Research paper

Antibacterial potential of *Urtica dioica* and *Lavandula angustifolia* extracts against methicillin resistant *Staphylococcus aureus* isolated from diabetic foot ulcers

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Despite the antibacterial potential of plant extracts against several bacterial infections, until now, no major studies have been published about the effect of lavender and nettle leaves against methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus* (MSSA and MRSA, respectively). Thus, the authors studied their antibacterial potential against MSSA and MRSA from diabetic foot ulcers in order to find alternatives to the systematic use of antibiotics. Phenolic acids, flavonoids, flavones and flavonols were extracted from lavender and nettle leaves and characterized by HPLC-DAD/Vis. Disc diffusion assay and minimum inhibitory concentration (MIC) were used to assess their antibacterial activity. A direct association between the high content of hydroxycinnamic acids (chlorogenic acid, caffeic acid, rosmarinic acid) and flavonoids (quercetin) and decreasing bacterial growth activity was noted. The fact that lavender and nettle are rich in hydroxycinnamic acids and quercetin seems to explain the high antibacterial potential of these plant and the differences between them.

1. Introduction

Diabetes mellitus is a disease with multifaceted complications and diabetic foot wounds, and subsequent foot ulceration, is one of the most common, with a prevalence that ranges from 4% to 10% (Singh et al., 2005). Diabetic foot ulcers frequently become infected and are a major cause of global hospital admissions and account for more than half of non-traumatic lower limb amputations (Dang and Boulton, 2003; Pinzur et al., 2005). It is well recognized that diabetic foot infection is polymicrobial, and *Staphylococcus aureus* is the pathogen most frequently isolated (Kim et al., 2012; Dunyach-Remy et al., 2016; Reveles et al., 2016). Due to the frequent use of antibiotics, *S. aureus* strains have evolved resistance against the most abundantly used therapeutics and the treatment of methicillin-resistant *S. aureus* (MRSA), infections is polymicrobial and *Staphylococcus aureus* is the pathogen most frequently isolated. The World Health Organization, has developed a global priority list of antibiotic-resistant bacteria, to help in prioritizing the research and development of new and effective antibiotic treatments (World Health Organization, 2017); MRSA is classified as high priority. Also, epidemiological studies showed that MRSA, besides being multidrug resistant, has emerged as a serious and common problem in patients with diabetic foot ulcers (Kim et al., 2012), therefore, the research for alternative measures to antimicrobials is imperative. Recent strategies have been the research for natural compounds largely present in plants with recognized antimicrobial properties (González-Lamothe et al., 2009). Thousands of plant species have been tested for antimicrobial properties, but still the vast majority has not been adequately evaluated against MRSA, such as lavender (*Lavandula angustifolia* Mill) and nettle (*Urtica dioica* L). Lavender, a plant, from the lamiaceae family is recognized to act as an antioxidant and to be anti-inflammatory, as well as having antimicrobial effects (Moon et al., 2006; Field et al., 2008; Kritsidima et al., 2009; Dobesberger and Buchbauer, 2011). Similar reports have been made about nettle leaves, a perennial plant belonging to the urticae family. Nettle extracts can have beneficial effects due to their levels of flavonoids, carotenoids, sterols and minerals (Aksu and Kaya, 2004; Sebranek et al., 2005; Karabacak and Bozkurt, 2008) and have shown antimicrobial activity against several microorganisms (Gülçin et al., 2003; Serafim et al., 2006; Köksal et al., 2009; Alper et al., 2011; Kritsidima et al., 2009; Sebranek et al., 2005; Karabacak and Bozkurt, 2008). Therefore, the authors studied their antibacterial potential against MSSA and MRSA from diabetic foot ulcers in order to find alternatives to the systematic use of antibiotics. Phenolic acids, flavonoids, flavones and flavonols were extracted from lavender and nettle leaves and characterized by HPLC-DAD/Vis. Disc diffusion assay and minimum inhibitory concentration (MIC) were used to assess their antibacterial activity. A direct association between the high content of hydroxycinnamic acids (chlorogenic acid, caffeic acid, rosmarinic acid) and flavonoids (quercetin) and decreasing bacterial growth activity was noted. The fact that lavender and nettle are rich in hydroxycinnamic acids and quercetin seems to explain the high antibacterial potential of these plant and the differences between them.
2004; Tahri et al., 2000; Rieheman et al., 1999). Despite the beneficial properties of lavender and nettle, no studies have been conducted into their effects on MRSA, particularly against strains isolated from diabetic foot ulcers. Therefore, the aim of this study was to evaluate the biological potential of nettle and lavender, as natural sources of bioactive compounds with antibacterial effect against methicillin-sensitive S. aureus (MSSA) and MRSA, isolated from diabetic foot ulcers. Results from this study could contribute to clarify whether these plants can be useful as alternatives or in combination with traditional antibiotic therapy.

2. Material and methods

2.1. Plant material

Lavender (Lavandula angustifolia Mill) and nettle (Urtica dioica L.) were collected in a field located in Northern Portugal (460 m, 41° 17’N and 7°44’W) in spring and identified by Dr. A. Aires, University of Trás-os-Montes and Alto Douro (UTAD), Portugal. Field collection standards were also stored in the botanical collection of the Department of Botany (UTAD). Plants leaves were freeze-dried (Ultra-dry Systems™) and powdered prior to be used in experimental work.

2.2. Extraction

The powder was extracted in triplicate with ethanol 70%, in a water bath (70°C, 1 h). Extracts were then centrifuged at 1500 rpm (5 min 4 °C), filtered and 1 mL aliquots of each extract were collected and filtered again (Spartan Ø 0.13 mm) before being placed in HPLC vials and stored at 20 °C until analysis. Remaining hydro-alcoholic extracts were dried in the rotary evaporator at 40 °C until complete dehydration. Residues were stored at −20 °C for antibacterial experimental assay.

2.3. Total phenolic and flavonoid content

Total phenolic content (TPC) and total flavonoid content (TFC) were determined colorimetrically using a 96-well microplate (Costar, Corning, New York, USA). The TPC was measured based on the Folin–Ciocalteau assay (Singleton et al., 2002), with some modifications. Briefly, 20 μL of plant extract was added to each microplate well. Then 100 μL of Folin-Ciocalteu phenol reagent (1:10 in dd H2O) was added, followed by addition of 80 μL of 7.5% Na2CO3. Microplates were incubated for 15 min at 45 °C (warm-bath) and absorbance was read at 765 nm against a blank (solvent extraction) in a microplate reader (Multiskan™, FC Microplate Photometer, Thermo Scientific). A calibration curve, using gallic acid, was used for quantifications. Values were expressed as mg gallic acid equivalent (GAE) g⁻¹ dry weight (mg GAE g⁻¹ d w).

The TFC in samples was measured based on AlCl3 spectrophotometer method (Shin et al., 2008) with some modifications. To each microplate well, 100 μL of ultra-pure water was added, followed by 10 μL of NaNO2 and 25 μL of each plant extract. The mixture was left to stand in the dark at room temperature for 5 min. Then, 15 μL of AlCl3 10% was added and left to stand in the dark at room temperature for 6 min. After, 100 μL of NaOH 1 M and 50 μL of ultra-pure water were added to each well. Absorbance was measured at 510 nm in microplate reader, against blank. A calibration curve, using catechin, was used for quantifications. Values were expressed as mg catechin equivalent (CAE) g⁻¹ dry weight (mg CAE g⁻¹ d w). The mean and standard deviation within samples were calculated for all cases.

2.4. Phytochemical composition

Phytochemical composition was evaluated by HPLC-diode array detector (DAD)/Vis system. In order to increase chromatogram accuracy, HCl (2 M) in 50% methanol and tertiary butylhydroquinone (TBHQ) were added to 200 μL of each plant extract and placed in the heater at 80 °C for 2 h and then centrifuged (model 2–16 K, Sigma, Osterode, Germany) at 13000 rpm for 20 min. Supernatant was collected and injected in HPLC. The eluent was constituted by water with 1% of trichloroacetic acid (TFA) (solvent A) and acetonitrile with 1% TFA (solvent B). Chromatograms were recorded at 280, 320, and 370 nm with a C18 column. Phytochemical identification was carried out using peak retention time and UV spectra. The quantification was carried out using external calibration curves with commercial phenolic standards (Extrasynthese, Cedex, France).

2.5. Bacteria strains

Samples were taken from diabetic foot infections at Hospital Centre of Trás-os-Montes e Alto Douro, Vila Real, Portugal (under a protocol with UTAD university). Isolates were identified by morphological and biochemical tests, followed by Kirby-Bauer antibiotic sensitivity assays. Different antibiotics such as Oxacillin, Levofloxacin and Vancomycin (10 μg) were used. Ten isolates of MSSA and ten isolates of MRSA were used in this study.

2.6. Antibacterial activity of plants extracts

Stored plant extracts were diluted with 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich, Germany) to a concentration of 100 mg mL⁻¹ to be used in disc diffusion and minimum inhibitory concentration (MIC) assays.

Disc diffusion assay (Jorgensen and Turnidge, 2007) was used to screen the antibacterial effect of plant extracts. Test isolates were grown on nutrient agar plates and later inoculated on nutrient broth to log phase. Test bacterial suspensions turbidity was adjusted to 0.5 MacFarland units. Petri dishes were prepared with 20 mL of Mueller-Hinton Agar (Oxoid, UK) and seeded with bacterial suspensions. Sterile paper discs were impregnated with 15 μL of each previous extract solution in DMSO (corresponding to a 1.5 mg of extract) and placed on an agar plate. Incubation was at 37 °C for 24 h. Discs of gentamicin (CN10 – Oxoid CT0024B, UK) were used as positive control and discs impregnated with DMSO were used as negative control. After incubation, the diameter (mm) of the inhibitory zone around the disc was recorded. All the tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced ± SD (standard deviation).

Antibacterial activity index (Aai) was used for efficiency comparison between plant extracts and positive control (antibiotic), using the following formula: Aai = (A1 - A0)/A1, where A1 is diameter of inhibitory zone of compound tested, A0 is the diameter of sterile paper disc. The rate values below 0.14 (representing the boundary near the halo limit) were considered insignificant and thus ignored when specifying MIC.

Minimum inhibitory concentration (MIC) was evaluated by a resazurin microdilution assay (Sarker et al., 2007). Bacteria tested were picked from overnight cultures in brain heart agar (Oxoid, UK). A small portion of bacteria was transferred into a bottle with 50 mL of Mueller-Hinton broth (Oxoid, UK), capped and placed in an incubator overnight at 37 °C. After 16 h of incubation, bacterial suspension was adjusted to an optical density of 0.5 measured at OD500 nm. The resazurin solution (Sigma-Aldrich, Germany) was prepared as 3.4 mg mL⁻¹ in sterile distilled water. A 96-wells sterilized microplate was used. A volume of 100 μL of Mueller-Hinton broth was used in each well together with 100 μL of extract solution, or positive control. From the first well (belonging to the first horizontal line) 100 μL was taken and added to the next well and then this step was repeated to each of the following wells in the vertical line, allowing a serial fold dilution of decreasing concentration (range of 50 mg/mL to 0.024 mg/mL). In

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addition, 20 μL of bacterial suspension and 20 μL of resazurin solution was added to each well. Microplates were incubated at 37 °C for 18–24 h. All tests were performed in triplicate and MIC was then evaluated visually by the color change of resazurin in each well (blue to pink in the presence of bacteria growth). After each MIC identi
cation, 20 μL of resazurin solution was added to each well. Microplates were incubated at 37 °C for 24 h. All tests were performed in triplicate and MIC was then evaluated visually by the color change of resazurin in each well (blue to pink in the presence of bacteria growth). After each MIC identification, 20 μL of resazurin solution was added to each well. Microplates were incubated at 37 °C for 24 h. All tests were performed in triplicate and MIC was then evaluated visually by the color change of resazurin in each well (blue to pink in the presence of bacteria growth).

2.7. Data analysis

The data were analyzed using the statistical program SPSS version 17.0 (Statistical Package for the Social Sciences). Data was compared applying nonparametric Wilcoxon test (p < 0.05).

3. Results and discussion

Table 1 shows the phenolics (TPC) and flavonoids (TFC) quantified in lavender and nettle leaf extracts. Generally, for nettle TPC was higher than TFC, and among phenolic acids, caffeic acid was the most representative and protocatechuic acid and p-coumaric acid were found at moderate levels. A high content of flavonoids, such as rutin, isoorcezetin and quercetin, was also found. Lavender samples had the hydroxycinnamic and hydroxybenzoic acids, such as caffeic, ferulic, syringic and rosmarinic acids, all non-flavonoids, as the most representative.

Generally, lavender had a higher quantity and diversity of phenolic compounds, and lower levels of flavonoids, than nettle leaves, which can be important to endorse the type of biological activity of each extract. It is well accepted that higher content of hydroxycinnamic acids, such as chlorogenic and caffeic acids, as well as flavonoids, such as quercetin and quercetin-related compounds, are important since they are highly correlated with antioxidant, anti-inflammatory, antimicrobial and anticancer activities. In fact, quercetin has been reported as having antibacterial activity against S. aureus and Pseudomonas aerugi
nosa (Woénicka et al., 2013). Chlorogenic and caffeic acids are also reported to be important antimicrobial and antioxidant agents (Sung and Lee, 2010). Rosmarinic acid is a caffeic acid ester found in a variety of plants of the lamiaceae family such as lavender (as we observed in this work), and has been reported to be one compound with anti-
oxidative, anti-inflammatory and anti-tumour effects (Zhang et al., 2010). Therefore, based on their composition, nettle and lavender may have significant potential, not only as a source of phytochemicals for the human diet, but more importantly, as a source of natural compo
unds with antimicrobial, anti-oxidative and anti-inflammatory activity.

The antibacterial activity of plant extracts was evaluated by the presence of an inhibition zone and results showed that lavender and nettle are effective against MSSA and MRSA (Table 2). The bacterial inhibition halos varied between 11.3–19.3 mm for nettle, and from 8.7–21.3 mm for lavender, although effectiveness was not significantly different between extracts. For gentamicin (positive control), inhibition halos varied between 17.7 and 24.7 mm, showing effectiveness that was
significantly different from the extracts. However, considering MRSA mean values, the relative difference between antibiotic and plant extracts does not exceed 17%. This similarity between antibiotic and both, lavender and nettle extracts, reveals that these plants can be a source of phytochemicals with antimicrobial activity, as shown by antibacterial activity index (Aai) (Fig. 1A and B). The Aai was used for efficiency comparison between plant extracts and antibiotics, and values obtained were quite similar, reinforcing this idea. Except for one MSSA isolate (MJMC 001), the average difference of Aai between gentamicin and nettle was 8.1% and 9% for MRSA and MSSA isolates, respectively, whilst between gentamicin and lavender was 8.6% and 10.1% for MRSA and MSSA, respectively. These results mean that nettle and lavender, at the tested concentrations, show an interesting antimicrobial activity, compared with the antibiotic gentamicin.

Results from MIC assay also showed parallelism between these plant extracts (Table 3). It was noted that for nettle extracts MIC values ranged between 0.0625–0.500 mg mL\(^{-1}\) and for lavender extracts ranged between 0.0625–1.0 mg mL\(^{-1}\), closer to the MIC values of the positive control gentamicin, which were lower than 0.039 mg mL\(^{-1}\). In addition, the main bacterial effect observed was bacteriostatic, for both lavender and nettle extracts. It seems that these extracts, rather than eliminate, limit exponential bacterial growth, which is also important from a pharmaceutical point of view.

Based on HPLC results, it seems that the high content of identified phenolics, such as chlorogenic acid, caffeic acid, rosmarinic acid and rutin in nettle and lavender extracts, could be the main reason for the antibacterial capacity of these extracts against \textit{S. aureus} isolates. Recent experiments with pure phenolic compounds (Cueva et al., 2010, 2012) showed that phenolic acids, such as caffeic acids, were very effective against both gram negative and gram positive bacteria. Thus, plant extracts with a higher content of caffeic acid, as we have observed, could increase efficiency in bacteria growth inhibition. In fact, nettle extracts present an average 163 μg g\(^{-1}\) dry weight; whereas lavender extracts present 61 μg g\(^{-1}\) dry weight of caffeic acid. Rodríguez-Vaquero et al. (2007) also found that caffeic acid inhibited the growth of \textit{P. aeruginosa} and \textit{S. aureus} which is in agreement with our results for \textit{S. aureus} isolates. Furthermore, previous studies reported that other phenolics, like ferulic acid, rosmarinic acid, rutin and quercetin, can be effective against other gram positive bacteria like \textit{Listeria monocytogenes} (Liu, 2003; Saavedra et al., 2010). Antibacterial effects of these plant extracts were also observed in different situations (Akbari et al., 2015; Sasaki et al., 2015; Motamedi et al., 2014; Shahin et al., 2014).

However, previous researchers have stated that pure phenolic compounds are sometimes ineffective or less effective than phenolic...
plant extracts, probably because of the additive/synergistic effects of the extracts. Additive and synergistic effects of phytochemicals in plants are responsible for their potent bioactive properties and the benefit of them is attributed to the complex mixture of phytochemicals present in the whole plants (Lowy, 2003). This seems to be the case here, since nettle and lavender presented similar antibacterial activity, despite the differences in average content of each phenolic identified in both extracts.

It is interesting to note that both MRSA and MSSA showed susceptibility towards the extracts tested. MRSA is resistant to all β-lactam antibiotics and this ability is due to the acquisition of the mecA gene (Lowy, 2003). This gene encodes PBP2a protein and when it is challenged by β-lactams, MRSA will use transpeptidase functionality of PBP2a to synthesize the cell wall (Wilke et al., 2005). However, specific compounds can affect this ability, causing cell wall disruption. Shintani et al. (2000) found that in presence of licoricidin and triazine dye, MRSA PBP2a synthesis during bacteria multiplication was affected. Also, the phenolics epicatechin gallate and epigallocatechin gallate observed against MRSA and MSSA. In fact, the presence of carboxylic MRSA, but phenolics present in both could play a similar role.

Also, the structure of phenolic compounds, such as hydroxybenzoic and hydroxycinnamic acid derivatives, could contribute to the activity observed against MRSA and MSSA. In fact, the presence of carboxylic acid (COOH), two hydroxyl (OH) groups in the para and ortho positions of the benzene ring and also a methoxyl (OCH3) group in the acid (COOH), two hydroxyl (OH) groups in the observed against MRSA and MSSA. In fact, the presence of carboxylic MRSA, but phenolics present in both could play a similar role.

4. Conclusions

Our results highlight the antibacterial activity of nettle and lavender extracts against MRSA and MSSA. Based on this, we can conclude that these extracts can be used alone or in combination with antibiotics to limit the infections of these bacteria, or to prevent secondary opportunistic infectious agents. They can easily be used prophylactically in pharmaceutical formulations for topical applications, preventing the appearance of infections. In addition, they can be used in combination with lower doses of traditional antibiotics, reducing the time and severity of infections and occurrence of any possible antibiotic resistance, particularly in patients with diabetic foot ulcers.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgments

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Table 3

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Nettle</th>
<th>Lavender</th>
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<tbody>
<tr>
<td></td>
<td>MIC (mg mL$^{-1}$)</td>
<td>Bacterial activity</td>
</tr>
<tr>
<td>MJMC 001 (MSSA)</td>
<td>0.500 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 002 (MSSA)</td>
<td>0.250 ± 0.000</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>MJMC 003 (MRSA)</td>
<td>0.188 ± 0.088</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 004 (MSSA)</td>
<td>0.500 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 007 (MSSA)</td>
<td>0.188 ± 0.088</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 008 (MSSA)</td>
<td>0.500 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 009 (MRSA)</td>
<td>0.063 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 010 (MRSA)</td>
<td>0.125 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 011 (MRSA)</td>
<td>0.125 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 014 (MSSA)</td>
<td>0.125 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 016 (MSSA)</td>
<td>0.250 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 018 (MSSA)</td>
<td>0.250 ± 0.000</td>
<td>Bacteriostatic</td>
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<tr>
<td>MJMC 020 (MRSA)</td>
<td>0.125 ± 0.000</td>
<td>Bacteriostatic</td>
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<tr>
<td>MJMC 021 (MSSA)</td>
<td>0.188 ± 0.088</td>
<td>Bacteriostatic</td>
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<tr>
<td>MJMC 022 (MRSA)</td>
<td>0.500 ± 0.000</td>
<td>Bacteriostatic</td>
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<tr>
<td>MJMC 024 (MSSA)</td>
<td>0.375 ± 0.177</td>
<td>Bacteriostatic</td>
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<tr>
<td>MJMC 025 (MRSA)</td>
<td>0.250 ± 0.000</td>
<td>Bacteriostatic</td>
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<tr>
<td>MJMC 026 (MSSA)</td>
<td>0.250 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>MJMC 027 (MRSA)</td>
<td>0.250 ± 0.000</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>MJMC 029 (MRSA)</td>
<td>0.250 ± 0.000</td>
<td>Bacteriostatic</td>
</tr>
</tbody>
</table>

$^a$ Results are expressed as mean ± SEM (standard error of the mean) of 3 replicates.

References


toxins and diabetic foot ulcers: role in pathogenesis and interest in diagnosis. Toxins 8 (7), 209.


Liu, R.H., 2003. Health bene...