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Potentiality of standardized extract and isolated flavonoids from *Zuccagnia punctata* for the treatment of respiratory infections by *Streptococcus pneumoniae*: *In vitro* and *in vivo* studies

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ABSTRACT

Ethnopharmacological relevance: Zuccagnia punctata Cav. (Fabaceae) is a monotypic species distributed in western Argentina and is traditionally used for the treatment of bacterial and fungal infections. The aim of this study was to demonstrated the antibacterial activity of the *Zuccagnia punctata* standardized extract and the structurally related non-methoxylated flavonoids with similar pattern of substitution and differences in ring C present in this plant species: 7-hydroxyflavanone (HF), 2′,4′-dihydroxychalcone (DHC) and 3,7-dihydroxyflavone (DHF), against *Streptococcus pneumoniae* clinical isolates using *in vitro* and *in vivo* models.

Materials and Methods: MIC values of natural products were determined by agar macrodilution method. *In vivo* activities were investigated in a *Streptococcus pneumoniae* infection model in mice. Lung and blood samples were obtained for bacterial cell counts. The serum was used by biochemical analysis (alanine transaminase, aspartate transaminase, urea and creatinine) in order to evaluate the toxicity of natural products.

Results: All samples showed antimicrobial activity *in vitro* with MIC values between 50 and 500 μg/ml. *Zuccagnia punctata* extract (1 mg/mice) and HF (1 mg/mice) significantly reduced the number of viable *Streptococcus pneumoniae* in lung ($p < 0.01$) while lower quantities has not effect. Therefore, the present study has shown that intake once or twice a day of 1 mg of *Zuccagnia punctata* extract or HF for seven days did not result in toxicity.

Conclusions: Our results showed that *Zuccagnia punctata* extract as well as one of its isolated flavonoids, 7-hydroxyflavanone, could be useful for the development of a novel respiratory infections treatment.

1. Introduction

Streptococcus pneumoniae is a clinically important bacterial pathogen that causes significant morbidity and mortality worldwide. This bacterium infects the upper respiratory tract in approximately 50% young children under the age of 2 years (Bogaert et al., 2004). *Streptococcus pneumoniae* is a leading cause of bacterial meningitis, sepsis, pneumonia and otitis media in young children and elderly (Hausdorff et al., 2000; Jackson, 2002). Antibiotic resistance to nosocomial Gram-positive bacteria has been increasing at an alarming rate, especially in developing countries (Borkow and Gabbay, 2010). Consequently, alternative therapies are necessary. Recently, there has been considerable interest in the use of plant extracts as multi-drug complex as an alternative method to control pathogenic microorganisms (Zampini et al., 2005, 2007, 2009a; Wagner and Ulrich-Merzenich, 2009), and many compounds of plant products have been shown to be specifically targeted against resistant pathogenic bacteria (Zampini et al., 2005, 2009b).

Zuccagnia punctata Cav., which belongs to the family of Fabaceae, is commonly known as jarilla pispito, puspus and jarilla macho, is a monotypic species widely distributed in western Argentina (Cabrera, 1971). *Zuccagnia punctata* has been used extensively as a traditional medicine in Argentina for the treatment of bacterial

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and fungal infections, asthma, arthritis and rheumatism (Ratera and Ratera, 1980; Toursarkissian, 1980). In addition, *Zuccagnia punctata* is reported to have antioxidant (Morán Vieyra et al., 2009), antibacterial (Zampini et al., 2005), antifungal (Quiroga et al., 2001; Svetaz et al., 2004, 2007; Agüero et al., 2010), antiulcer (De la Rocha et al., 2003), and antigenotoxic (Zampini et al., 2008) properties. The constituents of *Zuccagnia punctata* include phytochemicals such as flavonoids (flavanones, flavones, chalcones) and caffeoyl esters (Pederiva et al., 1975; Pederiva and Giordano, 1984; Svetaz et al., 2004; Agüero et al., 2010). In addition, it was reported that the standardized extracts of *Zuccagnia punctata* and chalcone isolated from this plant possesses antibacterial activity against Gram-negative bacteria (Zampini et al., 2005) including several multiple drug-resistant clinical strains such as *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and others. However, to date, no *in vivo* studies regarding the antimicrobial activity of the extracts or the flavonoids isolated from *Zuccagnia punctata* have been conducted. Therefore, the aim of this study was to evaluate the antimicrobial activity of the *Zuccagnia punctata* standardized extract and three compounds with similar pattern of substitution and differences in ring C (7-hydroxyflavanone, 2′,4′-dihydroxychalcone and 3,7-dihydroxyflavone) isolated from aerial parts of them against diverse *Streptococcus pneumoniae* clinical isolates using *in vitro* and *in vivo* models.

2. Materials and methods

2.1. Material plant

Zuccagnia punctata aerial parts (leaves and stems) were collected from January to February, 2008–2009 at 2000 m above sea level (masl) in Amaicha del Valle, Tucumán, Argentina. The samples were dried in a dark place at room temperature. Voucher specimens (IML 605935) were deposited at Herbarium of Fundación Miguel Lillo, Tucumán, Argentina. *Zuccagnia punctata* was authenticated by Lic. Nora Muruaga, Botany Department, Fundación Miguel Lillo, San Miguel de Tucumán, Tucumán, Argentina.

The flavonoids 7-hydroxyflavanone (HF), 3,7-dihydroxyflavone (DHF) and 2′,4′-dihydroxychalcone (DHC) were isolated from *Zuccagnia punctata* and obtained from Indofine Chemical Company (New Jersey, USA).

2.2. Preparation of Zuccagnia punctata extract

Ground air-dried plant material was macerated in ethanol 80% (1 g/5 ml) for 7 days with stirring (40 cycles/min) at room temperature. The extract was filtered through Whatman No. 4 filter paper.

The extract was standardized by the determination of total phenolic compounds content using Folin-Ciocalteu reagent (Singleton et al., 1999). The results were expressed as μg gallic acid equivalent/ml extract (μg GAE/ml).

The solvent was then removed under reduced pressure in a rotary evaporator and dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) prior to use.

2.3. Fractionation of ethanol extract of Zuccagnia punctata

The ethanolic extract was evaporated and the aqueous solution was extracted with Et_2O. The organic fraction was subjected to column chromatography (CC) (80 mm × 5 mm) using silica gel (0.063–0.200 mm) and eluted stepwise with C_6H_6 and then with mixtures of C_6H_6—$CHCl_3$, $CHCl_3$, $CHCl_3$—CH_3OH and CH_3OH. Fractions were collected and combined according to the TLC analysis (Kieselgel G60 F254 0.2 mm, Merck; mobile phase: ethyl acetate:chloroform 2:1, v/v). Fractions 8, 9, 15, 17 and 18 were further purified by repeated CC on Sephadex LH-20 with MeOH as mobile phase. The column fractions were analyzed by TLC and assayed for antibacterial activity by bioautography (Nieva Moreno et al., 1999).

2.4. Identification of bioactive compounds

Three bioactive compounds obtained from Sephadex LH-20 were analyzed by UV–visible spectra. Absorption spectra (200–600 nm) of isolated compounds (in MeOH, MeOH·MeONa, MeOH·$AlCl_3$ or MeOH·$AlCl_3$/HCl) were recorded using a Beckman DU 650 spectrophotometer (Mabry et al., 1970). NMR spectra were recorded in a Bruker AC 200 (200 MHz) spectrometer at room temperature.

2.5. Determination of antibacterial activity

2.5.1. Microorganisms

Different serotypes of *Streptococcus pneumoniae* clinical isolates, AV3 (serotype 3), AV6 (serotype 6B), AV14 (serotype 14) and AV23 (serotype 23F) were used. The strains were kindly provided by Dr. M. Regueira from the Laboratory of Clinical Bacteriology, National Institute of Infectious Diseases, Argentina. The strains were maintained at −70 °C in Brain Heart Infusion (BHI) containing 30% (v/v) glycerol in CERELA (CONICET).

Bacterial inocula were prepared from an overnight culture in Columbia Agar 5% sheep blood (bioMérieux Brasil S.A.) at 37 °C until the log phase was reached. Then, each suspension was diluted in sterile 0.9% NaCl solution to obtain a cell suspension of 10^7 CFU/ml (colony forming units/ml).

2.5.2. Determination of minimum inhibitory concentrations (MIC)

MIC values of samples were determined against the different *Streptococcus pneumoniae* strains by agar macrodilution method in accordance with the Clinical Laboratory Standards Institute (CLSI, 2006) guidelines. *Zuccagnia punctata* extract, HF, DHF or DHC were serially diluted in DMSO and added to Mueller Hinton Agar supplemented with 5% sheep blood medium at concentrations of 12.5, 25, 50, 100, 200 and 400 μg GAE/ml for crude extract and concentrations of 0.1, 1, 10, 100 and 500 μg/ml for the isolated compounds. After cooling and drying, the plates were inoculated in spots with 2 μl of each bacterial cell suspension (5×10^4 CFU). A growth control of each tested strain and the control of solvent were carried out. The inoculated plates were incubated for 20–24 h in 5% CO_2 at 37 ± 2 °C. MIC was defined as the lowest concentration of sample capable of inhibiting visible growth after incubation. MIC values were also determined for commercial antibiotics (amoxicillin, GlaxoSmithKline S.A). MIC determinations were performed in triplicate.

2.5.3. Animals

Infant mice were selected because their high susceptibility to pneumococcal respiratory infection (Villena et al., 2010). All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals and approved by the Ethical Committee of Animal Care at CERELA under the bioethical allowance number BIOT-CRL/11.

Swiss albino mice (weight, 20 ± 3 g each one) at the age of 3 weeks were used for the *in vivo* experiments. They were kept in a temperature-controlled room under a 12 h light 12 h dark cycle. Animals had free access to commercial solid food and water *ad libitum*.

Table 1
Antibacterial activity (MIC) of *Zuccagnia punctata* extract and isolated flavonoids against *Streptococcus pneumoniae* strains.

Streptococcus pneumoniae strains	MIC (μg/ml)				
	Zp	DHC	HF	DHF	Amoxicillin
AV3	400	100	500	R	R
AV6	50	100	100	100	R
AV14	R	500	R	R	R
AV23	50	100	100	100	R

MIC: minimum inhibitory concentrations, Zp: *Zuccagnia punctata* crude extract, DHC: 2′,4′-dihydroxychalcone, HF: 7-hydroxyflavanone and DHF: 3,7-dihydroxyflavone were serially diluted in DMSO. R: resistant until 400 or 500 μg/ml for *Z. puntata* extract or flavonoids, respectively. Breakpoints for amoxicillin, are ≤2.0 μg/ml (susceptible), 4.0 μg/ml (intermediate), and ≥ 8.0 μg/ml (resistant).

2.5.4. Experimental Streptococcus pneumoniae infection

The therapeutic effects of the standardized *Zuccagnia punctata* extract and isolated flavonoids from them were determined in a *Streptococcus pneumoniae* infection model in mice (Racedo et al., 2006).

Freshly grown colonies of *Streptococcus pneumoniae* strains AV6 (serotype 6B) were suspended in Todd Hewitt Broth (THB) (Laboratorios Britania, Argentina) and incubated at 37 °C until the log phase was reached. Mice were challenged nasally with the pathogen by dropping 25 μl of an inoculum containing 10^6 CFU of *Streptococcus pneumoniae* into each nostril (Villena et al., 2010).

2.5.5. Treatment protocol

Mice were distributed into groups of nine mice each one: G1 control (C), G2 *Streptococcus pneumoniae*-infected (SI), G3 *Streptococcus pneumoniae*-infected + *Zuccagnia punctata* extract (0.25 mg/mice) (SIZp 0.25), G4 *Streptococcus pneumoniae*-infected + *Zuccagnia punctata* extract (0.5 mg/mice) (SIZp 0.5), G5 *Streptococcus pneumoniae*-infected + *Zuccagnia punctata* extract (1.0 mg/mice) (SIZp 1.0), G6 *Streptococcus pneumoniae*-infected + HF (1 mg/mice) (SIHF) and G7 *Streptococcus pneumoniae*-infected + DHC (1 mg/mice) (SIDHC). Mice without infections treated with different concentrations of extract and flavonoids were included.

The antibiotics (*Zuccagnia punctata* extract or flavonoids) in DMSO were administered orally once a day for 7 days post-infection (p.i.) and twice a day for 7 days p.i. Treatment of positive control was performed with two doses of amoxicillin sodium salt (Clamoxyl® 1 g; GlaxoSmithKline S.A.): 0.5 and 2.0 mg/mice. Amoxicillin was administered once a day for 7 days. In all groups, the treatments of animals with drug started at 24 h p.i. At days 3, 5 and 7 p.i. three mice of each group were sacrificed and lung and blood samples were obtained for bacterial cell counts. The serum was used by biochemical analysis. The results are means of three independent experiments.

2.5.6. Bacterial cell counts in lung and blood

The lungs of the animals were prepared for the quantitative bacteriological examination as described previously (Racedo et al., 2006). Briefly, the lungs were excised, weighed and homogenized in 5 ml of sterile peptone water. A total of 0.1 ml of the homogenate was plated in duplicate for bacterial culture to determine the numbers of CFU. *Streptococcus pneumoniae* colonies were counted and the results were expressed as log CFU/g of organ.

Progression of bacterial growth to the bloodstream was monitored by blood samples obtained by cardiac puncture with a heparinized syringe. Samples were plated and bacteremia was reported as negative or positive hemocultures, after incubation for 18 h at 37 °C (Villena et al., 2010).

2.6. Biochemical analysis

The blood was collected in a small plastic tube through cardiac puncture. The blood was centrifuged at 2000 × *g* for 20 min. The blood serum was collected. Alanine transaminase (ALT) and aspartate transaminase (AST), and urea and creatinine levels were measured to assess the liver and renal function, respectively. All biochemical assays were done spectrophotometrically using Hitachi-917 Autoanalyser (Mannheim, Germany) with kits supplied by Roche Diagnostics (Mannheim, Germany). To obtain data with good sensitivity and validity, samples were analyzed in triplicates and blindly.

2.7. Statistical analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). After verification of a normal distribution of data, 2-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Statistical significance was determined at $p < 0.05$.

3. Results

Zuccagnia punctata ethanolic extracts have showed high content of phenolic compounds (54 mg/g of dry plant material). From the fractionation of extract, three bioactive flavonoids were obtained: DHC, 2′,4′-dihydroxychalcone (Fr 8 and Fr 9), DHF, 3,7-dihydroxyflavone (Fr 15), and HF, 7-hydroxyflavanone (Fr 17 and Fr 18). The compounds were identified by ^{1}H NMR, UV spectra and co-chromatography with the authentic samples obtained commercially, and the data were in agreement with the previously reported in the literature (Braga de Oliveira et al., 1972; Pederiva et al., 1975; Wollenweber and Seigler, 1982).

3.1. In vitro antibacterial activity

The antimicrobial efficacy of *Zuccagnia punctata* extract, DHC, HF and DHF, and commercial antibiotic against the four different serotypes of *Streptococcus pneumoniae* strains was evaluated by the agar macrodilution method. The MIC values of the samples against *Streptococcus pneumoniae* strains are shown in Table 1. All natural products showed *in vitro* antimicrobial activity against each of the tested strains with MIC values between from 50 to 500 μg/ml. *Streptococcus pneumoniae* AV6 and AV23 strains were more sensitive to natural products while *Streptococcus pneumoniae* AV14 was the more resistant strain. The higher antibacterial activity against the different *Streptococcus pneumoniae* serotype was achieved by DHC and followed by HF and DHF. The MIC values of DHC were similar to those observed with the *Zuccagnia punctata* extract.

Table 2
Effects of treatment with *Zuccagnia punctata* crude extract and isolated flavonoids in mice infected with *Streptococcus pneumoniae*.

Group	Log_{10} CFU/g of organ		
	Days post-infection		
	Day 3	Day 5	Day 7
SI	5.49 ± 0.27	5.29 ± 0.12	5.36 ± 0.01
SIZp 0.25–24 h	5.52 ± 0.20	5.45 ± 0.22	5.33 ± 0.29
SIZp 0.25–12 h	5.47 ± 0.16	5.31 ± 0.18	5.37 ± 0.08
SIZp 0.5–24 h	5.50 ± 0.40	5.40 ± 0.18	5.44 ± 0.19
SIZp 0.5–12 h	5.40 ± 0.17	5.38 ± 0.13	5.45 ± 0.11
SIZp 1–24 h	4.97 ± 0.41*	4.39 ± 0.14*	5.14 ± 0.33
SIZp 1–12 h	4.64 ± 0.25*	4.09 ± 0.03**	4.30 ± 0.31**
SIHF 1–24 h	5.50 ± 0.48	3.96 ± 0.28**	4.54 ± 0.13**
SIHF 1–12 h	4.43 ± 0.33*	4.13 ± 0.07**	4.35 ± 0.10**
SIDHC 1–24 h	5.51 ± 0.19	5.30 ± 0.25	5.45 ± 0.09
SIDHC 1–12 h	5.35 ± 0.09	5.25 ± 0.20	5.35 ± 0.29
Amox 0.5–24 h	4.88 ± 0.13*	4.33 ± 0.34*	4.02 ± 0.16*
Amox 2–24 h	4.34 ± 0.22*	3.67 ± 0.21**	3.41 ± 0.14**

SI: *Streptococcus pneumoniae*-infected, Zp: *Zuccagnia punctata* crude extract, HF: 7-hydroxyflavanone, DHC: 2′,4′-dihydroxychalcone. Amox: amoxicillin. The data are means ± SD.

* Statistical significance ($p \leq 0.05$).

** Statistical significance ($p \leq 0.01$).

3.2. In vivo antibacterial efficacy

The antibacterial activities of different quantities of *Zuccagnia punctata* extract, DHC and HF (flavonoids with the higher anti-*Streptococcus* activity) were examined by using a *Streptococcus pneumoniae* infection model in mice. In fact, mice were infected with 10^6 CFU of *Streptococcus pneumoniae* and one-day later, natural products were orally administered to infected mice. As shown in Table 2, treatment with higher concentrations of *Zuccagnia punctata* extract (1 mg/mice) significantly reduced the number of viable *Streptococcus pneumoniae* in lung as compared to the controls. Comparable efficacy against infection was obtained with HF treatment. In both cases, the efficacy was better when the natural products were administered twice a day. Crude extract at dose of lower than 1 mg/mice was not therapeutically effective against the pneumococcal infection as well as DHC. All treated group showed negative hemocultures as well as amoxicillin, a commercial antibiotic, therefore, were able to prevent the progression of bacterial growth to the bloodstream.

3.3. Biochemical analysis

The possible hepatotoxic and nephrotoxic effects of active treatment (Zp-1 mg/mice and HF-1 mg/mice) were analyzed in mice with and without streptococcal infections. In both cases, the activities of AST and ALT enzymes and the levels of creatinine and urea in blood were not changed by *Zuccagnia punctata* extract or HF as compared to the control values (Table 3). Therefore, the present study has shown that intake once or twice a day of 1 mg of *Zuccagnia punctata* extract or HF for seven days did not result in toxicity.

4. Discussion

In the last time, a number of antibiotics have lost their effectiveness due to the development of resistant bacteria. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. In this sense, the natural products constitute an interesting option. *Zuccagnia punctata* is a plant used extensively in argentine traditional medicine for the treatment of bacterial and fungal infections. In the present study, the *Zuccagnia punctata* crude extract and three flavonoids present in the plant (DHC, HF and DHF) exhibited *in vitro* antibacterial activity against

Table 3
Effect of *Zuccagnia punctata* extract and 7-hydroxyflavanone on hepatotoxicity and nephrotoxicity related biochemical variables in infected mice serum.

Parameters	ALT			AST			Urea			Creatinine		
Group	Days post-infection											
	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7
SI	5.3 ± 2.0	6.0 ± 1.8	7.3 ± 0.9	119.0 ± 28.8	126.5 ± 8.6	139.0 ± 24.5	570.0 ± 40.0	710.0 ± 40.0	690 ± 40.0	3.5 ± 0.2	3.7 ± 0.2	3.8 ± 0.1
SIZp 1–24 h	5.0 ± 1.4	6.5 ± 0.7	5.0 ± 1.7	111.5 ± 7.7	107.0 ± 12.0	101.6 ± 20.8	620.0 ± 10.0	600.0 ± 70.0	670 ± 50.0	4.0 ± 0.1	3.4 ± 0.2	3.6 ± 0.1
SIZp 1–12 h	7.6 ± 2.2	5.7 ± 2.0	7.0 ± 1.1	150.3 ± 13.4	103.0 ± 3.0	149.0 ± 18.8	590.0 ± 60.0	580–0 ± 30.0	660 ± 40.0	2.9 ± 0.6	3.0 ± 0.1	3.5 ± 0.1
SIHF 1–24 h	7.0 ± 1.7	5.0 ± 1.4	6.5 ± 0.6	147.0 ± 21.4	106.0 ± 0.5	122.0 ± 12.3	630.0 ± 20.0	660.0 ± 40.0	640 ± 80.0	3.6 ± 0.3	3.8 ± 0.1	3.7 ± 0.2
SIHF 1–12 h	7.0 ± 1.4	5.5 ± 2.1	4.5 ± 0.7	141.0 ± 16.7	110.0 ± 8.2	137.6 ± 36.5	580.0 ± 30.0	510.0 ± 20.0	570 ± 20.0	3.2 ± 0.3	4.0 ± 0.1	3.7 ± 0.7

SI: *Streptococcus pneumoniae*-infected, Zp: *Zuccagnia punctata* crude extract, HF: 7-hydroxyflavanone; ALT: alanine transaminase as IU/l; AST: aspartate transaminase as IU/l; urea as mg/l; creatinine as mg/l. The data are means ± SD. Significant differences between groups were not observed.

the strains of four different *Streptococcus pneumoniae* serotypes. The highest *in vitro* activity of isolated compounds was observed for assayed chalcone and flavanone (DHC and HF, respectively). The results indicate that these structures are the most favourable for antibacterial activity within the flavonoid family (Zampini et al., 2005; Agüero et al., 2010; Vera et al., 2011). The same effect was obtained with other chalcones and flavanones previously investigated (Olivella et al., 2001; Nowakowska, 2007; Agüero et al., 2010; Vera et al., 2011).

In this work, the anti-streptococcal *in vitro* activities of *Zuccagnia punctata* crude extract and HF were also observed *in vivo* when their activities were evaluated in a *Streptococcus pneumoniae* infection model in mice. However, DHC was not active in the assayed conditions. Important sites of flavonoid metabolism are the gastrointestinal lumen, cells of the intestinal wall, and the liver (Erlund, 2004). Due to extensive phases I and II metabolism during and after absorption, a major fraction of the absorbed flavonoid is excreted back into the intestine through the bile as glucuronides and/or sulfates and reaches the colon where bacterial β-glucuronidases and sulfatases can release the flavonoids aglycon. Absorption of the released aglycon leads to enterohepatic circulation. Some flavonoids that reached the colon directly or indirectly can subsequently be absorbed from the colon or act as substrate for the indigenous bacterial community with their extensive metabolic potential (Possemiers et al., 2011). However, the *in vivo* antimicrobial activity observed for HF was similar to that produced by the extract of *Zuccagnia punctata* allowing inference that the antibacterial activity of them might be related, at least in part, to the action of HF present in the extract.

In the present study was investigated also the hepato and nephrotoxic effect of the natural products in mice and was observed a normal level of AST, ALT, urea and creatinine in blood, suggesting absence of hepatic or renal damage. In addition to the variety of health-promoting activities of *Zuccagnia punctata* extract, and their active flavonoid, HF, these natural products have not shown oral toxicity. Flavonoids in general are believed to have no or little toxicity and have a long history of human consumption. Very large doses of these compounds (up to 500 mg/kg) have been administered to animals, with little or no toxicity reported (Morris and Zhang, 2006).

Based on these promising findings, we believe that *Zuccagnia punctata* extract as well as one of its isolated flavonoids, 7-hydroxyflavanone, could possibly be useful for the development of a novel antimicrobial treatment for respiratory infections. On the other hand, these natural products could be useful in order to improve the conventional antibiotic therapy. Currently, the study to determine the possible mechanism of action responsible for antibacterial activity is in progress.

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