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The inhibition of methicillin-resistant *Staphylococcus aureus* by essential oils isolated from leaves and fruits of *Schinus areira* depending on their chemical compositions*

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Schinus areira **L. is a native plant from South America used for centuries in traditional medicine. Here, we investigate the antimicrobial activity of four essential oils extracted from leaves and fruits of *S. areira* exhibiting different chemical profiles. The antibacterial activity against the human pathogenic bacteria *Staphylococcus aureus* susceptible as well as methicillin resistant strain was assessed by the broth microdilution assay. The results showed that the limonene-rich oil extracted from the leaves and fruits have potent antibacterial effect on *S. aureus* ATCC 25923, while the α-phellandrene-rich fruit oil having a lower content of limonene showed the lowest antibacterial efficacy. In this work, for the first time, we demonstrated the bactericidal activity of essential oils isolated from fruits and leaves of *S. areira* against susceptible and methicillin resistant *S. aureus* strains. All results point out the potential use of the *S. areira* oils as antimicrobial agents to be used, at least against *Staphylococcal* infections.**

Key words: *Schinus areira*; essential oils; antibacterial; Methicillin-resistant *Staphylococcus aureus*

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INTRODUCTION

Plant-based essential oils (EO) are well known to exhibit a wide range of biological activities as well as they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Dorman & Deans, 2000). The use of plant compounds as antimicrobial agents is enjoying great popularity in the late 1990s (Cowan, 1999). Since the middle ages, the EO has been widely used in pharmaceutical, sanitary, cosmetic, and agricultural and food industries (Bakkali *et al.,* 2008). A recent overview study indicates that EO can be used as effective antiseptics against many species, including multidrug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Kon & Rai, 2012). These *S. aureus* strains resistant to numerous antibiotics as methicillin, penicillin as well as quinolones and aminoglycosides are a worldwide problem increasing the probability of unfavorable infection outcome (Grundmann *et al.,* 2006; Chen *et al.,* 2010). Therefore, one of the proposed methods to cope with MDR bacteria is the use of natural antimicrobial substances such as the plant EO (Langeveld *et al.,* 2014).

Molle, aguaribay, pepper tree (USA.), among others, refer to two species of *Schinus* (Anarcadiaceae family) closely taxonomically related as *S. molle* L. and *S. areira* L. native from South America (Muñoz, 2000). The essential oil of these aromatic plants is obtained by distillation and contains a variety of volatile molecules such as terpenes and terpenoids, derivatives of phenol compounds and other aromatic and aliphatic components. The fruits and leaves of these plants are used in traditional medicine as antibacterial, antifungal and antirheumatic, anti-conjunctivitis, tuberculosis, bronchitis, cough (Molina-Salinas *et al.,* 2007).

Schinus areira L. [synonymous: *Schinus molle* L. var. *areira* (L.) DC.] is distributed principally in Perú, Bolivia, Northern Chile. It grows naturally in Argentina, from 0 to 3000 m altitude, in central and northwest of the country (Catamarca, San Luis, Córdoba, La Rioja, Salta, Tucumán and Jujuy provinces). All parts of this species have been used medicinally by indigenous people as a digestive, stimulant, diuretic, astringent, and antidepressant and for topical use as wound healer and skin antiseptic (Martínez & Barboza, 2010).

Chemical compositions of essential oil of *S. areira* from aerial parts (leaves and flowers) collected from Mendiolaza, Córdoba, Argentina revealed that the chromatographic profile was predominantly composed by monoterpenes compounds (66.4%); the main components were α-pinene (13.80%), limonene (12.81%) and camphene (12.62%) (Bigliani *et al.,* 2012). Other chemical compositions of essential oils from aerial parts of *S. areira* were reported by Scrivanti *et al.* (2003), Wannaz *et al.* (2003) and Murray *et al.* (2005). Extensive studies concerned with the chemical composition of EO isolated from *S. molle* have been reported (Marongiu *et al.,* 2004; Hayouni *et al.,* 2008; Atti dos Santos *et al.,* 2009; Mendonça Rocha *et al.*, 2012) showing the basic constituents α-phellandrene, β-phellandrene, myrcene and α-pinene. These essential oil compositions are different from Chilean species as *Schinus ploygamus* and *Schinus longifolia* (Mur-

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Abbreviations: EO, essential oils; MRSA, methicillin-resistant *Staphylococcus aureus*; MDR, multidrug-resistant bacteria; Py1F, Pinchayoc 1 fruits; Py1L, Pinchayoc 1 leaves; Tg2F, Tilcara 2 fruits; Tg2L, Tilcara 2 leaves

ray *et al.,* 2009). The chemical compositions of *Schinus* oils, are focused generally on fruits, however, correlation between their constituents and the biological action are issues that have not been totally investigated.

Previously, we reported the chemical composition of EO from fruits of *S. areira* at the same state of development which showed similar qualitative chemical profile, but containing different amounts of the main bioactive compounds (limonene, α- and β-phellandrene, sabinene and camphene) in relation to the geographic origin (Viturro *et al,*. 2010). The aim of the present study was to evaluate the antibacterial activity of EO isolated both from leaves and fruits of two specimens of *S. areira* growing in Jujuy, Argentina displaying different chemical profiles against the human pathogenic bacteria *S. aureus* susceptible as well as resistant to antibiotics.

MATERIAL AND METHODS

Plant material. Aerial parts (leaves and fruits) of *S. areira* L. were collected in May 2013 in Jujuy, Argentina from over ten-year-old trees. Sample HN° 13–12 was collected from the specimen Pinchayoc (Py1) in Humahuaca region (altitude above 2900 m). Sample HN° 13–16 was collected from the specimen Tilcara (Tg2) in Tilcara region (altitude above 2500 m). The plant material was identified by Professor Gustavo Giberti (University of Buenos Aires, Argentina).Voucher specimens have been deposited in Herbarium of PRONOA-UNJu (Faculty of Engineering from National University of Jujuy, Argentina) and herbarium BAF (Buenos Aires Farmacobotánica, University of Buenos Aires, Argentina). The harvested materials were air-dried at room temperature (20±2°C) for 1 week (fruits) and 3–5 days (leaves).

EO extraction. Oils were extracted from 200g dried material (grinded fruits or leaves) by steam distillation for 2.5–3.5 h with a Clevenger trap. Py1 and Tg2 dried leaves yielded 0.49 and 0.52% (w/w on dry weight basis) of colorless essential oil, respectively, while the steam distillation of Py1 and Tg2 grinded fruits yielded 4.16 and 3.32% (w/w on dry weight basis), respectively, of pale yellowish oils with a distinct smell.

The collected oil samples were dried (Na_2SO_4) and stored at 4°C until analysis by GC/MS and GC/FID.

Chemical characterization of EO. GC/MS analysis was carried out on a Hewlett Packard GC 6890/MDS 5972 apparatus equipped with a HP-5 MS column (30 m × 0.25 mm; 0.25 μm film thicknesses) and a N_2 carrier gas flow of 0.7 mL/min. The oven temperature program was 60°C (5 min), 60–230°C (6°C/min). The temperature of the transfer line (300°C) and of the injector (250°C) was held constant during analysis. GC/FID analysis was carried out on a Konik KNK 3000 G with a unique injector connected by a flow splitter Y shaped to two columns: a) Innowax 20 M and b) HP5, both 60 m × 0.25 mm with 0.25 0.25 μm film thickness. Carrier gas H2; flow rate 1.2 mL/min. The oven temperature program was the same as above. Injector and both FID temps were set at 25°C and 300°C respectively. The components of the EO were identified by comparison of their Retention indices (RI) and mass spectra with those from literature data (Adams, 2007; Jennings, 1980), those recorded in the MS library (NBS 75K, NIST98), and those of a spectra library built up from pure substances and components of known oils. The RI was determined relative to a series of *n*-alkanes (C_7–C_{24}). The quantification of each compound was performed on the basis of their GC/FID peak areas without the use of response factor corrections.

Antibacterial activity. The *in vitro* antibacterial activity of the *S. areira* oils was evaluated against the human pathogenic bacteria *S. aureus* ATCC 25923 and a nosocomial multidrug-resistant strain of *S. aureus* (MRSA, 1977 strain). MRSA 1977 (mec+, nuc+) was isolated from a pediatric patient in the Pediatric Hospital S.A.M.I.C. "Prof. Dr. Juan P. Garrahan" and identified by standard methods according to the recommendations of the Manual of Clinical Microbiology (Versalovic et al. 2010). The resistance to antibiotics (oxacyllin, rifampicin, erythromycin, clindamycin and ciprofloxacin) was assessed according to the clinical and laboratory Standards Institute (CLSI) guidelines (CLSI 2012).

The overnight cultures were standardised by dilution with sterile Mueller-Hinton (MH) broth (Difco, MD, USA) to an absorbance of 0.07–0.1 at 625 nm according to Ojeda *et al.* (2013). The antibacterial activity of the EO was estimated by the microplate bioassay with slight modifications (CLSI, 2006). Dilutions in MH medium containing 0.5% Tween 80 were prepared from an 80% (v/v) EO in ethanol, as reported (González *et al.,* 2003). Each oil (3–50 μL/mL, 200 uL per well) was incubated in 96-well flat bottom microplates together with each bacterial strain. The microplate was aseptically sealed, and incubated at 37°C for 16–24 h under constant shaking (100 rpm). All experiments were performed in triplicate. The antibacterial activity was expressed as percentage of bacterial growth inhibition: % inhibition 100 × (Ab control- Ab Sample)/Ab Control where Ab Control is the absorbance of bacteria cultured alone. The minimum inhibitory and minimum bactericidal concentrations (MIC and MBC) were determined by a microplate-based assay. The MIC was defined as the lowest concentration of the oil that was able to inhibit the visible bacterial growth after 24 h incubation at 37°C. The MBC was determined from the well of microplate with no visible bacterial growth. The viable colony forming unit CFU/mL was estimated employing Trypticase soy broth (TSB) (Merck, Darmstadt, Germany) agar plates incubated at 37°C (Aguilar *et al.,* 2012).

RESULTS AND DISCUSSION

Chemical composition of the EO of *S. areira* by GC-MS

Leaves and fruits of two specimens of *S. areira* (Py1*;* and Tg2) growing in Jujuy were collected and the chemical characterizations of their EO were performed. Table 1 shows the EO composition (% total peak area) of leaves and fruits of *S. areira* by GC/FID-MS. Forty-five compounds representing 77–98% of the total EO were identified. Results showed that the composition of the oils from fruits and leaves for the same specimen was different. The major components vary in a wide range: limonene (2.5 to 35.7%), myrcene (1.7 to 38.7%), α-phellandrene (1.2 to 30.9%), β-phellandrene (1.8 to 15%), sabinene (4.1 to 10.9%) and camphene (0.1 to 4.1%). It is well known that the content of the EO from aromatic plants depends on several factors such as the geographical origin, the part of the plant from which the oil was obtained and the genetic background of the plant from which the oil was taken.

In an earlier poblational study, the analyzed EO from fruits of individual trees of the Humahuaca region (Jujuy, Argentina) could be grouped in clusters according to their chemical composition (Viturro *et al.,* 2006).

Table 1. Chemical composition of EO expressed as percentages obtained by distillation of leaves (L) and fruits (F) of *S. areira* from Py1 and Tg2 specimen

Compound[c]	RI[b]	Content [%][a]			
		Py1F	Py1L	Tg2F	Tg2L
Tricyclene	926	tr	0.7	tr	0.1
α-Thujene	931	0.2	0.2	0.2	0.3
α-Pinene	939	1.4	2.6	2.3	3.0
Camphene	954	0.2	4.1	0.1	0.9
Sabinene	977	8.0	10.9	4.1	6.9
β-pinene	980	0.2	1.7	0.2	0.5
Myrcene	992	13.3	1.7	38.7	3.3
α-Phellandrene	1005	1.2	6.4	30.9	24.7
α-Terpinene	1019	tr	0.3	0.1	0.5
p-Cymene	1029	5.5	0.8	0.9	4.7
Limonene	1035	35.7	15.5	2.5	5.8
β-Phellandrene	1036	2.4	1.8	15.0	13.0
cis-β-Ocimene	1043	tr	0.1	tr	tr
trans-β-Ocimene	1054	tr	tr	tr	0.1
γ-Terpinene	1063	tr	0.5	0.2	0.8
Terpinolene	1090	tr	0.2	0.1	0.3
Linalool	1098	0.4	tr	tr	0.1
Methyl octanoate	1129	1.0	0.1	0.7	–
Terpinen-4-ol	1186	tr	tr	tr	tr
α-Terpineol	1188	0.7	0.1	tr	0.1
Cryptone	1192	0.3	tr	tr	tr
Bornyl acetate	1288	tr	0.7	tr	0.1
para-Cymen-7-ol-	1292	0.3	tr	tr	tr
δ-Elemene	1340	1.6	0.2	tr	0.2
β-Elemene	1393	1.2	0.7	0.1	0.8
β-Caryophyllene	1419	0.9	0.8	0.2	1.1
α-Humulene	1455	0.3	0.8	0.1	0.9
Germacrene-D	1480	0.2	0.3	1.0	0.4
γ-Muurolene	1482	tr	0.3	tr	0.4
Bicyclogermacrene	1500	0.5	12.3	tr	3.0
α-Muurolene	1501	0.7	-	0.1	1.0
β-Bisabolene	1506	tr	0.5	0.1	0.4
γ-Cadinene	1516	0.6	0.9	0.1	1.0
Bourbonanol-endo-1	1521	tr	0.3	–	–
δ-Cadinene	1526	1.2	3.0	0.4	4.3
Elemol	1552	0.2	0.9	–	0.2
Germacrene-D-4-ol	1578	4.1	3.9	0.2	1.8
β-Oplopenone	1608	tr	tr	tr	tr
Cubenol-1,10-di epi	1624	tr	0.2	tr	tr
Cubenol-1-epi	1631	tr	tr	tr	0.3
γ-Eudesmol	1634	tr	0.1	tr	–
epi-a-Muurolol	1644	1.2	1.4	0.1	1.7
β-Eudesmol	1653	0.3	0.3	–	0.1
α-Eudesmol	1655	1.2	1.9	0.2	2.4
α-Cadinol	1658	tr	tr	tr	tr

[a]Percentage peak area of EO components. [b]Experimental retention indices on HP5 MS capillary column in reference to C_7-C_{24} *n*-alkanes; the compounds are listed in order of elution. [c]All compounds were identified by comparison of their RI and mass spectra with literature data, the MS library (NBS 75K, NIST98), and a spectra library built up from pure substances and components of known oils. –not detected; tr, traces (<0.1%)

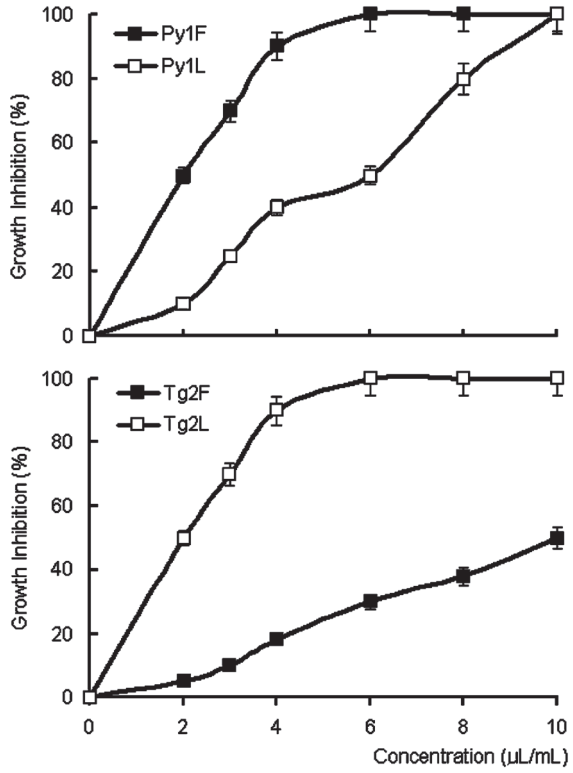

Figure 1. Antibacterial activity of *S. areira* EO from Py1 specimen (A) and Tg2 specimen (B) against *S. aureus* ATCC 25923. EO from fruits (filled squares) or leaves (empty squares).

Moreover, these chemical profiles were detected in these Argentinean *S. areira* specimens for over 7 years; therefore they are mainly determined by the genotype of the plants.

Wannaz *et al.* (2003) studied the chemical composition of volatile compounds sampling in Mendoza city, Argentina and reported that limonene and α-phellandrene show the higher percentages while the greatest variability belonged to sabinene, myrcene and tricyclene. A different chemical composition was obtained for other EO of plants collected from Mendiolaza, Córdoba, Argentina, finding as the main components: α-pinene (13.80%), limonene (12.81%) and camphene (12.62%) (Bigliani *et al.,* 2012).

It is well known that whole EO tend to vary in their exact composition due to factors such as seasonal variation, climate, humidity, sun exposure and even the oil-extraction method (Edris, 2007). Therefore, due to the large chemical variability observed in the EO of *S. areira*, it is likely to find different antibacterial efficiencies depending on the chemical composition of the oils.

Table 2. The minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) of EO from the fruits (F) and leaves (L) of Py1 and Tg2 specimens against *S. aureus* ATCC 25923

Treatment	MIC (µL/mL)	MBC
EO Py1F	6	20
EO Py1L	10	30
EO Tg2F	30*	n.a.
EO Tg2L	6	30

*Corresponded to a growth inhibition of 70%. n.a., not active

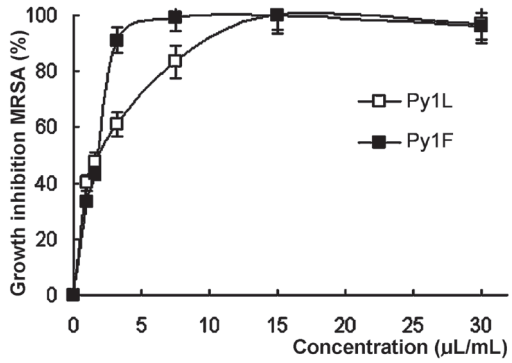

Figure 2. Antibacterial activity of *S. areira* EO from Py1 from fruits (filled squares) or leaves (empty squares) against MRSA evaluated by the microplate bioassay.

Antibacterial activity of fruits and leaves of four EO of *S. areira* (Py1 and Tg2) against *S. aureus* ATCC 25923

First, the antibacterial activity of fruits and leaves of both EO of *S. areira* (Py1 and Tg2) was evaluated against a susceptible to antibiotics *S. aureus* strain by the microplate bioassay testing different amounts of them. Figure 1 shows the antibacterial activity expressed as a percentage of bacterial growth inhibition and the results displayed dose dependent curves for the four oils assayed. The oil of Py1 fruits had higher antibacterial activity than the EO of leaves, although both were able to inhibit the bacterial growth in a 100% (Fig. 1A). A different behavior was observed when the oils of fruits and leaves obtained from Tg2 specimen were assayed. In this case, the EO obtained from the fruits exhibited a lower efficacy than the oils obtained from the leaves, since it was unable to induce a total inhibition of the growth of *S. aureus* ATCC 25923 (Fig. 1B). Table 2 shows the MICs values of the EO of fruits and leaves of the specimen Py1 against the susceptible strain of *S. aureus* (6 µL/mL and 10 µL/mL, respectively), while the oil of the leaves of Tg2 was 6 µL/mL. Later, we examined whether bacteriostatic or bactericide actions were performed by the EO of *S. areira*. Bactericide effects were obtained after treatment with 3X 5X MIC values of leaves of both specimens, although the EO of fruits of Py1 exhibited the higher bactericide concentrations (20 µL/mL) (Table 2). Again, Tg2 specimen oil showed the lowest antibacterial potency because it showed no bactericidal effects assaying up to 40 µL/mL.

Given the observed difference in the antibacterial efficacy of the four oils from *S areira* studied, we hypoth-

Table 3. Colony forming unit (CFU) recovered after treatment with *S. areira* EO isolated from leaves (L) and fruits (F) of Py1 and Tg2 specimens against MRSA 1977

Treatment	Mean bacterial count (CFU ±S.D.)
Inoculum, 0 h	$5.5 \times 10^5 \pm 1.5 \times 10^4$
Bacterial culture, 24 h	$2.3 \times 10^7 \pm 0.5 \times 10^6$
20 µL/mL EO Py1F	0
30 µL/mL EO Py1L	2.3×10^2
20 µL/mL EO Tg2F	n.a.
30 µL/mL EO Tg2L	0

n.a., not active

esized that the antibacterial activity of these EO could, in part, be to their major components (limonene and α-phellandrene). The oil of Py1 fruits containing higher amount of limonene (35.5%) was more potent than Py1L oil bearing half of the content (15.5%) of this active compound. Limonene has been demonstrated to have bacteriostatic activity against several microorganisms and against *Staphylococcus aureus* CCTCC AB91053 (Bakkali *et al.,* 2008; Donsì *et al.,* 2011; Dai *et al.,* 2013). It can also be speculated that Tg2F would have a lower antibacterial activity than Tg2L, possibly due to the fact that it contained a lower amount of limonene. On the other hand, the antimicrobial efficacy of Tg2 specimens may be also attributed to α-phellandrene. The antimicrobial activities of α-phellandrene have been also reported (Simic *et al.,* 2002).

Antibacterial activity of the EO of *S. areira* against MRSA

MRSA infections are a major public health problem producing a large number of deaths every year worldwide (NNIS, 2004). This important nosocomial and community-acquired pathogen has developed resistance to various antibiotics (β-lactams, quinolones, and aminoglycosides) (Chen *et al.,* 2010). For this reason we evaluated the EO isolated from *S. areira* against a nosocomial MDR strain of *S. aureus* isolated from a Pediatric Hospital in Argentina. We observed a dose–response curve for the antibacterial activity of EO from fruits and leaves of Py1 specimen (sample 13-12) against MRSA (Fig. 2). Results obtained also showed that the 100% of growth inhibition (MICs value) of the EO from fruits and leaves 13–12 were 3.2 μL/mL and 15 μL/mL, respectively. Importantly, both leaves and fruits oils exhibited bactericidal action after incubation for 24 h with 20 μL/mL and 40 μL/mL, respectively (Table 3). In addition, EO from fruits of Tg2 (sample 13–16) is inactive against SAMR, while that of the leaves are active assessing 30 μL/mL.

It is difficult to attribute the antibacterial effect of the oils to particular compounds, because they contain a mixture of different chemical compounds where synergy and antagonism phenomenon may occur. However, taking into account the results presented herein, it could be inferred that the antibacterial effectiveness of *S. areira* essential oils depend on an adequate number and content of key bioactive/s compound/s. In fact, the antibacterial action of the *S. areira* oils against *S. aureus* strains is probably due to the content of limonene. However, it cannot be ruled out that the antibacterial action of the oils from Tg2 specimens can be the result of the synergistic effects of α-phellandrene and limonene. Further studies are needed to address this possibility.

The mechanism of action of this class of compounds has not been completely elucidated to date; however, the antimicrobial activity of the essential oils of herbs and spices or their components could be the result of disruption of bacterial membrane integrity or disturbance of several enzymatic cell systems, including energy production and synthesis of structural components (Burt, 2004).

CONCLUSIONS

The essential oils from many plants are of great interest to food, cosmetic, and pharmaceutical industries because of their possible use as natural additives to replace synthetic antimicrobial agents. This is the first study showing the effective killing activity of susceptible and multidrug-resistant *S. aureus* strains of the EO isolated from fruits and leaves of *S. areira*. As the inherent activity of these oils may be related to their chemical composition and the proportions of the main components, our finding revealed that the oil from leaves and fruits of *S. areira* may be a new potential source of natural antimicrobial agent. In particular, results point out the use of the *S. areira* oils against *Staphylococcal* infections. However, further studies need to be conducted to obtain more information on the safety and toxicity of these oils.

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