SHORT COMMUNICATION

**Antimicrobial activity of *Annona muricata* leaf oleoresin**

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ABSTRACT

Bioactive compounds extracted from plants such as antimicrobials have attracted the attention of consumers and the food industry. This study aimed to determine the antimicrobial activity and chemical composition of *Annona muricata* leaf oleoresin obtained by supercritical CO₂ extraction. The oleoresin was obtained by supercritical CO₂ extraction and the chemical identification by gas chromatography coupled to mass spectrometry. Antimicrobial activity was evaluated by broth microdilution method against 14 foodborne fungi and bacteria. The oleoresin major chemical class was phytosterols (22.7%) and the major compounds were γ-sitosterol (15.7%), α-tocopherol (13.7%), phytol (13.1%), and hexadecanoic acid (11.5%). Minimum inhibitory concentration against bacteria ranged from 0.0025 to 0.010 mg mL⁻¹. The oleoresin had high bactericidal activity against all bacteria, mainly *Enterobacter cloacae* and *Pseudomonas aeruginosa* with 0.005 mg mL⁻¹ minimum bactericidal concentration. However, it had low fungicidal activity. The leaf oleoresin of *A. muricata* has promising applications in food, cosmetic, and pharmaceutical industries.

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1. Introduction

Food deterioration caused by microbial contamination is one of the biggest challenges for ensuring food safety in industry (Bhat et al. 2010). Antimicrobial compounds such as sodium sulfite, potassium metabisulfite and sulfur dioxide are frequently added to foods for preservation. However, there are concerns about the safety of sulfur dioxide consumed daily (EFSA ANS Panel 2016). To minimize this problem, the food industry has used natural substances and extracts with antimicrobial activity in order to control foodborne and spoilage microorganisms (Munekata et al. 2020).

*Annona muricata* L. is a tropical plant found in several countries, including Brazil. The leaves are thick and shiny on the upper surface and the fruit is dark green, spiny, and ovoid with a juicy, acid, whitish, and aromatic pulp (Moghadamtousi et al. 2015). Ethnopharmacological applications of alcoholic extracts of *A. muricata* include treatment of bacterial infections, fever, respiratory and skin diseases, diabetes, parasites, and inflammation (McLaughlin 2008; Ioannis et al. 2015). These extracts also showed antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella* sp. (Tojola et al. 2019).

There are reports of antifungal activity against yeasts of the *Candida* genus (Simo et al. 2018), but studies on the antifungal activity of this plant against filamentous fungi are scarce.

Furthermore, the antimicrobial activity of *A. muricata* leaf oleoresin obtained by supercritical CO₂ extraction has not yet been investigated. Supercritical extraction has numerous advantages, including the feasibility of process scale-up; production of high purity extracts; ability to alter extraction conditions, leading to extracts with different compositions; lack of organic solvent residues; low extraction and oxidation temperatures; low environmental impact; and reduced solid waste and wastewater production (Mazutti et al. 2006; Baldino et al. 2021). Thus, this study aimed to determine the antibacterial and antifungal activities against 14 foodborne disease microorganisms and chemical composition of *A. muricata* leaf oleoresin obtained by supercritical CO₂ extraction.

2. Results and discussion

A total of 0.09 g of oleoresin per 1 g of *A. muricata* leaves (dry basis) was obtained, representing a 9% yield. Thirty-eight from forty-eight compounds were identified and the major chemical class was phytosterols (22.7%). The major compounds were γ-sitostanol (15.7%), α-tocopherol (13.7%), phytol (13.1%), and hexadecanoic acid (11.5%) (Table S1), as assessed by gas chromatography coupled to mass spectrometry (GC-MS).

There are no reports of the chemical composition of *A. muricata* leaf oleoresin in the literature; however, the main components of the essential oil are reported such as β-caryophyllene, β-pinene, limonene, germacrene D and α-caryophyllene (Pélissier et al. 1994; Kossouoh et al. 2007; Thang et al. 2013). It might not be valid to compare these results, as oleoresins and essential oils are different types of extracts. Moreover, hydrodistillation, which is one of the most used methods for extracting volatile compounds, can change the essential oil composition by oxidation and hydrolysis (Yousefi
et al. 2019). Supercritical extraction protects thermolabile compounds of molecular change processes because the extraction temperature is lower between 30 and 50 °C (Mazutti et al. 2006). High hydrodistillation temperatures might be the cause of reduced antimicrobial activities found in essential oils than in oleoresins (Wetwityayaklung et al. 2009; Yousefi et al. 2019).

The minimum inhibitory concentration (MIC) of A. muricata leaf oleoresin against different bacteria ranged from 0.0025 to 0.010 mg mL\(^{-1}\); and controls streptomycin and ampicillin from 0.05 to 0.50 mg mL\(^{-1}\) and potassium metabisulfite and sodium sulfite from 0.50 to 4.00 mg mL\(^{-1}\) (Table S2). The oleoresin MIC values for controlling Bacillus cereus and Enterobacter cloacae were 17 and 200 times lower than streptomycin, 33 and 120 times lower than ampicillin, 333 and 800 times lower than sodium sulfite, and 333 and 200 times lower than potassium metabisulfite, respectively. For P. aeruginosa and Salmonella enterica the oleoresin MIC values were 40 and 60 times lower than ampicillin and 20 times lower than streptomycin. For S. aureus the oleoresin MIC values were 25 times lower than streptomycin, 20 times lower than ampicillin, 800 times lower than sodium sulfite, and 200 times lower than potassium metabisulfite, respectively. It suggests that the oleoresin is much more effective in controlling bacteria than the controls.

The oleoresin of A. muricata had lower minimum bactericidal concentration (MBC) values (0.005–0.04 mg mL\(^{-1}\)) than streptomycin and ampicillin (0.1–0.5 mg mL\(^{-1}\)) and sodium sulfite and potassium metabisulfite (0.5–4.0 mg mL\(^{-1}\)), indicating a greater bactericidal activity (Table S3). The oleoresin was very effective to all bacteria, mainly E. cloacae and P. aeruginosa with MBC values 20 times lower than streptomycin, 100 and 40 times lower than ampicillin, 800 times lower than sodium sulfite, and 100 and 400 times lower than potassium metabisulfite, respectively. The oleoresin was efficient for controlling all Gram-negative and -positive bacteria tested. It suggests that A. muricata leaf oleoresin has high capacity to control a broad spectrum of bacteria and it is much more effective than the controls.

Some Bacillus spp. are capable of forming biofilms, preventing the action of the antimicrobials (Branda et al. 2004). Merzougui et al. (2014) found that the antibiotics ampicillin (10 mg mL\(^{-1}\)), tetracycline (30 mg mL\(^{-1}\)), oxacillin (1 mg mL\(^{-1}\)), cefepime (30 mg mL\(^{-1}\)), and penicillin (10 IU mL\(^{-1}\)) did not exhibit antimicrobial activities against B. cereus. Moreover, B. cereus is characterized by the formation of endospores, which increases its resistance to synthetic antimicrobials (Valero et al. 2006). It indicates that the antibacterial activity of A. muricata leaf oleoresin against this species is of importance as a microbial control alternative.

The multidrug-resistant and Gram-negative bacterium P. aeruginosa is frequently implicated in severe infectious processes in human and non-human animals (Bjarnsholt et al. 2010; Magiorakos et al. 2012). This bacterium has several genes dedicated to modulating responses to environmental changes and regulating transcription, increasing its adaptive capacity (Stover et al. 2000). It suggests that the antibacterial activity of A. muricata leaf oleoresin against this species is an alternative to control this species.

The MIC values of A. muricata leaf oleoresin against fungi ranged from 2.4 to 10 mg mL\(^{-1}\), and the controls bifonazole and ketoconazole were from 0.1 to 2.5 mg mL\(^{-1}\),
and sodium sulfite and potassium metabisulfite from 0.50 to 2.00 mg mL\(^{-1}\) (Table S4). The oleoresin had a minimum fungicidal concentration (MFC) from 5 to 40 mg mL\(^{-1}\), and the controls MFC values from 0.2 to 3.5 mg mL\(^{-1}\) (Table S5). The fungistatic and fungicidal activities of \(A. \) muricata oleoresin were not so effective compared to bacteriostatic and bactericidal activities (Tables S2–S5). It indicates that the oleoresin has selective control of bacteria but not fungi and could be used in growth media for this purpose. However, against \(P. \) funiculosum and \(P. \) ochrochloron the MIC values were 2.5 and 2.4 mg mL\(^{-1}\) and MFC values were of 5 mg mL\(^{-1}\), respectively (Tables S4 and S5). It means that the best results against these fungi had oleoresin MIC values 12 times lower than streptomycin, 0.08 and 1.04 times lower than ampicillin, 0.4 and 0.8 times lower than sodium sulfite, and 0.2 times lower than potassium metabisulfite, respectively. It indicates that oleoresin is less effective against fungi than controls, but in proper amounts could control some fungi.

The mechanism of action of medicinal plants against microorganisms has been related to the major chemical compounds of the plant. Plant oils have a heterogeneous composition and several compounds may act synergistically, increasing biological activity (Simić et al. 2004). \(\gamma\)-Sitosterol (Table S1) is a phytosterol found in many plants that are traditionally used as antibacterial, antifungal, and antiangiogenic agents (Zhang and Zhou 2011). Moreover, it has anti-diabetic activity by protein-binding interactions (Balamurugan et al. 2015). \(\alpha\)-Tocopherol is a type of vitamin E, preferentially absorbed and accumulated in humans, and the first line of defense against peroxidation of lipids contained in cell membranes. As an antioxidant, it breaks free-radical chain reactions and may also prevent some hypertension complications such as atherosclerosis, ischemic heart disease, and cerebral ischemia (Bartolini et al. 2021). These major compounds of \(A. \) muricata oleoresin, \(\alpha\)-tocopherol and \(\gamma\)-sitosterol, are food additives and/or antimicrobials without having an intake limitation and therefore with the possibility of using a larger quantity if needed (Bertolami and Bertolami 2018; Silva et al. 2019).

The diterpenoid phytol (Table S1) has antioxidant, anti-inflammatory, antimicrobial, cytotoxic, and neuroprotective activities (Islam et al. 2015). Its mechanisms of antimicrobial action may include DNA synthesis inhibition, blockage of G1 progression, and inhibition of microtubule formation (Majewska et al. 2003; Islam et al. 2015). Phytol can also inhibit prometaphase by stopping cell division at the metaphase stage because of its interactions with microtubules (Pandey 2008). The microbial cytotoxicity of phytol is presumably due to the presence of a hydroxyl group at the \(\alpha\)-carbon position (Gyawali and Ibrahim 2014; Shah et al. 2014). In addition, the antimicrobial activity of plant oils is increased when they are composed of phenolic compounds, oxygenated terpenes (mainly alcohols and aldehydes) or the phytols found in \(A. \) muricata leaf oleoresin. These compounds may interact with lipid components of the bacterial membrane lipid bilayer, accumulating and increasing membrane permeability, resulting in loss of cell components, ion leakage, depletion of intracellular ATP concentration, and, ultimately microbial death (Delespaul et al. 2000; Kumar et al. 2008).

One of the challenges of oleoresins is the high cost of extraction, which can be up to six times higher than the cost of a chemical antimicrobial product (Kouassi et al. 2012). However, the increasing demand for natural compounds such as oleoresins may
promote the use of bioengineering techniques for their production and/or large-scale processes to reduce production costs (Burt 2004). According to the European Pharmacopoeia, the minimum oil extraction yield is 2 mL kg\(^{-1}\) plant to enable the develop of applications (Nemeth and Bernath 2008). In our study, A. muricata leaf oleoresin yield was 0.09 g g\(^{-1}\) and considering the approximate wax density is 0.9 g mL\(^{-1}\) (He et al. 2004) it was estimated that the oleoresin yield was 100 mL kg\(^{-1}\), which is more than adequate for industrial application development (Nemeth and Bernath 2008). Furthermore, oils have low risk of triggering resistance in pathogenic microorganisms because of their complex mixture of compounds with different antimicrobial properties and mechanisms of action (Oke et al. 2009). Bacteria, for instance, undergo several adaptations to decrease susceptibility to lipophilic compounds such as fatty acid saturation to improve lipid bilayer structure, modification of cell wall hydrophobicity, active excretion by energy-consuming transport systems, and modification of outer membrane lipopolysaccharides (Sikkema et al. 1994).

In conclusion, phytosterols are the predominant compound class (22.7%) in A. muricata leaf oleoresin and the major compounds are \(\gamma\)-sitosterol (15.7%), \(\alpha\)-tocopherol (13.7%), phytol (13.1%), and hexadecanoic acid (11.5%). The oleoresin has high antimicrobial activity against all Gram-positive and -negative bacteria studied but it not as effective against fungi. The antibacterial activity of A. muricata leaf oleoresin is a potential alternative for the pharmaceutical, cosmetic, and food industries.

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