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Eucalyptus spp. outer bark extracts inhibit *Helicobacter pylori* growth: *in vitro* studies

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ABSTRACT

Helicobacter pylori is the etiologic agent of several gastric disorders. The growing rates of unsuccessfulness of available antibiotics-based therapy led to the need for non-antibiotic compounds for *H. pylori* treatment. The antibacterial activity of *Eucalyptus nitens* and *Eucalyptus globulus* outer bark lipophilic extracts were evaluated against a panel of *H. pylori* strains, as well as the role of pure triterpenic acids and synthetic mixtures mimicking the natural extracts. Best results were obtained with *E. nitens* outer bark extract (MIC = 64 μg/mL) and it was demonstrated that after 6 h of exposure to the MIC, there was a significant decrease (> 50%) in *H. pylori* growth. Moreover, it was determined that the antibacterial effect of *Eucalyptus* spp. outer bark extracts was due to the synergetic effect of four triterpenic acids, namely betulinic, betulonic, ursolic and oleanolic acids. Our findings open new perspectives for development of anti-*H. pylori* therapies based on the use of these plant extracts.

1. Introduction

Helicobacter pylori (*H. pylori*) is one of the most successful human pathogens, colonizing the gastric mucosa of over half the world population (Pinho et al., 2013). Gastric carcinoma, the 5th most common cancer and the 3rd leading cause of cancer-related deaths worldwide (American Cancer Society, 2016), has been associated with persistent gastric infection with *H. pylori*, being the only classified as a class I carcinogen by the World Health Organization (Correa and Houghton, 2007; Piazuelo et al., 2010; Jemal et al., 2011; Ferlay et al., 2013). Approximately 75% of the global gastric cancer burden is attributable to *H. pylori* infection, but there are other related gastric disorders with high morbidity, such as chronic active gastritis and peptic ulcer disease (Peek and Blaser, 2002; Suerbaum and Michetti, 2002).

The success rate of the current therapeutic scheme against *H. pylori*, which consists in a combination of antibiotics and proton pump inhibitors, has declined over the years, mostly due to bacterial resistance to antibiotics (Malfertheiner et al., 2012; Yang et al., 2014; Ermis and Senocak Tasci, 2015). As a consequence, millions of people worldwide are left without effective treatment and, therefore, the development of anti-*H. pylori* therapeutics based on non-antibiotic substances is of the utmost importance (Vakil, 2006; Gonçalves et al., 2013).

Treatment of gastrointestinal disorders such as dyspepsia, gastritis and peptic ulcer disease has for centuries been based on the use of natural plant extracts/compounds (Nostro et al., 2005; Parreira et al., 2016) and, despite the focus in the last decades on synthetic drugs, the use of phytopharmaceuticals has regained strength in the scientific community in more recent times (Nostro et al., 2005; Parreira et al., 2016).

Recently, our research group reviewed several classes of bioactive

Abbreviations: cagA, cytotoxin associated gene A; TAs, triterpenic acids; BA, betulinic acid; BOA, betulonic acid; OA, oleanolic acid; UA, ursolic acid; GC–MS, gas chromatography–mass spectrometry; DMSO, dimethylsulfoxide; CLSL, clinical and laboratory standards institute; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; MIC index, mechanism of antibiosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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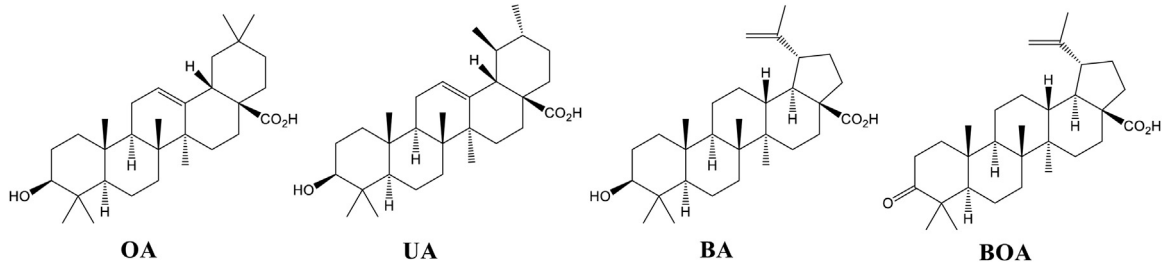

Fig. 1. Chemical structures of triterpenic acids: oleanolic (OA), ursolic (UA), betulinic (BA) and betulonic (BOA) acids.

compounds, *i.e*, compounds known to efficiently interact with the cellular membrane and with biological macromolecules to produce a desired biological outcome (Parreira et al., 2016). These compounds presented themselves as promising candidates for the development of novel/coadjutant therapies against *H. pylori*, being triterpenic acids (TAs) among those promising candidates (Parreira et al., 2016). TAs from lupane, oleanane and ursane classes are regularly found in different plant parts, such as stem bark, leaves and fruit waxes (Jäger et al., 2009). Previously, in the search for natural sources of TAs, our research team described *E. nitens* and *E. globulus* outer bark fractions as rich sources of TAs, namely betulinic (BA), ursolic (UA), betulonic (BOA) and oleanolic (OA) acids (Fig. 1), their acetylated forms, namely 3-O-acetylbetulinic, 3-O-acetyloleanolic and 3-O-acetylursolic, as well as betulonic acid (BOA) (Domingues et al., 2010; Domingues et al., 2011; Domingues et al., 2014).

Those outer bark fractions can easily be obtained in large scale as biomass by-products from pulp and paper industry, an important economic sector in the Iberian Peninsula, which currently are only burned for energy production. By extraction of high-added value compounds, such as TAs, *Eucalyptus* spp. bark may find further economical valorizations within other industries linked to human health improvement, while also contributing to the quest related to the achievement of novel and/or more efficient antibacterial compounds (McChesney et al., 2007).

In this work the antibacterial activity of *Eucalyptus* spp. outer bark-TAs rich extracts against a panel of *H. pylori* strains with distinct degrees of virulence, characterized by cagA status was accessed. The aim was to evaluate the biological potential of these extracts within the frame of development of novel non-antibiotic therapeutic strategies targeting the gastric human pathogen *H. pylori*.

2. Material & methods

2.1. Eucalyptus spp. outer bark lipophilic extracts

E. nitens and *E. globulus* barks were collected from clone plantations described elsewhere (Domingues et al., 2011). The outer part of the barks was separated by hand, air dried until a constant weight was achieved, and grounded to a granulometry lower than 2 mm prior to extraction, as previously described (Domingues et al., 2011; Freire et al., 2002a).

Then, the outer bark samples (15 g each) were Soxhlet extracted with dichloromethane (99% purity, Sigma Chemical) for 7 h (Freire et al., 2002b). Afterwards, the solvent was evaporated to dryness, extracts weighed and the results expressed in percentage of dry outer bark.

2.2. Gas chromatography–mass spectroscopy (GC–MS) analysis

Approximately 20 mg of each dried sample were converted into trimethylsilyl derivatives according to the literature (Freire et al., 2002b). GC–MS analysis was performed with a Trace Gas Chromatograph 2000 Series equipped with a Thermo Scientific DSQ II mass spectrometer, using helium as carrier gas (35 cm/s) and equipped with a DB-1 J & W capillary column (30 m × 0.32 mm *i.d.*, 0.25 mm film thickness). The thermal ramp chromatographic conditions were: initial temperature 80 °C/5 min with a temperature rate of 4 °C/min up to 260 °C and 2 °C min/till the final temperature of 285 °C, maintained for 10 min; injector temperature: 250 °C; transfer-line temperature: 290 °C; split ratio: 1:50. The MS was operated in the electron impact mode with electron impact energy of 70 eV and data collected at a rate of 1 scan/s over a range of *m/z* 33–700. The ion source was maintained at 250 °C. For quantitative analysis, GC–MS was calibrated with pure reference compounds, representative of the major lipophilic extractives components: palmitic acid (99%; Sigma-Aldrich), nonacosane-1-ol (98%; Sigma-Aldrich), β-sitosterol (98%, Sigma–Aldrich), BA (Molekula), UA (Molekula), OA, (Molekula), BOA (Chemos GmbH), relative to tetracosane (99%, Sigma-Aldrich), the internal standard used. The respective multiplication factors needed to obtain correct quantification of the peak areas were calculated as an average of six GC–MS runs. Two aliquots of each extract were analyzed and each aliquot was injected in triplicate. Results are presented as the average of concordant values (less than 5% variation between injections of the same aliquot and between aliquots of the same sample).

2.3. Eucalyptus spp. outer bark extracts and eucalyptus synthetic mixtures

Extracts were dissolved with different solvents, due to their distinct solubility behaviors: *E. nitens* outer bark was solubilized in absolute ethanol (99%, Merck) and *E. globulus* outer bark extract dissolved in dimethylsulfoxide (DMSO; AppliChem), both to a final concentration of 50 mg/mL (w/v). Synthetic mixtures mimicking the *Eucalyptus* spp. outer bark extracts composition, regarding TAs, were prepared with pure standards (BA, UA, OA and BOA) using DMSO as solvent. For the purpose of the antibacterial performance characterization herein described, only the TAs and not their acetylated forms were studied.

2.4. Helicobacter pylori strains

A panel of *H. pylori* strains with distinct virulence degrees was used: two strains expressing the cytotoxin-associated gene A ($cagA^+$), associated with poorer prognosis, *H. pylori* J99 and *H. pylori* 101UK; and the less virulent $cagA^-$ *H. pylori* 094UK and *H. pylori* 131UK (Marcos et al., 2008).

H. pylori strains were grown in tryptic soy agar supplemented with 5% sheep blood (BioMérieux) at 37 °C, 72 h, under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2; GenBox system, BioMérieux). Afterwards, bacterial cells were transferred to Pylori agar (BioMérieux) and incubated another 48 h in the same settings (Magalhães et al., 2015).

2.5. Antibacterial activity analysis

2.5.1. Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and mechanism of antibiosis (MIC_{index}) determination

MIC assays were performed in accordance with Clinical and Laboratory Standards Institute (CLSL) guidelines (CLSI, 2015). Briefly, *H. pylori* cells in exponential growth phase were suspended in Brucella