthetic ones which puts the use of bioactive ingredients in topical products on the rise (Cushnie et al., 2020; Mauricio et al., 2020). This can be achieved through the inclusion of bioactive vegetable extracts in the pharmaceutical and cosmetic

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https://doi.org/10.1016/j.scp.2022.100739
Received 20 December 2021; Received in revised form 23 May 2022; Accepted 4 June 2022
Available online 11 June 2022

formulations (Mahesh et al., 2019). These extracts can act, for example, as preservatives or as antiaging agents, if they exhibit antimicrobial or antioxidant properties, respectively. Another area of cosmetic science where natural extracts are being investigated is in oral and intimate hygiene (Sá et al., 2012; Leo and Benvenuti, 2015). *Myrtus communis* L., or myrtle, is an evergreen shrub, typical of the Mediterranean flora, which grows wildly across most of Portugal. It is, since ancient times, considered as a medicinal plant and used as such, which is probably related to its antioxidant and antimicrobial activities (Aleksic and Knezevic, 2014; Elfellah et al., 1984; Ghasemi et al., 2011; Messaoud et al., 2012). In order to achieve an altogether environmentally friendly process, supercritical fluid extraction (SFE) was used to obtain the myrtle extracts, using supercritical CO_2 as a solvent. SFE is a process that complies with a number of the Green Chemistry's 12 principles, namely, as far as the safety of solvent and prevention of waste is concerned. The elimination of hazardous organic solvents and the search for useful non-hazardous solvents is a prime goal of green chemistry. Techniques that use supercritical fluids as solvents have been considered green solvents by several authors in the last few years (Poliakoff and Licence, 2015; Sankula et al., 2014; Wang et al., 2018, 2019; Vidović et al., 2021) as they do not use harmful organic solvents which is an important environmental advantage given the tremendous environmental and economic costs that organic solvents used in chemical processes have. Organic solvents can lead to health problems, adversely impact the environment, are flammable, contribute to smog formation and are eco toxic (Kaziunas and Schlake, 2016). In contrast, supercritical CO_2 exhibits a range of properties that are in accordance with the characteristics of an ideal green solvent. It is non-toxic, non eco toxic, non-flammable, it leaves no residues in the extract, it is inert, and it comes at a relatively low cost due to its abundance. It is easily removed from the extract, it is recyclable, it is non-eutrophying and does not contribute to global warming as the process uses CO_2 but does not generate it (Rozzi et al., 2002; Vidovic et al., 2021). It has a low polarity, however, making it necessary to use a cosolvent for the extraction of the large, polar molecules (Grigonis et al., 2005; Mandana et al., 2011). Since the objective was to obtain an extract containing large, polar antioxidant molecules, it was necessary to use a cosolvent in the extraction process. Ethanol was the chosen cosolvent because of its low level of toxicity, when compared to other organic solvents, and for being, in general, considered benign and compatible with the Ecocert label (Ecocert, 2012).

In previous works (Pereira et al., 2013, 2016), the antioxidant capacity (AOC) of the supercritical fluid myrtle extracts were evaluated using the TEAC and ORAC methods and the polyphenols content was measured using the Folin-Ciocalteu method. The correlation studies that were carried out showed a strong positive correlation between AOC, for both methods, and the polyphenols content. These results indicate that the AOC measured in myrtle extracts is extensively due to its polyphenolic compounds. The supercritical fluid extracts of *M. communis* were also characterized by HPLC-MS and their major constituents identified, namely, myricetin-*O*-galactoside, myricitin-3-*O*-rhamnoside and quercetin-*O*-rhamnoside. For myrtle, myricetin glycosides seemed to be the polyphenols with the major role in its antioxidant capacity. As reported before, it was observed that the extraction methods have a significant effect on the polyphenols composition of myrtle leaves extracts. For instance, the SFE extracts contained only the conjugated glycoside forms as opposed to liquid phase extraction (LPE) which contained mainly the aglycone forms of the same polyphenols. Myricetin-3-*O*-rhamnoside was the only antioxidant flavonoid extracted by both methods, although the quantities extracted by SFE were much higher than in LPE.

In one of the previous studies (Pereira et al., 2013), the antimicrobial properties of the SF extract were also investigated. The results obtained showed antibacterial activity against Gram-positive bacteria, being inactive against the Gram-negative studied. This finding was in agreement with reports in the literature stating that the inhibitory effects of phenolic compounds from natural extracts are more effective against Gram-positive than against Gram-negative bacteria (Adámez et al., 2012; Kao et al., 2010). Therefore, in this study, only Gram-positive bacteria were investigated.

All things considered, the previous findings pointed towards the supercritical extract of *M. communis* being a very interesting candidate for use as an antimicrobial cosmetic ingredient which could help in the preservation of the final formula (Pereira et al., 2013, 2016). As is known, cosmetics formulations must include a preservative so that their shelf life can be extended. However, some of the synthetic preservatives habitually used in most formulations are associated with causing allergies. For this reason, the cosmetics industry is increasingly looking for alternatives, such as natural plant extracts, to include in their formulations.

The microorganisms of this study were chosen because they can cause skin infections, or mucous membranes infections, more or less severe depending on the overall health of the host and the pathogenicity of the microorganism.

Staphylococcus aureus is a type of bacteria that develops on the skin due to pre-existing lesions, follicular obstruction or weakened immune system, causing skin infections, which can be treated via the administration of topical or oral antibiotics, according to the severity of the infection. Researchers also found that a large percentage of microorganisms belonging to the genus *Staphylococcus* isolated in hospitals were mainly methicillin-resistant *Staphylococcus aureus* (MRSA) which, compared to the most common *Staphylococcus aureus*, have a much more complicated and difficult-to-treat clinical condition, with high mortality rates (Harvey et al., 2007).

Enterococcus faecalis are part of the normal faecal flora. They can also colonize oral mucous membranes and skin under conditions in which host resistance is lowered, or the integrity of gastrointestinal or genitourinary tract has been disrupted, causing urinary tract infections (Harvey et al., 2007). They can also be a cause of hospital acquired infections, which can be exceptionally difficult to heal because of multiple antibiotic resistance of many *E. faecalis* isolates (Brown et al., 2021; Kau et al., 2005).

Propionobacterium acnes also appears frequently associated with skin infections. This bacteria in conjunction with *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*, seems to be associated with acne pathology (Hassanzadeh et al., 2008). Acne is one of the most common skin diseases that affects more than 45 million individuals worldwide and is usually associated with hormonal changes. Treatments are usually time-consuming, expensive and, sometimes, cause severe adverse effects to patients (Lehmann et al., 2002; Harper, 2004; Ördögh et al., 2010).

In addition, two pathogenic bacteria known to cause caries and oral infections were also tested: *Streptococcus mutans* and *Streptococcus mitis* (Sá et al., 2012). These microorganisms can lead to disease which can have tooth loss as consequence and in patients with

abnormal or damaged heart valves, they can also cause bacterial endocarditis and other infections (Nomura et al., 2020).

The *Bacillus cereus* is an endospore-forming microorganism that can be found in soil, water and can be frequently found in medical laboratory as airborne contaminant. Can also be implicated in opportunistic lesions, particularly following trauma, or hospital interventions (Premkrishnan et al., 2021).

Finally, *Candida albicans* was chosen for being the most commonly occurring fungal opportunistic pathogen which can be found in normal body flora, in skin, mouth, vagina and intestines. Infections occur, usually, in debilitated individuals and patients treated with broad-spectrum antibiotics and other substances, when competing bacterial flora are eliminated (Harvey et al., 2007; Ponde et al., 2021).

In terms of safety compliance, myrtle extracts obtained through conventional extraction methods are already considered safe for use in cosmetics by regulatory authorities (EU Cosing, 2018). However, the myrtle extract used in this study was obtained through a method different than the ones considered there, SFE, therefore its safety needs to be proved. Since a human dermatological application is sought, the cytotoxicity and mutagenicity of the extract were investigated. For an initial safety assessment of this extract, *in vitro* assays were carried out.

The aim of this study was, therefore, to investigate the possible application of a supercritical fluid myrtle extract as an antimicrobial ingredient or as a co-preservative in oral, intimate, cosmetics and pharmacological formulations.

2. Materials and methods

2.1. Plant material

Samples of wild myrtle leaves were collected from the Sintra area (Portugal) in the spring of 2019 at the pre-flowering stage. No soil tillage, fertilization and pest treatments were carried out in this area. A specimen of this plant was identified and deposited in the Herbarium of the Superior Agronomy Institute of the Technical University of Lisbon. The plant material was air dried for two months out of the sunlight, and then sealed in black bags and kept at −20 °C. Before extraction the plant material was ground using a food chopper (A327R1, Moulinex, France). Sieving was carried out using a vibratory sieve shaker (AS 200 basic, Retsch, Haan, Germany) with the appropriate sieves to achieve the desired average particle diameter (Dp = 696 μm). Final moisture of the plant material was determined as (9.94 ± 0.04) wt%.

2.1.1. Reagents

SFE: Carbon dioxide (N48–99.998%) for SFE was supplied in cylinders by Air Liquide (Lisbon, Portugal). Absolute ethanol p.a., used as co-solvent, was obtained from Merck (Darmstadt, Germany).

***In vitro* antimicrobial activity**: Positive controls vancomycin, tetracycline, ketaconazole, were purchased from Sigma-Aldrich (St. Louis, MO, USA), chlorhexidine gluconate solution 20% (w/w) was purchased from Medichem, S. A. (Barcelona, Spain) and negative control propylene glycol was purchased from Univar Iberia, S.A. (Barcelona, Spain). Sodium chloride and glycerol were purchased from Panreac (Barcelona, Spain). Tryptone USP, Mueller Hinton Agar and Mueller Hinton Broth were purchased from Biokar (Allone, France). Brain Heart Agar was purchased from Merck (Darmstadt, Germany), Reinforced Clostridial Medium and Reinforced Clostridial Agar were purchased from Oxoid (Hampshire, U.K.).

Cell viability assays: Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Biowest (Nuaillé, France). Trypsin, penicillin-streptomycin solution, fetal bovine serum, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulphoxide (DMSO) was purchased from Merck (Darmstadt, Germany).

Mutagenic activity: Citric acid monohydrate, di-sodium hydrogen phosphate dihydrate and sodium dihydrogen phosphate monohydrate were purchased from Panreac (Barcelona, Spain). Ammonium sodium phosphate dibasic tetrahydrate, di-potassium hydrogen phosphate anhydrous and sodium chloride were purchased from Fluka (Seelze, Germany). D-Biotin, D-(+)-glucose monohydrate, dimethyl sulfoxide, 2-nitrofluorene and *tert*-butyl hydroperoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-Histidine monohydrochloride monohydrate was purchased from Merck (Darmstadt, Germany). Magnesium sulfate heptahydrate was purchased from LabChem Inc. (Zelienople, PA, USA). Sodium azide was purchased from J.T. Baker Chemical Company (Phillipsburg, NJ, USA). Bacto™ Agar was purchased from Becton Dickinson & Co (Sparks, MD, USA). Nutrient broth nº 2 was purchased from Oxoid (Basingstoke, UK).

2.2. Supercritical fluid extraction

SFE experiments were carried out in the SFE apparatus, already detailed described in Pereira et al. (2013). In each extraction run, 30 g of finely ground dried myrtle leaves was placed, wrapped in a filling of cotton wool, inside the extraction cell with a volume of 278 cm^3, which was then placed inside a temperature-controlled oven. The experimental conditions used in the current study were those reported as optimal in that previous study: temperature: 45 °C, pressure 23 MPa, $SCCO_2$ flow rate 0.3 kg h^{-1}, and cosolvent flow rate (ethanol) 0.09 kg h^{-1}. Each extraction was run for 2 h. The yield of the extraction was 2.25 wt%.

2.3. In vitro antimicrobial activity

The *in vitro* antimicrobial activity was determined based on Clinical and Laboratory Standards Institute guidelines (CLSI document M07-A9, 2012; CLSI document M02-A11, 2012), using the well diffusion and the broth microdilution methods. All assays were performed in triplicate, and positive (vancomycin, tetracycline, and ketaconazole) and negative (propylene glycol) controls were included in both methods. These controls were used at 1 mg/mL concentration. Chlorhexidine was tested at 0.2%, through dilution of the 20% commercial solution, since this is the normal concentration found in oral elixirs.

Propylene glycol has been widely used in the preparation of plant extracts and in topical products as a permeation promoter, emollient, humectant and emulsion stabilizer (Herai et al., 2007; Hosmer et al., 2009). According to the European Committee for the Safety Assessment of Cosmetic Ingredients, propylene glycol is considered a safe ingredient for the skin and is present in numerous cosmetic and pharmaceutical products on the market (Fiume et al., 2012).

2.3.1. Microorganisms and media

The antimicrobial activity of extract was assessed against the Gram-positive bacteria *Enterococcus faecalis (*ATCC® 29212), methicillin-resistant *Staphylococcus aureus (*MRSA ATCC® 33591), *Propionibacterium acnes* (ATCC® 6919), *Staphylococcus aureus (*ATCC®6538)*, Streptococcus mutans* (ATCC ®25175), *Streptococcus mitis* (NCIMB®13770), *Bacillus cereus* (ATCC ®11778)) and the yeast *Candida albicans* ATCC®10231. Microorganisms were kept frozen at – 80 °C in broth containing glycerol 15% (v/v). To prepare the working culture of the microorganisms, a subculture was made from the stock culture in plates with recommended media for each microorganism, according to strains suppliers and Clinical and Laboratory Standards Institute (CLSI document M07-A9, 2012; CLSI document M02-A11, 2012).

2.3.2. Well diffusion method

The antimicrobial growth inhibition was made by *in vitro* conventional well diffusion method and was employed for the initial assessment of the antimicrobial potential of the extract (Balouiri et al., 2016; Maurício et al., 2017; CLSI document M44-A2, 2009). A saline suspension, with 1% tryptone USP and 0.85% sodium chloride, corresponding to 1–2 x 10^8 CFU/mL for bacteria, and 1–5 x 10^6 CFU/mL for yeasts, was prepared and used to inoculate the Mueller-Hinton Agar plates. For *Streptococcus mitis* and *Streptococcus mutans*, a saline suspension was also carried out under the same conditions as above and used to inoculate the Brain Heart (BHI) Agar. For *Propionibacterium acnes,* a bacterial suspension was prepared in fresh Reinforced Clostridial Medium corresponding to 1–2 x 10^8 CFU/mL and used to inoculate Reinforced Clostridial Agar plates.

Wells with 6.0 mm diameter were, then, made and in each of them 50 μL of extract at 10 mg/mL in propylene glycol was added. All the plates were kept in an incubator at 35 ± 2 °C during 24 h for the growth of bacterial and yeast strains, except for *P. acnes* that was incubated for 48 h at 35 ± 2 °C under anaerobic atmosphere. The antimicrobial activities were determined by measuring, in mm, the diameter of the growth inhibition zone.

2.3.3. Determination of minimum inhibitory concentrations

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method in 96-well microtiter plates (CLSI document M07-A9, 2012). The extract was prepared at 50 mg/mL in propylene-glycol and a dilution of 1:10 was introduced in the first line, followed by a series of 2-fold dilutions in Reinforced Clostridial Broth for *Propionibacterium acnes,* Brain Heart infusion (BHI) broth for both *Streptococcus* strains and in Mueller-Hinton broth for the remaining microorganisms. Propylene glycol was used as negative control. A standardized saline suspension, in the same conditions as described in 2.4.2., was prepared and used to inoculate microtiter plates. The microplates were incubated at 35 °C ± 2 °C, during 24 h, except for *P. acnes,* that was incubated for 48 h at 35 °C ± 2 under anaerobic atmosphere. After the incubation period microplates were visually observed in order to determine the minimal inhibitory concentration which is defined as the lowest extract concentration at which no visible growth could be detected.

2.4. Cell viability assays

Cell viability assays were carried out in human keratinocytes (HaCat cell line). HaCat cells were routinely cultured in DMEM supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cells were kept at 37 °C, under an atmosphere containing 5% CO_2 in air.

Approximately 5000 cells were cultured in 200 μL of culture medium per well in 96-well plates and incubated at 37 °C for 24 h. Cell cultures were then exposed to 7 concentrations of the extract from 0.1 to 60 μg/mL, for a 24 h-period. The extract was initially solubilized in DMSO and then further diluted in PBS. The final DMSO concentration in culture medium was 0.5%, except for the positive control (DMSO 5%). Cell viability was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay, according to a previously published protocol (Wagemake et al., 2015). Two independent experiments were performed, each comprising four replicate cultures.

2.5. Mutagenic and antimutagenic activities

2.5.1. Mutagenic activity

The mutagenic activity of myrtle extract was evaluated by the Ames test, according to the plate incorporation assay described by Mortelmans and Zeiger (2000). Mutagenicity was evaluated using *Salmonella* Typhimurium tester strains TA98, TA100 and TA102. The strains were kindly provided by the Genetic Department of the Nova Medical School of the New University of Lisbon (Portugal). The extract was solubilized in DMSO and the mutagenicity was assayed from 0 up to 5 mg/plate, according to the OECD Guidelines (1997). Test mixtures composed by 50 μL of different concentrations of myrtle extract or 50 μL of DMSO, 100 μL of each tester strain and 500 μL of phosphate buffer 0.1 M, pH 7.4, were mixed with 2 mL of molten Top agar with biotin and trace of histidine and plated in glucose minimal agar. After incubation at 37 °C, during 48 h, manual counting of His^+ colonies revertants for each concentration was performed. Sodium azide, 2-nitrofluorene and *tert*-butyl-hydroperoxide (t-BHP) were used as positive controls for TA100, TA98 and TA102, respectively. At least three independent experiments were performed for each assay.

2.5.2. Antimutagenic activity

The antimutagenic activity was also evaluated by the Ames test, according to the plate incorporation assay previously described

(Mortelmans and Zeiger, 2000). The antimutagenic activity of myrtle extract was assessed against the mutagenic effect of *t*-BHP in *Salmonella* Typhimurium strain TA 102. This strain has been shown to be highly sensitive to reactive oxygen species, including those generated by *t*-BHP. A concentration of 50 μg t-BHP/plate was chosen from the linear portion of the dose–response curve of this compound, using strain TA102 (data not shown). The anti-mutagenic activity, expressed as inhibition percentage of mutagenicity was calculated by the following expression (Yen and Chen, 1995):

$$\% \text{ Inhibition} = [(RI\text{-}RE) - (RIM\text{-}RE)] \times 100/(RI\text{-}RE)$$

RI represents and the number of revertants induced by the mutagen, RE the number of spontaneous revertants and RIM the number of revertants induced by the mutagen in the presence of myrtle extract. The assay was performed in triplicate.

2.5.3. Statistical analysis on antimutagenic assays

After verifying the assumption of normality and variance homogeneity, one-way analysis of variance (ANOVA) followed by Tukey's test was used to identify statistical significance. All statistical analyses were performed at 0.05 level of probability with the software STATISTICA™ 7.0 (StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. In vitro antimicrobial activity

Our previous study on the supercritical fluid extract of myrtle (Pereira et al., 2013) showed that the extract possessed significant antibacterial activity against Gram-positive bacteria, being particularly effective towards bacteria such as *S. aureus* and *S. epidermidis*.

In this study, a different set of Gram-positive bacteria and one yeast were studied to better evaluate the efficacy of this extract against microorganisms capable of causing various skin and mucous membranes infections. The microorganisms were tested against suitable antibiotics (positive controls) to ensure the proper sensitivity of each strain. As to the solvent used (negative control) propylene glycol, it did not exhibit inhibition against the growth of microorganisms tested.

The results for the antibacterial activity of the myrtle SF extract measured as inhibition zone diameter (IZD) are shown in Table 1. The highest values were obtained for the *S. aureus* (28.0 ± 1.4 mm), MRSA (28.3 ± 2.1 mm) and *P. acnes* (28.3 ± 0.6 mm), followed by *B. cereus* (27.0 ± 1.0 mm), *E. faecalis* (26.7 ± 2.9 mm), *S. mitis* (18.3 ± 0.2 mm) and finally *Candida albicans* with an inhibition zone of 14.7 ± 1.5 mm. The extract has, therefore, a higher antimicrobial activity against the Gram-positive bacteria than against the yeast (*C. albicans*).

Table 2 shows the MIC values obtained from the microdilution test. It can be seen that the SF extract exhibits a significant antibacterial activity against the Gram-positive bacteria under study, showing the best inhibitory properties against *S. aureus* and MRSA, with similar MIC's (156 μg/mL), followed by *P. acnes*, *S. mutans* and *B. cereus* all three with MIC values of 312 μg/mL, *S. mitis*, *E. faecalis* both with MIC values of 612 μg/mL and finally *C. albicans* with a MIC value of 2500 μg/mL. The MIC values confirm the result already hinted by the IZ results, that the SF extract possesses a higher activity against Gram-positive bacteria than against yeasts.

Several authors have classified the antimicrobial activity of natural extracts as strong inhibitors (MIC up to 500 μg/mL), moderate inhibitors (MIC between 600 and 1500 μg/mL) and weak inhibitors (MIC above 1600 μg/mL) (Duarte et al., 2007; Vieitez et al., 2018; Espinoza et al., 2019). Using this classification, this SF extract exhibited strong activity against five of the seven bacterial strains tested (*S. aureus*, MRSA, *P. acnes*, *S. mutans* and *B. cereus*) and moderate activity against the remaining two (*S. mitis* and *E. faecalis*).

According to some authors, the phenolics composition of the extracts may contribute to the observed antimicrobial effects. It seems that main flavonoids identified in the analysed myrtle extract, myricetin and quercetin, may be related to the demonstrated actimicrobial activity, especially against Gram-positive bacteria (Cushnie and Lamb, 2005; Krisch et al., 2008; Álvarez-Martínez et al., 2020).

The results obtained show that myrtle extract possesses antibacterial activity against all the tested microorganisms with a particular significant inhibitory effect against the *S. aureus* and MRSA bacteria.

The results for the yeast, show a lower activity than that obtained for bacteria, for both the well diffusion test and the MIC determination. These findings are consistent with those reported in the literature by other authors as can be observed in Table 3 where the results obtained in this work are compared with those obtained by several different authors for the antibacterial activity of *M. communis* extracts and essential oil.

Table 1
Inhibition zone diameter (IZD) of the myrtle extract in the well diffusion assay (mean ± SD).

IZD (mm)	Extract 10 mg/mL	Propylene glycol[a]	Vancomycin[b] (1 mg/mL)	Tetracycline[b] (1 mg/mL)	Ketaconazole[b] (1 mg/mL)	Chlorhexidine[b] 0.2%
S. aureus	28.00 ± 1.41	0	27.00 ± 0.00	–	–	–
MRSA	28.33 ± 2.08	0	26.50 ± 0.71	–	–	–
E. faecalis	26.67 ± 2.89	0	23.00 ± 0.00	–	–	–
P. acnes	28.33 ± 0.58	0	–	35.67 ± 0.58	–	–
S. mutans	23.33 ± 0.15	0	19.33 ± 0.58	–	–	22.00 ± 0.00
S. mitis	18.33 ± 0.20	0	25.67 ± 0.58	–	–	22.50 ± 0.00
B. cereus	27.00 ± 1.00	0	33.50 ± 0.71	–	–	–
C. albicans	14.67 ± 1.53	0	–	–	29.33 ± 0.58	.

[a] Negative control.
[b] Positive control; - not tested.

Table 2
Minimum inhibitory concentration (MIC) of *Myrtus* extract against tested microorganisms.

Test strains	Minimum inhibitory concentration (MIC) (μg/mL)					
	Myrtle extract	Propylene-glycol[a]	Vancomycin[b]	Tetracycline[b]	*Ketaconazole*[b]	*Chlorhexidine*[b]
S. aureus	156	$>5 \times 10^3$	1.56	–	–	
MRSA	156	$>5 \times 10^3$	1.56	–	–	
E. faecalis	625	$>5 \times 10^3$	3.12	–	–	
P. acnes	312	$>5 \times 10^3$	–	6.25	–	
S. mutans	312	$>5 \times 10^3$	12.5	–	–	15.6
S. mitis	625	$>5 \times 10^3$	3.12	–	–	31.2
B. cereus	312	$>5 \times 10^3$	0.78	–	–	
C. albicans	2500	$>5 \times 10^3$	–	–	0.039	–

[a] Negative control.
[b] Positive control; - not tested.

The antimicrobial activity (AMA) against *S. aureus* is, by far and understandably, the most widely studied given the range of infections that these bacteria can cause, from skin infections to respiratory infections and food poisoning. Analyzing the results in Table 3 no clear trend can be detected in terms of method of extraction or solvent. Nevertheless, SFE with CO_2 as solvent is among those with lower MIC's and higher IZD's. Another interesting observation is that the MIC and the IZD values do not always follow the same trend. For example, Mir et al. (2020) report an extremely low MIC for the extract of *M. communis* but a fairly low IZD for the given concentration of the extract, 400 mg/mL (a rather high concentration). Thus, overall, we can conclude that the extract of *M. communis* obtained by SFE exhibit a significant AMA towards *S. aureus*, aligned with the other results reported in the literature. As to the AMA of *M. communis* towards MRSA strains, the results in the literature are not as abundant as for the methicillin sensitive *S. aureus* strains. Nonetheless, comparing the results here reported with those of Abdulqawi and Quadri (2021), the MIC and IZD values for the SFE extract are significantly better than the values obtained by those authors for ethanolic and methanolic extracts.

The extract activity against *S. mutans* was compared with those reported by Sateriale et al. (2020), and Gortzi et al. (2008). The latter report only IZD results, for a methanolic extract obtained from leaves, with a significantly lower value than that of this work. The results of Sateriale et al. (2020) are for a hydroalcoholic extract and show a considerably higher MIC and lower IZD than those of the extract obtained by SFE.

As to the extract activity against *E. faecalis*, the results obtained in this work were compared with the findings of Raoof et al. (2019), Behbahani et al. (2016) and Mir et al. (2020). With regard to the first two authors, the reported MIC values are considerably higher and the IZD's much lower than those reported in here. As to the results of Mir et al. (2020), they report excellent MIC values but surprisingly low IZD values. This, once again, shows that the two methods of evaluating the antimicrobial activity not always concur (Moreno et al., 2006; Klančnik et al., 2009; Balouiri et al., 2016). The well diffusion method is often described as a preliminary assay, while the microdilution method is the appropriate quantitative assay as it measures the concentration of the tested antimicrobial agent. Moreno et al. (2006) and Klančnik et al. (2009) report that fewer polar compounds can diffuse more slowly into the culture medium. This means that the diameter of the inhibition zone is influenced both by the rate of diffusion of the antimicrobial agent through the agar and by the hydrophobic nature of most plants, so the antimicrobial activity in those two methods might not always concur.

Interestingly, these same authors using the same extract did not detect any activity towards the yeast *C. albicans* whereas the SF extract shows a significant activity against this strain, shown both in the MIC and IZD values. Gortzi et al. (2008) report only IZD values which are lower than those obtained in this work. Hence, overall, the SF extract exhibits a superior AMA than the other extraction methods.

There is a dearth of data concerning the activity of *M. communis* extract towards the *S. mitis*, *B. cereus* and *P. acnes* bacteria. For *S. mitis*, there is no data whatsoever that could be used for comparison. Nevertheless, the results of the AMA determined for the SF extract show that it possesses a moderate to strong activity (Trindade et al., 2021). This is a very important result since the *S. mitis* is associated to many oral infections.

As to *B. cereus*, we could not find results for *M. communis* extract, but Hsouna et al. (2014) report results of the essential oil (EO) activity of *M. communis* against this strain. They show a higher MIC and lower IZD than those obtained by SFE which means that the SF extract is more active against this strain than the EO of the plant.

For the *P. acnes* bacteria, again there is almost no data for comparison. We could only find a report by Fiorini-Puybaret et al. (2011) on a registered purified ethanolic myrtle extract called Myrtacine®, which is standardised to 0.75% of myrtucommulones (potential active compounds). There is no IZD value in this study, but the MIC reported is considerably lower than that of the SF extract. However, this can be due to the extra purification steps undergone to obtain this registered extract. That aside, the results obtained in these two studies both show that the myrtle extracts possess antimicrobial activity against the bacteria *P. acnes*. This could point to the possibility of myrtle extract being considered as an active ingredient in the fight against acne and, therefore, the possibility of being incorporated in topical products for these skin condition.

Additionally, the antimicrobial activity detected in this and in previous published studies (Pereira et al., 2013) demonstrate that the SF extract is active against some of the reference strains that can contaminate the cosmetics formulations, usually *S. aureus* and *Candida albicans*. However, this extract has not shown sufficient efficacy against other three essential strains in the conservation of cosmetics, *E. coli*, *P.aeruginosa* and *A. niger,* referenced in ISO 11930 standards (2012).Therefore, these results indicate that myrtle extract can be used as a co-preservative in cosmetics formulations helping their conservation and safety. This addition may help to reduce the final

Table 3
Comparison of the results obtained in this work on the microbial activity of *Myrtus communis* extracts with results from other authors. (HD-hydrodistillation; EO- essential oil).

Microorganism	Type	Method	Solvent	MIC	IZD	Extract conc.	Extract conc.	Extract conc.	Ref.
				(μg/mL)	(mm)	(mg/mL)	(mg/well)	μL/well)	
Staphylococcus aureus	**ATCC 6538**	**SFC**	**CO_2**	**156**	**28**	**10**	**0.5**		
	PTCC 1112	Percolation	Ethanol	125	30.1	8			Raeiszadeh et al. (2018)
	PTCC 1112	Conventional extraction	Methanol	100	18	0.5			Mansouri et al. (2001)
	Clinically isolated	Conventional extraction	Hydroalcoholic	200	11	10			Taheri et al. (2013)
	ATCC25923	Conventional extraction	Methanol		14	1			Gortzi et al. (2008)
	ATCC 20231	WE	Hydroalcoholic		10–30	100			Casaburi et al. (2015)
	ATCC 25923	Conventional extraction	Aqueous		0				Besufekad et al. (2017)
	ATCC 25923	Conventional extraction	n-Hexane		5.5				Besufekad et al. (2017)
	ATCC 25923	Conventional extraction	Methanol		1				Besufekad et al. (2017)
	ATCC 25923	Conventional extraction	Chloroform		2.2				Besufekad et al. (2017)
	Not indicated	Soxhlet	Methanol	781	17.1		2.5		Abdulqawi & Quadri (2021)
	Not indicated	Conventional extraction	Aqueous	781	15		2.5		Abdulqawi & Quadri (2021)
	Not indicated	Soxhlet	Ethanol	9.7	25	400			Mir et al. (2020)
	Not indicated	Conventional extraction	Hydroalcoholic	200	11	10			Taheri et al. (2013)
MRSA (*Staphylococcus aureus)*	**ATCC 33591**	**SFC**	**CO_2**	**156**	**28.33**	**10**	**0.5**		
	ATCC 43300	Soxhlet	Methanol	781	18		2.5		Abdulqawi & Quadri (2021)
	ATCC 43300	Conventional extraction	Aqueous	781	14.1		2.5		Abdulqawi & Quadri (2021)
Streptococcus mutans	**ATCC 25175**	**SFC**	**CO_2**	**312**	**23.33**	**10**	**0.5**		
	ATCC 25175	Conventional extraction	Hydroalcoholic	10	19.8		2		Sateriale et al., 2020
	ATCC 25175	Conventional extraction	Hydroalcoholic		10.5		1		Sateriale et al., 2020
	ATCC31989	Conventional extraction	Methanol		14	1			Gortzi et al. (2008)
Streptococcus mitis	**NCIMB13770**	**SFC**	**CO_2**	**625**	**18.33**	**10**	**0.5**		
Bacillus cereus	**ATCC11778**	**SFC**	**CO_2**	**312**	**27**	**10**	**0.5**		
	ATCC 14579	HD (EO)	Water	625	26			50	Hsouna et al. (2014)
Enterococcus faecalis	**ATCC 29212**	**SFC**	**CO_2**	**625**	**26.67**	**10**	**0.5**		
	PTCC1237	Maceration	Methanol	12500	7.6	100			Raoof et al. (2019)
	ATCC 29212	Maceration	Methanol	4000	14.3	10			Behbahani et al., 2016
	ATCC 29212	Maceration	Aqueous	16000	11.1	10			Behbahani et al., 2016
	ATCC29212	Soxhlet	Ethanol	78	9	400			Mir et al. (2020)
	not indicated	Soxhlet	Ethanol	19.5	9	400			Mir et al. (2020)
Propionibacterium acnes	**ATCC 6919**	**SFC**	**CO_2**	**312**	**28.33**	**10**	**0.5**		
	not indicated	Myrtacine	Ethanolic	24					Fiorini-Puybaret et al. (2011)
Yeast *Candida albicans*	**ATCC 10231**	**SFC**	**CO_2**	**2500**	**14.67**	**10**	**0.5**		
	not indicated	Soxhlet	Ethanol	no detection	no detection	400			Mir et al. (2020)
	ATCC10231	Conventional extraction	Methanol		10	1			Gortzi et al. (2008)

concentration of synthetic preservatives that can be added to the formulations, making them more ecological and natural.

3.2. Cell viability assays

Potential intended uses of the myrtle extracts include skin applications, either as cosmetics or topical medicines. Therefore, it is fundamental to evaluate its cytotoxicity in human normal-like keratinocytes. The obtained results are depicted in Fig. 1.

The myrtle extract did not show cytotoxicity at lower concentrations (up to 10 μg/mL), while concentrations of 30 and 60 μg/mL decreased cell viability to around one half of that of non-treated control cells ($p < 0.05$). Under the same experimental conditions, DMSO 5% (v/v) used as positive control decreased cell viability to 2.9 ± 1.2% ($p < 0.01$).

A previous report (Fiorini-Puybaret et al., 2011) has evaluated the anti-proliferative effect of an ethanolic myrtle extract and its potential active compounds (myrtucommulones) in HaCat cells, using the BrdU incorporation assay. The authors observed a concentration-dependent inhibition of proliferation, highly significant for concentrations of 3 μg/mL of extract or 5 μg/mL of myrtucommulone A or B. Despite the different methodologies used, the supercritical fluid extract obtained in the present work appears to have a better safety profile than this previous ethanolic extract.

As keratinocyte proliferation seems to contribute to the pathological process of comedogenesis (Fiorini-Puybaret et al., 2011), the decrease in cell viability observed for higher concentrations could be advantageous for a potential application of myrtle extract for acne treatment.

3.3. Mutagenic and antimutagenic activities

Mutations are significant early steps in carcinogenesis processes, thus the evaluation of the mutagenicity is a necessary aspect of the preliminary safety evaluation of plant-derived extracts and compounds in order to avoid possible deleterious effects (Eloff and McGaw, 2006). Gene mutations are readily measured in bacteria and other cell systems when they cause a change in the growth requirements of the cell (Mortelmans and Zeiger, 2000), therefore bacterial reverse mutation assays have been commonly used as an initial screening to detect the mutagenicity of pure compounds or mixtures such as plant extracts (OECD, 1997). Different bacterial strains, namely from *Salmonella* Typhimurium and *Escherichia coli*, have been developed and validated to detect efficiently mutagens with different mode of action (Maron and Ames, 1983; Duarte et al., 2005, 2007; Duarte et al., 2005a,b), being the Salmonella mutagenicity assay (Ames assay) used for submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides (Mortelmans and Zeiger, 2000).

When tested up to 5.0 mg/plate, which correspond to the recommended maximum test concentration according to the OECD (1997) guidelines for testing of chemicals, the extract did not induce an increase in the number of revertants per plate in any of the tested bacterial strains (Table 4). Thus, under the conditions tested, the extract did not show mutagenic activity, which is crucial to ensure its safety.

3.4. Antimutagenic activity

The antimutagenic activity of myrtle extract was evaluated in the Ames assay, by assessing its capacity to inhibit the mutagenicity induced by *t*-BHP using the strain TA102. Results obtained showed that myrtle extract dose-dependently reduced the *t*-BHP-induced mutagenicity in strain TA102. The highest percent inhibition reached was 46% at the highest assayed concentration (Fig. 2).

It is known that *t*-BHP generate free radical derivatives, thus the antimutagenicity of myrtle extract may be related to its antioxidant capacity, which in turn, is related to its content in phenolic compounds (Pereira et al., 2013). In addition to deactivating potential mutagens, polyphenols have been shown to exert antimutagenic activity through other mechanisms, such as protection of DNA nucleophilic sites, stimulation of DNA repair, inhibition of the enzymatic conversion of mutagen precursors into mutagens or inhibition of nitrosation reactions (Duarte et al., 2000; Ferguson and Philpott, 2008). Therefore, the protective effect of myrtle extract against *t*-BHP mutagenicity may be exerted by other mechanisms besides scavenging reactive oxygen species. Myricetin-O-galactoside and myricetin-3-O-rhamnoside, two of the main polyphenols identified in the supercritical fluid extract of myrtle analysed, may contribute to the observed antimutagenicity as these two compounds have already been shown to reduce the mutagenicity of nifuroxazide, aflatoxin B1 and hydrogen peroxide (Hayder et al., 2008a).

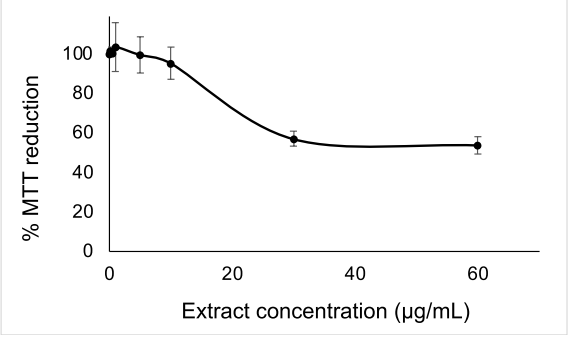

Fig. 1. – Cell viability of HaCat cells exposed to myrtle extract for 24 h, as evaluated by the MTT assay. Results are expressed as mean ± SD (n = 2).

Table 4
Mutagenic activity of *Myrtus communis* extract tested with *Salmonella* Typhimurium strains TA98, TA100 and TA102.

Sample	Concentration (mg/plate)	Revertants per plate (mean ± SD)		
		TA98	TA100	TA102
Myrtle extract	0	26 ± 7	154 ± 1	317 ± 6
	0.25	23 ± 8	161 ± 18	323 ± 8
	0.50	21 ± 2	170 ± 15	311 ± 8
	1.00	23 ± 3	155 ± 6	336 ± 14
	2.50	25 ± 6	140 ± 16	311 ± 2
	5.00	19 ± 4	133 ± 7	317 ± 18
Positive Control[a]		488 ± 30	1048 ± 53	881 ± 26

[a] Positive Control: TA98, 2-nitrofluorene (5 μg/plate); TA100, sodium azide (1.5 μg/plate); TA102 *tert*-butyl-hydroperoxide (50 μg/plate).

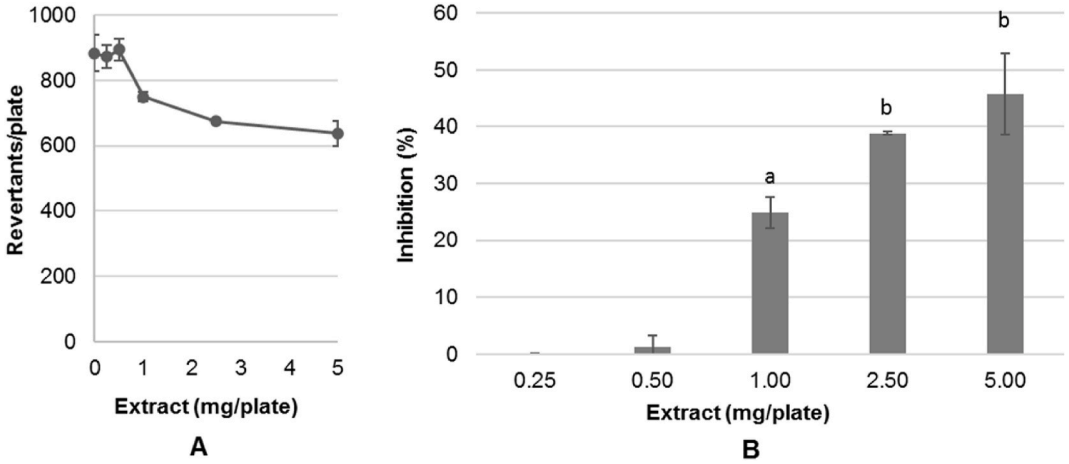

Fig. 2. Effect of *Myrtus communis* extract on the mutagenicity of *t*-BHP (50 μg t-BHP/plate) in TA102 tester strain of *Salmonella* Typhimurium. (A) Dose-response curve; (B) Percent Inhibition. Bars with different letters are significantly different ($p < 0.05$).

Previous studies have demonstrated antimutagenic activity for myrtle aqueous or organic (methanol, chloroform, hexane and ethyl acetate) Soxhlet extracts against several direct and indirect mutagens (Hayder et al., 2003, 2008b). Nonetheless, Hayder et al. (2003; 2008b) reported percent inhibitions higher than those obtained with the myrtle extract obtained using SFE. The differences observed in percent inhibitions may be due to differences in the constitution of the leaves resulting from their diverse geographical origin (Portugal and Turkey). Climate conditions influences the general metabolism of the plants, which affects the metabolic pathways responsible for the accumulation of secondary metabolites. For example, the concentrations of phenolic compounds and of other bioactive plant secondary metabolites seem to be enhanced when plants are grown in semi-arid regions, i.e., under drought conditions, comparing to plants growing in a moderate climate (Chiappero et al., 2021). Moreover, differences in the extraction method, namely differences in the temperature and polarity of the solvents used, as well as differences in the mechanism of mutagenicity induced by the mutagens tested or in the experimental conditions (plate assay or pre-incubation assay) may also explain the discrepancy observed (Chandel et al., 2012; Hayder et al., 2008b). The percent inhibition obtained is in line with the reported for other natural extracts against direct mutagens tested in the same conditions, namely methanol root extract from *Rubia cordifolia* L. (Chandel et al., 2012) or chloroform or methanol leaf extracts of *Nitraria retusa* (Boubaker et al., 2010).

Results suggest that the studied myrtle extract is safe and could reduce the genotoxic damage induced by reactive species. The antimutagenic activity detected could make this extract a promising candidate for future applications in human healthcare. However, further studies, namely cytogenetic assays must be conducted in order to totally ensure the extract safety.

4. Conclusion

The results clearly demonstrated that myrtle extract obtained using SFE, a technique considered environmentally friendly, exhibits antimicrobial activity against all tested microorganisms since the minimum inhibitory concentration (MIC) and inhibition zones values obtained showed significant inhibitory effect against the Gram-positive bacteria and yeast tested.

Having in mind a potential skin application of *Myrtus* extract, its cytotoxicity was evaluated in normal-like human keratinocytes. No toxic effects were observed for concentrations up to 10 μg/mL, while higher concentrations decreased cell viability.

The mutagenicity tests carried out have also indicated that the myrtle SF extract is safe to use in topical applications.

This work constitutes a step forward towards the potential use of this extract as an ingredient for topic products and as a co-preservative in the final cosmetics formulations, although further studies are needed to duly assess the safety of SFE myrtle extract.

For example, Challenge Test Standards (ISO 11930, 2012) could be performed to determine the amount of preservative needed to ensure the correct preservation of a cosmetic product. Synergy studies could also be performed using this extract together with other preservatives so that the quantity of synthetic preservatives in formulations could be decreased, for both economic and consumer safety reasons. It is important to perform these types of tests in final formulations to ensure total safety before the product reaches the market.

The tested extract, sourced from a wildly available plant, seems a promising low-cost antimicrobial agent. The myrtle extract obtained through SFE could turn out to be an alternative to conventional antimicrobial treatments that usually consist of more polluting and less safe for human health synthetic substances such as antibiotics, chlorohexidine, phenoxyethanol and parabens.

Author contributions

Paula Pereira: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Elisabete Muchagato Mauricio:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Maria Paula Duarte:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Katelene Lima:** Investigation, Validation, Formal analysis. **Ana S. Fernandes:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Gabriela Bernardo-Gil:** Resources, Writing - review & editing. **Maria-João Cebola:** Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

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.

Acknowledgements

The authors gratefully acknowledge the support of Fundação para a Ciência e a Tecnologia (FCT) through the grants UIDB/04567/2020 and UIDP/04567/2020 to CBIOS, UIDB/04028/2020 to CERENA and UIDP/04077/2020 to MEtRICs.

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