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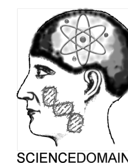

Specific Gram-Positive Antibacterial Activity of 4-hydroxy-3-(3-methyl-2-butenyl) Acetophenone Isolated from *Senecio graveolens*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JS and CEC developed the hypothesis. Author CEC identified the S. graveolens plant and conserves the voucher specimen at CODECITE-CIHDE, Arica Chile. Author JS determined the antibacterial activity of 4-H-3-(MB) AP and designed the assays. Author CO carried out membrane permeabilization and antibacterial assays. Authors JS and DL carried out the microscopy assays and authors MM and MC purified 4-H-3-(MB)AP and carried out NMR determinations. Authors CS, FR and CEC obtained the S. graveolens and prepared the ethanolic fractions. Authors JS, CO, CS and CEC analyzed the data and authors JS, CO, MM and CEC wrote the article. All authors read and approved the final manuscript.

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ABSTRACT

Background: Resistance to antimicrobial drugs has become an increasingly global problem, and is the main reason for an extended search for new drugs to treat microbial infections. *Senecioneae* is one of the largest tribes of *Asteraceae*, comprised of about 150 genera and 3000 plant species. *Senecio graveolens*, commonly called Chachacoma, is highly used as a medicinal plant for altitude sickness by the natives of the Andes Mountains around the Atacama Desert. Previous studies have demonstrated that *S. graveolens* extracts possess antibacterial properties, but its active compound and molecular mechanisms are still unknown.

Methods: Form the ethanolic extract of *S. graveolens* the main compound 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone (4-H-3-(MB)AP) was identify and purified by nuclear magnetic resonance (NMR). Antibacterial activity of (4-H-3-(MB)AP) was assayed on Gram-positive and Gram-negative bacteria by microbiological techniques. Possible mechanisms of action of (4-H-3-(MB)AP) were explored by microbiological, flow cytometry and electron microscopy techniques.

Results: Here we determined that *S. graveolens* extract has specific antibacterial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, and *Mycobacterium smegmatis*. The most abundant compound from *S. graveolens* extract, 4-H-3-(MB)AP, showed broad antibacterial activity against Gram-positive but no activity against Gram-negative strains. We determined that 4-H-3-(MB)AP permeabilizes bacterial membranes and precludes cell division by disrupting Gram-positive bacteria divisome, suggesting that the synthesis of teichoic acid is inhibited.

Conclusions: We conclude that 4-H-3-(MB)AP is one of the active compounds of *S. graveolens* extract responsible for its antibacterial activity. 4-H-3-(MB)AP is a candidate for further chemical modification studies and practical approaches to design antimicrobial drugs.

Keywords: Antimicrobial; Senecio graveolens; plant extract; gram-positive; 4-hydroxy-3-(3-methyl-2-butenyl) acetophenone.

1. INTRODUCTION

Plant extracts have been utilized for centuries to accelerate wound healing and to treat common infectious diseases. Such traditional medicinal plants are still utilized in the routine treatment many of those [1]. Because of their curative potential, plant extracts have been investigated for the development of novel drugs to control bacterial infections [2-4].

Due to the current increase in awareness of antibiotic resistance issues, self-medication with several plant products from herbal suppliers and natural-food stores is enjoying considerable popularity [5]. The World Health Organization (WHO) noted that the majority of the world's population depends on local traditional medicine for primary healthcare [6]. Indeed, in 2010, the global retail sale of botanical dietary supplements amounted to more than $25 billion in the United States [7].

Senecio is the largest genus in the family *Senecioneae* (*Asteraceae*) with more than 1500 described species distributed worldwide [8]. *Senecio* species have been used in folk medicine for wounds treatment, as an antiemetic, anti-inflammatory, and in vasodilator preparations [9]. Approximately 300 *Senecio* species are located in the Andes Mountains around the Atacama Desert [10,11]. *S. graveolens*, an endemic specie of the Atacama Desert highlands (over 3000 m of altitude) and known by the popular name of "Chachacoma", is commercialized as folk medicine mainly for altitude sickness syndrome [12]. Previous studies demonstrated that extracts from *Senecio* species has antibacterial and antifungal activities [13-16]. Here we corroborate that *S. graveolens* extract exhibits antibacterial activity and in addition showed that this antibacterial activity is specific to Gram-positive bacterial species.

Many plants produce antibacterial products as a defense mechanism in response to tissue disruption and pathogen attack, or are present constitutively, giving to the plant a characteristic odor, distinctive pigmentation, or flavor. Some of these plant-based antimicrobials can successfully fight infections and are being investigated for commercial development [1]. Here we purified the main component of *S. graveolens* extract, 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone (4-H-3-(MB)AP) also know as prenilatedacetophenone. 4-H-3-(MB)AP

was described for the first time by Bohlmann et al. [17] in *Helianthella uniflora* and later isolated from *Senecio nutans* [18], *Helichrysum italicum* [19], *Xenophyllum poposum* [20] and *Xenophyllum incisum* [21]. A previous study indicated that 4-H-3-(MB)AP is an effective antifungal agent possessing also a moderate antibacterial activity [19]. However, the specific antibacterial activity of 4-H-3-(MB)AP has not been determined. Here, we determined that 4-H-3-(MB)AP isolated from *S. graveolens* permeabilize bacterial membranes and has specific bactericidal activity against Gram-positive bacterial species by inhibiting cell division septum formation.

2. MATERIALS AND METHODS

2.1 Plant Material

S. graveolens was collected during 2010 from the highlands of the Atacama Desert, near to the Chungara lake Chile at 4,500 meters above the sea level (Fig. 1). Approximately 180.87 g of dry and ground plant material (principally flowers, leaves and stems) were macerated in 95% ethanol for 72h. The specimen collection is conserved at CODECITE-CIHDE, Arica Chile.

2.2 Phytochemical Extraction

Dry and ground *S. graveolens*plant material (180.87g) was macerated in 95% ethanol for 72 h at room temperature. The extract was filtered and concentrated under reducing pressure at 40°C. The plant extract was subjected to separation with a mixture of ethyl acetate/water (EtOAc/H2O) (500 ml each) in separation funnel. The resulting organic phase was concentrated under reducing pressure. A total of 54.86 g of *S. graveolens* extract was obtained from the original plant material.

2.3 Extraction and Compounds Isolation

The organic phase (20 g) was chromatographed on silica gel using a mixture of hexane and ethyl-acetate with increasing polarity (49:1 and 1:49) and monitored by thin layer chromatography (TLC). The samples with similar constitution were pooled and concentrated under reducing pressure. An impure solid product was obtained, which was recrystallized by using Et2O:MeOH (1:1) yielded a pure crystalline solid, identified as 4-H-3-(MB)AP (2.42 g). Pure compound (12.1% yield from the crude extract) was obtained from the original concentrated organic phase isolated form the crude plant material, mp 94-95°C. The 1H, 13C (DEPT 135), sel. 2D HSQC and 2D HMBC spectra were recorded in CDCl3 solutions on a Bruker Avance 400 Digital nuclear magnetic resonance (NMR) parameters spectrometer. Melting points were determined on a Stuart-Scientific SMP3 apparatus. Column chromatography (CC) used silica gel Merck 60 G (0,032-0,063 nm). The parameter for the NMR were (1H-NMR) (CDCl3): 7.77 (1H, s, H-2); 7.76 (1H, d, J = 8.0 Hz, H-6); 6.86 (1H, d, J = 8.0 Hz, H-5); 6.27 (1H, br s, HO); 5.32 (1H, t, J =7.0 Hz, H-2`); 3.40 (2H, d, J = 7,0 Hz, H-1`); 2.55 (3H, s, H-2``); 1,78 (6H, s, H-4`` + H-5``). 13C-RMN (CDCl3) δ: 197.5 (C-1``); 159.1 (C-4); 135.5 (C-3`); 130.8 (C-2); 130.2 (C-1); 128.9 (C-6); 127.0 (C-3); 121.0 (C-2`); 115.5 (C-5); 29.6 (C-1`); 26.3 (C-2``); 25.8 (C-4`)#; 17.9 (C-5`)#. # assignment may be interchanged in accordance to literature [22].

2.4 Bacterial Strains and Regents

The bacterial strains used in this work are listed in Table 1. Bacteriological media and components were purchased from Difco (Franklin Lakes, NJ). Luria Bertani (LB) broth [23], Bacto-Brain Heart Infusion (BHI), and tripticase soy broth (TSB), were used routinely. When required, the media was supplemented with 1.5% agar. Bacterial growth was monitored spectrophotometrically and/or by plating.

2.5 Minimal Inhibitory Concentration (MIC)

The MIC was determined by the microplate serial dilution assay [24]. This assay was performed using flat bottom 96-well clear microtitre plates. 10,000 µg/ml of 4-H-3-(MB)AP dissolved in ethanol 100% was serially diluted in 200 µl TSB and then inoculated with 2µl of mid-log-phase cultures (~1x10^8cfu/ml) of the respective bacterial strain.

2.6 Antimicrobial Assays

Antimicrobial assays were performed according Otto et al. [25]. Briefly, bacterial strains were grown overnight and diluted with fresh medium to achieve an approximate density of 1 x 10^7cfu/mL into 10ml. The bacteria culture was treated with 100 µg of 4-H-3-(MB)AP. The cultures were incubated at 37°C with constant rotary agitation (180 rpm) for 3 h. Positive controls consisted of

cultures not inoculated and negative controls consisted of cultures inoculated with 40% of isopropanol. These controls were included in each series of independent experiments. After incubation, the assays were subjected to successive 10-fold serial dilutions and plated to determine the number of viable bacteria. Three independent assays were performed per bacterial strain.

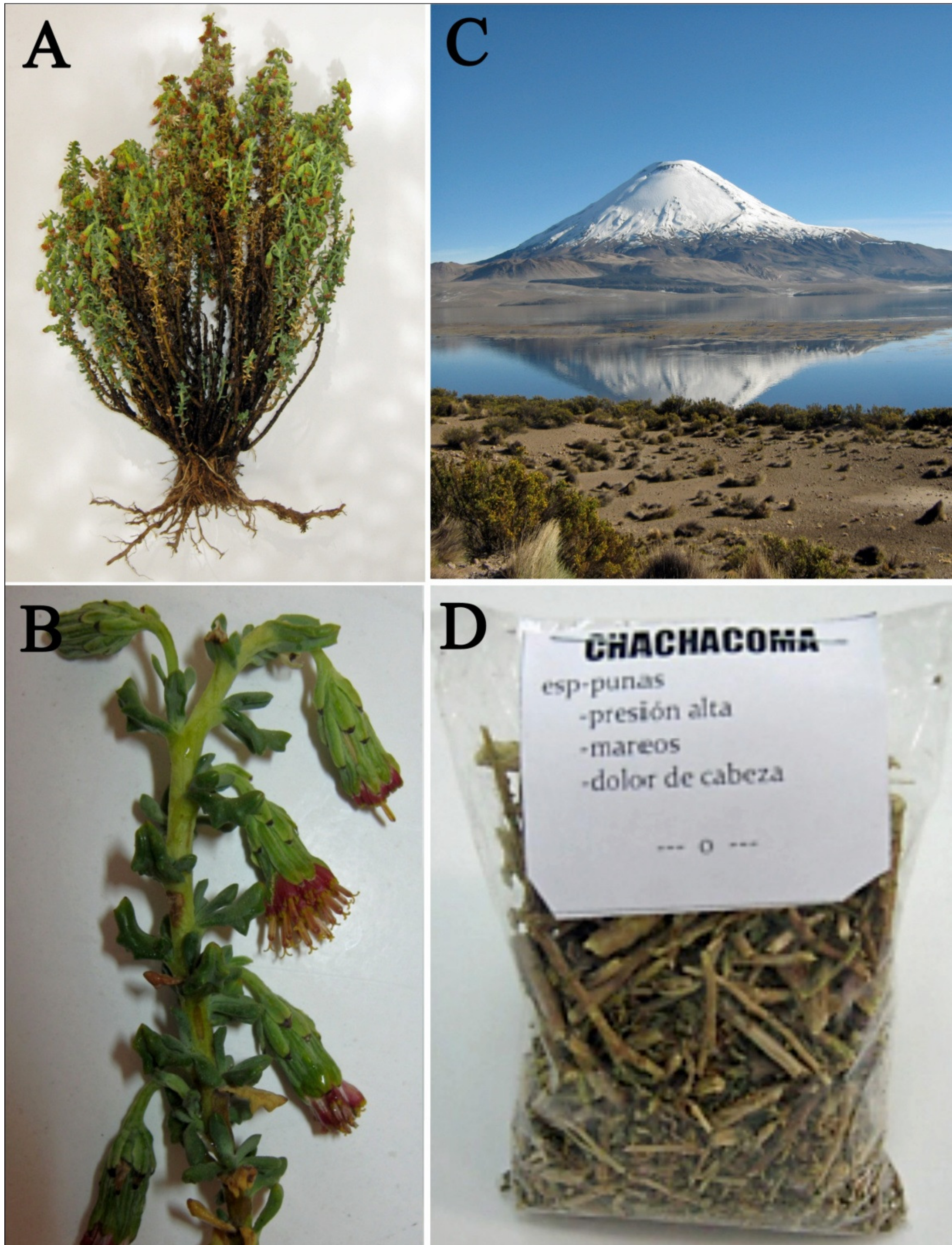

Fig. 1. Seneciograveolans environment. A. S. graveolans; B. S. graveolans leaves and flowers;

C. Lake Chungara area; D. Dry S. graveolans as folk medicinal "Chachacoma" plant

Table 1. Bacterial strains used in this study

Specie	Relevant characteristics	Source or reference
χ289 *Escherichia coli* K12	F^- *supE42* λ^- $T3^R$	Curtiss Lab
χ7122 Avian Pathogenic *E. coli*	APEC O78:K80:H9, *gyrA*Nal^r, Str^r	[47]
χ3761 *Salmonella enterica*serovar Typhimurium UK-1	Wild-type, smooth LPS	[41,42]
χ9944 *S.* Typhimurium UK-1	Δ*wzy-48,* semi-rough LPS	[28]
χ9945 *S.* Typhimurium UK-1	Δ*rfaH49,* rough LPS	[28]
χ11308 *S.* Typhimurium UK-1	Δ*waaG42,* rough LPS	[28]
χ11309 *S.* Typhimurium UK-1	Δ*waaI43,* rough LPS	[28]
χ11310 *S.* Typhimurium UK-1	Δ*waaJ44,* rough LPS	[28]
χ11311 *S.* Typhimurium UK-1	Δ*wbaP45,* rough LPS	[28]
χ11312 *S.* Typhimurium UK-1	Δ*waaL46,* rough LPS	[28]
χ3769 *S.* Typhi Ty2	Wild type	[48]
J100 *Edwardsiella ictaluri*	Wild-type 2003/c*;* smooth LPS	[43,44,45]
Shigella flexneri	Wild-type	Curtiss Lab
Vibrio vulnificus	Wild-type	Curtiss Lab
		Curtiss Lab
Vibrio parahaemolyticus	Wild-type	
G100 *Micrococcus luteus*	Wild-type	ATCC 4698
G101 *Bacillus subtilis*	Wild-type	ATCC 11714
G102 *Streptococcus epidermus*	Wild-type	ATCC 2228
G103 *Staphylococcus aureus*	Wild-type	ATCC 12600
G104 *Bacillus megaterium*	Wild-type	ATCC 14581
G105 *Streptococcus aureus*	Wild-type	ATCC 14581
G106 Methicillin-resistant *Staphylococcus aureus* (MRSA)	Wild-type	[46]
G107 Penicillin-resistant *Staphylococcus aureus* (PRSA)	Wild-type	[46]
M100 *Mycobacterium smegmatis*	Wild-type	ATCC 14468
Listeria monocytogenes	Wild-type	Curtiss Lab

2.7 Transmission Electron Microscopy (TEM)

E. coli and MRSA exponential phase cultures were normalized to an initial concentration of 10^6 CFU/mL. Following 30 min of exposure to 100 μg of 4-H-3-(MB)AP, the cells were fixed in 2% gluteraldehyde buffered in 50 mM phosphate, pH 7, for 2 h at room temperature. The cells were then washed in 50 mM phosphate and resuspended in 1% agarose (final concentration). The agarose-embedded cell pellets were fixed in 2% osmium tetroxide (buffered in 50 mM phosphate) for 2h at room temperature, washed three times in 50 mM phosphate buffer, washed three times in dH_2O, and en bloc stained in 0.5% uranyl acetate overnight at 4℃. The pellets were dehydrated in 10min washes with a sequential acetone series (20%, 40%, 60%, 80%, 3× 100%) and infiltrated with Spurr's resin. Thin sections (70 nm) were cut using an Ultracut R ultramicrotome (Leica Microsystems, Vienna, Austria). Sections were captured on formvar-coated, 300-mesh copper grids, post-stained in uranyl acetate and Sato's lead citrate, and observed on a Philips CM12 TEM at 80 kV. A minimum of 60 cells was counted from each of three independent replicates.

2.8 Flow Cytometric Measurements

To evaluate the membrane integrity of *E. coli* and MRSA following exposure to 4-H-3-(MB)AP, the BacLight LIVE/DEAD membrane permeability kit (Invitrogen, Carlsbad, CA, USA) was used following the manufacturer guidelines. *E. coli* and MRSA mid-logartithmic phase cultures were prepared as described above and harvested at an initial concentration of 10^8 CFU/mL. A standard curve was prepared by mixing live (0.85% saline-exposed) cells and dead (40% isopropanol-exposed) cells together at various

proportions of live:dead cells (100%, 75%, 50%, 25%, 0% alive). Following exposure to 4-H-3-(MB)AP or control conditions, cells were incubated in 5µM SYTO9 and 30 µM propidium iodide (PI) for 15 min in the dark and then immediately subjected to flow cytometric analysis. *E. coli* and MRSA cells were analyzed following 3 h exposure to 4-H-3-(MB)AP. A Cytomics FC 500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) fitted with a 488 nm excitation laser was used for membrane permeability analyses. Green fluorescence was detected on channel FL1 with a 525 nm bandpass filter. Red fluorescence was detected on channel FL3 with a 620 bandpass filter. Since the SYTO9 dye emits a strong signal at a wavelength of 600 nm, it overlaps with the PI emission [26]. Therefore, membrane permeabilization was determined by a horizontal population shift that occurs down the green fluorescent intensity axis. For each series of flow cytometric measurements, 50,000 cells were counted and analyzed.

2.9 Isolation of Strains Resistant to 4-H-3-(MB)AP

Concentration of 30, 50, and 100 µg/ml of 4-H-3-(MB)AP and high osmolarity (300 and 500 mMNaCl [27]) were used to prepare TSA. 10^8 CFU of the selected strain was added and incubate at 37℃ and lower temperatures (15 and 28℃). The strains used were MRSA, PRSA and *B. subtillis* (Table 1).

2.10 Statistical Analysis

Statistical analysis was performed using Prism 4 (GraphPad Software, San Diego, CA, USA) and was calculated using a two-tailed, paired Student's t-test. A *P* value of <0.05 was considered statistically significant.

3. RESULTS

3.1 Minimal Inhibitory Concentration of *S. graveolens* Extract

The *S. graveolens* organic extract did not present significant antibacterial activity against Gram-negative bacteria including *Salmonella*, *Escherichia,* *Edwardsiella*, *Shigella*and *Vibrio*(Table 2). In contrast, *S. graveolans* extract showed significant activity against the Gram-positive organisms *Listeria*, *Bacillus* and *Streptococcus* species, and against *Mycobacterium smegmatis* (Table 2).

3.2 4-hydroxy-3-(3-methyl-2-butenyl) acetophenone Purification and Identification

We determined that the main compound at the organic face was 4-H-3-(MB)AP ($C_{13}H_{16}O_2$; MW: 204.26884 g/mol) (Fig. 2). The crystal presented the characteristic aromatic smell of *S. graveolens*.

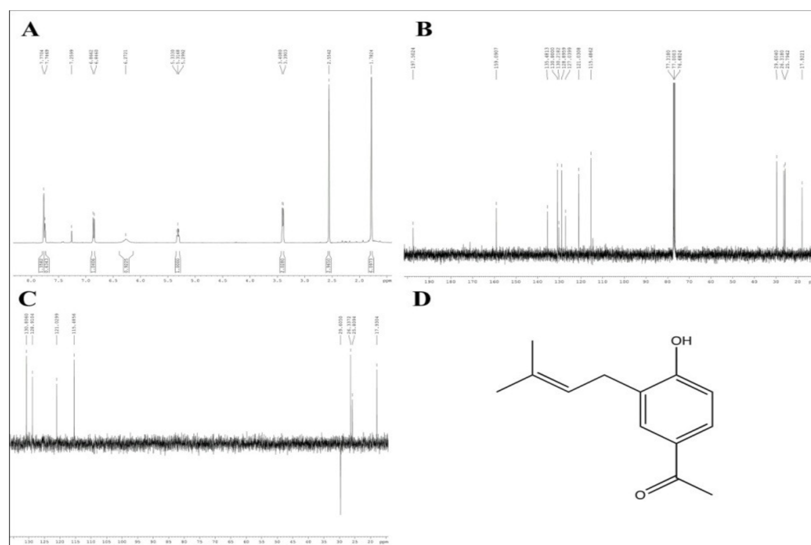

Fig. 2. Nuclear magnetic resonance (NMR) analysis. A. 1H-NMR; B. 13C-NMR; C. DEPT-135 NMR spectrum; D. 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone

3.3 Minimum Inhibitory Concentration (MIC) of 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone

The purified 4-H-3-(MB)AP resulted in a MIC against Gram-negative species over 1000 µg/ml with exception of *Vibrio* that had an MIC of 800 µg/ml (Table 3). We evaluated whether the lipolysaccharide (LPS) is involved in this resistance. We tested several *S. Typhimurium* LPS mutants with oligopolysaccharide, core, and lipid A modifications [28]. We found that LPS is not related to 4-H-3-(MB)AP resistance in Gram-negative bacteria (Table 3). In contrast, the MIC against Gram-positive species and *M. smegmatis* ranged between 25-200 µg/ml (Table 3). These data demonstrate that 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone has specific activity against Gram-positive bacteria.

3.4 Antibacterial Activity of 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone

The bactericidal activity of 100 µg/ml of 4-H-3-(MB)AP on *E. coli*, *S. aureus*, and MRSA was determined in agar and in liquid following a 3 h exposure (Fig. 3). *E. coli* viability was reduced by 2-$\log_{10}$ units after a 3 h exposure and MRSA and *S. aureus* viability was reduced by 6-$\log_{10}$ units, respectively, 3 h post treatment (Fig. 3). Together, these data demonstrate that, while *E. coli* is susceptible to some killing, the magnitude of killing does not meet clinical lab standards for bactericidal activity (minimum 3-$\log_{10}$ unit decrease). Alternatively, 100 µg/ml of 4-H-3-(MB)AP had bactericidal activity against both *S. aureus* and MRSA at clinically-relevant levels (Fig. 3).

3.5 Bacterial Membrane Permeabilization

E. coli treated with 4-H-3-(MB)AP was immediately permebilalized, affecting ~50% of the cell population (Fig. 4C). This effect in *E. coli* membrane permeabilization was constant during the entire assay (Fig. 4C). In contrast, MRSA and *S. aureus* treated with 4-H-3-(MB)AP exhibited delayed membrane permeabilization. MRSA and *S. aureus* membrane integrity decay was observed 3 h post treatment and almost all the cell population was affected (Figs. 4A-B).

3.6 TEM

Differences between *E. coli* treated with 4-H-3-(MB)AP and the non-treated were not detected (Fig. 5A-B). In contrast, MRSA treated with 4-H-3-(MB)AP for 30 min did not show membrane integrity damage, however, approximately 10% of the cells showed a disrupted division septum that was not observed in the non-treated MRSA cells (Fig. 6C-D). Similar results were observed at 3 h post 4-H-3-(MB)AP exposure. These results suggest that 4-H-3-(MB)AP affects the Gram-positive cell division process.

Table 2. Minimum inhibitory concentration (MIC) of *S. groevelans*

Bacterial strain	MIC (µg/ml)
Gram negative	
Salmonella Typhi	12,500
Salmonella Typhimurium	12,500
Salmonella Enteritidis	>25,000
Shigella flexneri	>25,000
Escherichia coli K-12	12,500
Escherichia coli APEC	>25,000
Vibrio parahaemolyticus	3,000
Vibrio vulnificus	3,000
Gram positive	
Listeria monocytogenes	1,500
Staphylococcus aureus	1,500
Bacillus subtilis	1,000
Acid Fast	
Mycobacterium smegmatis	1,000

Table 3. Minimal inhibitory concentration (MIC) of 4-H-3-(MB)AP purified from *S. groevelans*

Bacterial strain	MIC (µg/ml)
Gram negative	
S. Typhi Ty2	>1,000
S. Typhimurium UK-1	>1,000
S. TyphimuriumΔwaaG42, rough LPS	>1,000
S. TyphimuriumΔwaaI43, rough LPS	>1,000
S. TyphimuriumΔrfaH49, rough LPS	>1,000
S. TyphimuriumΔwaaJ44, rough LPS	>1,000
S. TyphimuriumΔwbaP45, rough LPS	>1,000
S. TyphimuriumΔwaaL46, rough LPS	>1,000
S. TyphimuriumΔwzy-48, semi-rough LPS	>1,000
Shigellaflexneri	>1,000
Escherichia coli K-12	>1,000
Escherichia coli APEC	>1,000
Vibrio parahaemolyticus	800
Gram positive	
Listeria monocytogenes	100
Bacillus megaterium	50
Bacillus subtilis	25
Staphylococcus aureus ATCC 12600	50
Staphylococcus aureus ATCC 11632	100
Methicillin-resistant *Staphylococcus aureus*	100
Penicillin-resistant *Staphylococcus aureus*	100
Staphylococcus epidermis	200
Micrococcus luteus	50
Acid Fast	
Mycobacterium smegmatis	100

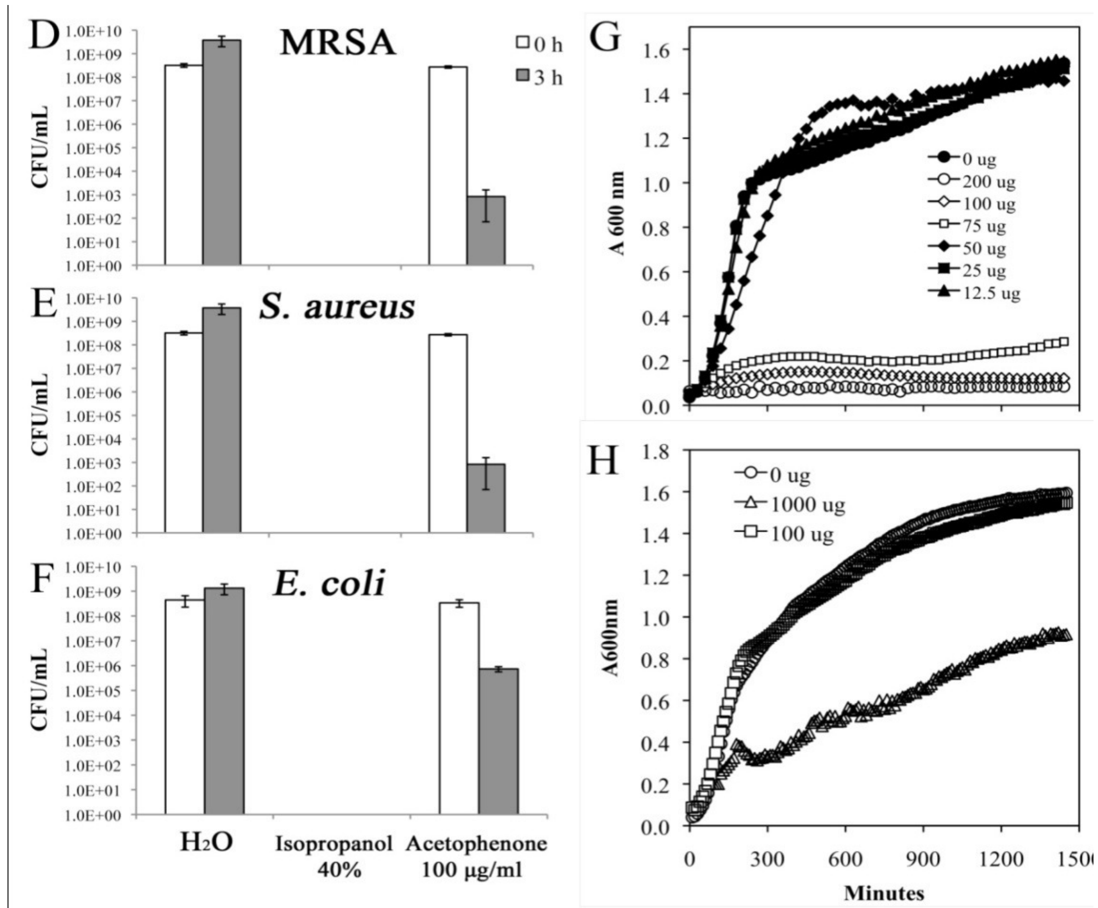

Fig. 3. Antibacterial activity of 4-H-3-(MB)AP. A. BHI agar; B. BHI agar supplemented with 100 µg/ml of 4-H-3-(MB)AP; C. BHI agar supplemented with 200 µg/ml of 4-H-3-(MB)AP. D-H. Growth inhibition of 3-(2-Hydroxy-3-methylbut-3-enyl)-4-hydroxyacetophenone in TSB at 37°C with aeration (180 rpm). D-F. Cell viability after 0 and 3 h post treatment. G-H. Growth inhibition. MRSA: Methicillin-resistant *Staphylococcus aureus*

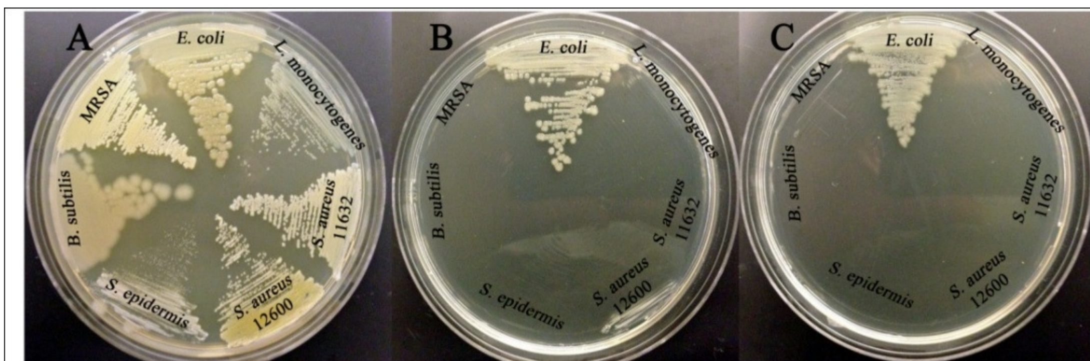

Fig. 4. Membrane permeabilization assay. A. MRSA; B. *S. aureus*; C. *E. coli*

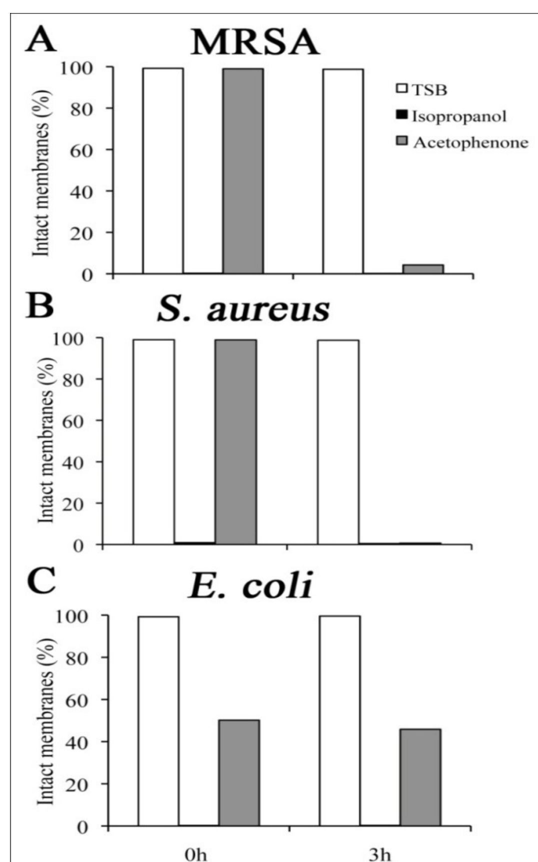

Fig. 5. TEM of cells non-treated and treated with 4-H-3-(MB)AP after 30 min. A. *E. coli* non-treated; B. *E. coli* treated with 100 µg/ml; C. MRSA non-treated; D. MRSA-treated.

3.7 Isolation of Strains Resistant to 4-H-3-(MB)AP

Using concentration from 30 to 200 µg/ml of 4-H-3-(MB)AP, high osmolarity conditions (300 and 500 mMNaCl [27]) and low temperatures (15 and 28ºC) in the solid media was not possible to isolate resistant mutants of MRSA, PRSA and *B. subtillis*. Suggesting that 4-H-3-(MB)AP affects essential processes for Gram-positive bacteria.

4. DISCUSSION

Since the pre-colonial period, a variety of native plants from the highlands of the Atacama Desert have been used as medicinal herbs by the Aymara culture and are currently used in popular folk medicine [29]. In particular, *S. graveolens* is known for its healing and anti-inflammatory properties that help to relive altitude sickness symptoms. Previously it has been determined that *S. graveolens* extract has antibacterial activity [13-16], but the specific antibacterial compound in the extract and the antibacterial mechanisms were unknown. We determined that the ethanolic extract of *S. graveolens* has a mild antibacterial activity against Gram-positive bacteria (Table 2). From this extract, we identified and isolated the molecule 4-H-3-(MB)AP as the main compound (Fig. 2) and showed that 4-H-3-(MB)AP has specific anti-bacterial activity against Gram-positive strains (Table 3). Plants produce many products as a mechanism of defense in response to tissue disruption or pathogen attack. Some of these compounds are known as phytoanticipins, which are constitutively synthesized in an inactive form, giving the plant a characteristic odor, distinctive pigmentation, and flavor (e.g., the terpenoid capsaicin from chili peppers) [1,30,31]. 4-H-3-(MB)AP has the characteristic odor of *S. graveolens*, suggesting that this compound might be related to a constitutive mechanism of plant defense.

As mentioned previously, 4-H-3-(MB)AP has been isolated from other *Senecio* species [12,13,22,32] and its antibacterial activity has been successfully evaluated. However its range of activity and mechanisms of action have not been determined. Here we showed that 4-H-3-(MB)AP has specific bactericidal activity against Gram-positive, including antibiotic-resistant strains (Table 3), and bacteriostatic activity against Gram-negative organisms (Fig. 3). 4-H-3-(MB)AP caused mild membrane permeabilization in *E. coli* (Fig. 4) , but did not result in correlatively killing. These data demonstrate that 4-H-3-(MB)AP affects Gram-negative membrane, but also suggest that membrane permabilization is not the antibacterial mechanism of action of 4-H-3-(MB)AP. The lipopolysaccharide (LPS) is the most external structure of the Gram-negative bacterial membrane, which is not present in Gram-positive bacteria. We evaluated whether the LPS is involved in the Gram-negative resistance to 4-H-3-(MB)AP (Table 3). We determined that LPS does not play a role in Gram-negative bacteria resistance to 4-H-3-(MB)AP, suggesting that its activity might be related to cell wall disruption or to another specific target present only in Gram-positive bacteria (Table 3). We unsuccessfully tried to isolate resistant mutants of MRSA, PRSA and *B. subtillis* under different conditions, including high and low osmolarity and low temperature. Suggesting that 4-H-3-(MB)AP affects essential processes for Gram-positive bacteria.

Teichoic acids are critical and abundant anionic glycopolymers found in Gram-positive cell envelopes, but not present in Gram-negative bacterial membrane [33]. Two kinds of these negative charge polymers are located on the surface of Gram-positive bacteria, lipoteichoic acids (LTA) and wall teichoic acids (WTA). Because teichoic acids are essential for bacterial viability [34,35], inhibition of the teichoic acid pathway is a promising antibacterial target [35-37]. Defective mutants of LTA or WTA are able to grow only under permissive temperature conditions and under non-permissive conditions these mutants present cell division defects in the divisome [38]. Depleted mutant of both LTA and WTA are not viable [34].

TEM showed that Gram-negativeand Gram-positive treated cells do not have membrane disruption (Figs. 5A-6B), but Gram-positive cells showed evident defects in the divisome after 30 min post treatment (Figs. 5C-6D) and 3 h post treatment. Compounds that preclude cell division through inhibition or disruption of the divisome typically cause cell elongation [38,39]. However, we did not observe cell elongation in the strains treated with 4-H-3-(MB)AP. The overall of these results suggests that 4-H-3-(MB)AP might have a pleiotropic effect in Gram-positive bacteria, perhaps affecting teichoic acid and cell wall synthesis. Recently, we evaluate the cytotoxicity effects of 4-H-3-(MB)AP [40]. We found that 4-H-3-(MB)AP is cytotoxic in human cell lines [40]. Further research is required to determine the precise antibacterial mechanism of action of 4-H-3-(MB)AP in Gram-positive bacteria and to modify this compound to maximize efficacy and minimize cytotoxicity activity.

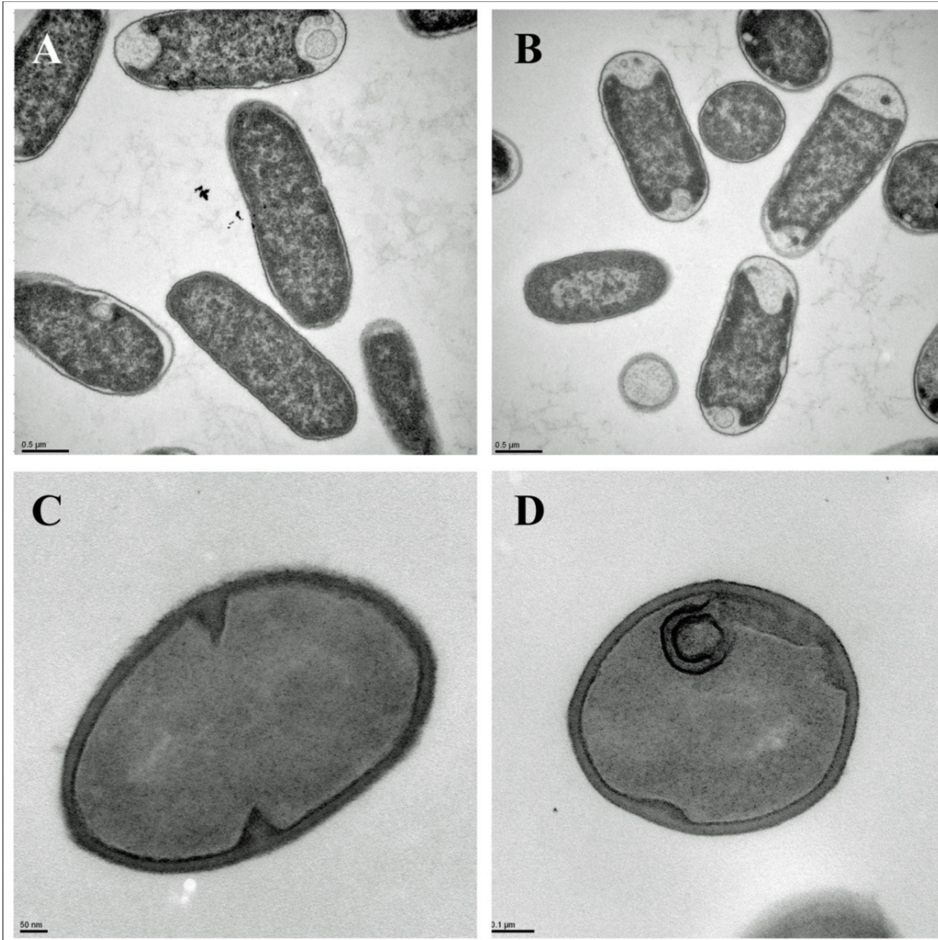

Fig. 6. TEM of cells non-treated and treated with 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone after 30 min. A.*E. coli* non-treated; B.*E. coli* treated with 100 µg/ml; C. MRSA non-treated; D. MRSA-treated

5. CONCLUSION

We conclude that 4-H-3-(MB)AP is one of the active compounds of S. graveolens extract responsible for its antibacterial activity. In light of the need of new drugs against multi-resistant bacteria, 4-H-3-(MB)AP is a candidate for further chemical modification studies and practical approaches.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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