



© 2016

Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 15 (6): 422 - 428

ISSN 0717 7917

www.blacpma.usach.cl

Artículo Original | Original Article

Antibacterial activity of cuticular components from *Heliotropium* species, against *Staphylococcus aureus* and *Salmonella typhimurium*

[Actividad antibacteriana de componentes cuticulares de especies de *Heliotropium* frente a *Staphylococcus aureus* y *Salmonella typhimurium*]

Mick Parra^{1,2}, Beatriz Valenzuela², Sarita Soto² & Brenda Modak²

¹Escuela de Ingeniería en Biotecnología, Facultad de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile

²Departamento de Ciencias del Ambiente, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile

Contactos / Contacts: Brenda MODAK - E-mail address: brenda.modak@usach.cl

Abstract: In this communication the antibacterial activity of filifolinol (1), naringenin (2) 3-O-methylgalangin (3) and pinocembrin (4) isolated from the resinous exudates of *Heliotropium filifolium* and *H. sinuatum*, were evaluated by flow cytometry against *Staphylococcus aureus* and *Salmonella typhimurium*. The results showed that filifolinol (1) and naringenin (2) were inactive in the range of concentrations used (10 to 1000 µg/mL). On the other hand, pinocembrin (4) produced a decrease in cell surface at 500 µg/mL and the total disappearance of both bacterial populations at 1000 µg/mL. Also, 3-O-methylgalangin (3) showed the total disappearance at 1000 µg/mL of both bacterial populations and a decrease at 200 µg/mL for *S. typhimurium* and at 500 µg/mL for *S. aureus*.

Keywords: antibacterial activity, *Staphylococcus aureus*, *Salmonella typhimurium*, *Heliotropium*, resinous exudates

Resumen: En esta comunicación, la actividad antibacteriana de filifolinol (1), naringenina (2), 3-O-methylgalangina (3) y pinocembrina (4) aislados de los exudados resinosos de *Heliotropium filifolium* y *H. sinuatum*, fueron evaluados por citometría de flujo frente a *Staphylococcus aureus* y *Salmonella typhimurium*. Los resultados mostraron que filifolinol (1) y naringenina (2) fueron inactivos en el intervalo de concentraciones usadas (10 a 1000 µg / mL). Por otro lado, pinocembrina (4) produce una disminución de la superficie de las células a 500 µg/mL y la desaparición total de ambas poblaciones bacterianas a 1.000 µg/mL. También, 3-O-metilgalangina (3) mostró la desaparición total a 1.000 µg / mL tanto de ambas poblaciones de bacterias y una disminución a 200 µg/mL para *S. typhimurium* y en 500 µg/mL para *S. aureus*.

Palabras clave: *Alpinia macroura*, hidrodestilación, aceite esencial, monoterpenos, sesquiterpenos.

Recibido | Received: Junio 19, 2016

Aceptado | Accepted: July 20, 2016

Aceptado en versión corregida | Accepted in revised form: August 30, 2016.

Publicado en línea | Published online: October 30, 2016.

Declaración de intereses | Declaration of interests: This work was supported by FONDECYT N°1140261.

Este artículo puede ser citado como / This article must be cited as: M Parra, B Valenzuela, S Soto, B Modak. 2016. Antibacterial activity of cuticular components from *Heliotropium* species, against *Staphylococcus aureus* and *Salmonella typhimurium*. *Bol Latinoam Caribe Plant Med Aromat* 15 (6): 422 – 428.

INTRODUCTION

Bacterial infections are among the most important infectious diseases worldwide. Two very important bacteria for their adverse effects and high prevalence in humans are *Staphylococcus aureus* and *Salmonella typhimurium*. *S. aureus* is a Gram-positive pathogen, with one of the highest prevalence in humans, colonizing the skin and mucous membranes in 20 to 30% of the world population (Otto, 2010). *S. aureus* is a dangerous human pathogen that can cause severe and life-threatening diseases, including: pneumonia, meningitis, endocarditis and septic shock syndrome (Mun et al., 2013) and cause a significant number of intra-hospital infections in susceptible patients (O'Neill, 2010). The negative impact of these pathogens on humans, has been increased because their ability to develop resistance to antibiotics (Chambers & Deleo, 2009). *Salmonella typhimurium* is a pathogenic Gram-negative bacteria predominately found in the intestinal lumen. Its toxicity is due to an outer membrane consisting largely of lipopolysaccharides (LPS) which protect the bacteria from the environment. This bacteria is the causative agent of salmonellosis (Kendall et al., 2003) and is able to colonize the digestive tract of poultry, resulting in fecal contamination and contamination of poultry and poultry products; being an important source of contamination for humans and result in a public health problem (Youn et al., 2016). Prevention of salmonellosis is difficult because of the persistence of *Salmonella* in poultry hosts, on the route of oral-fecal pollution and the wide range of chronic carriers of this pathogen (Henzler & Opitz, 1992). Antibiotic treatment of *S. aureus* and *S. typhimurium* infections often is problematic due to their slow response to therapy and the high frequency of recurrent infections. Therefore, the search for new specific antibacterial agents is due to a major clinical problem that is the multidrug resistance in pathogenic bacteria.

Plants have provided numerous novel compounds that have shown promising activity against infectious diseases caused by bacteria (Camporese et al., 2003; Ding et al., 2009; Dubey et al., 2012). The search for new active compounds where the natural products have an important role because from them have derived many of the drugs against pathogens (Demain & Sanchez 2009; Molinari, 2009).

Species of *Heliotropium* (Heliotropiaceae) section *Cochranea* (Miers) Reiche are found growing in the Pacific coastal region of Chile, in arid or semi-arid ecosystems, exposed to extreme environmental conditions. These species produce surface components (resinous exudates) that cover leaves and stem as a defense mechanism. These resinous exudate are characterized by the presence of flavonoid and aromatic geranyl derivatives. From *Heliotropium filifolium* Miers, filifolinol (1) (Figure 1), the first example of a *spiro*-benzodihydrofuranly terpene was isolated (Torres et al., 1994) and from *Heliotropium sinuatum* Miers the flavonoids naringenin (2), 3-O-methylgalangin (3) and pinocembrin (4) (Figure 1) were isolated (Torres et al., 1996).

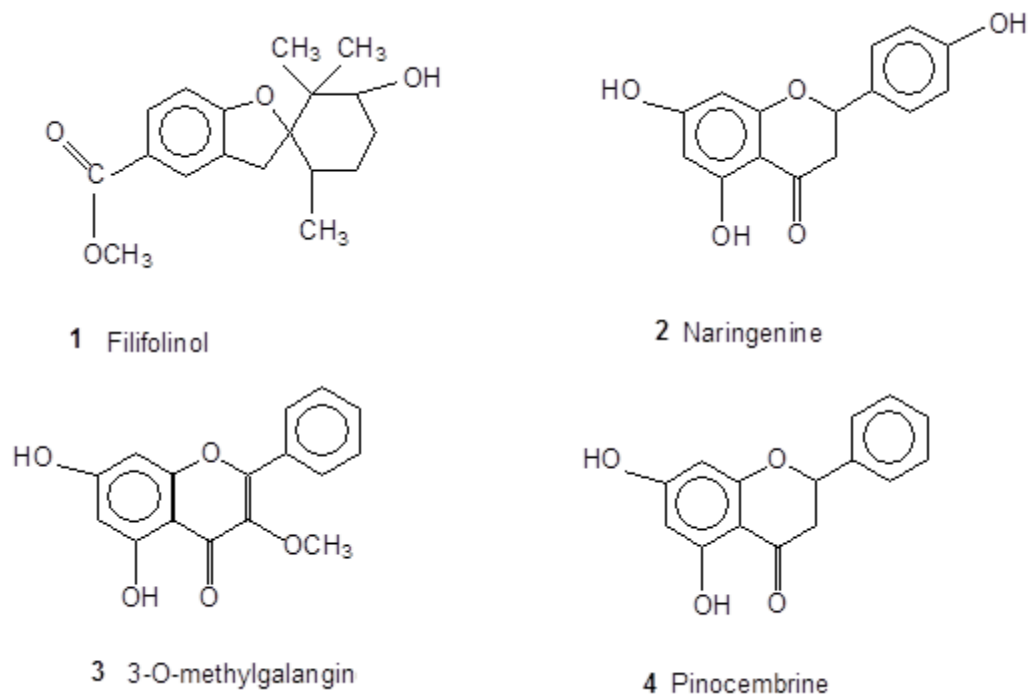
Previous studies showed that these compounds have interesting antimicrobial properties, against phytopathogenic bacteria (Modak et al., 2004) and also *Escherichia coli*, *Bacillus subtilis* and *Bacillus cereus* (Torres et al., 2002; Urzúa et al., 2008).

This work shows the results of the antibacterial activity of filifolinol (1), naringenin (2), 3-O-methylgalangin (3) and pinocembrin (4) against *Staphylococcus aureus* and *Salmonella typhimurium*. Two bacteria involved in serious health problems.

MATERIAL AND METHODS

Isolation of Filifolinol (1), Naringenin (2), 3-O-methylgalangin (3) and Pinocembrin (4).

The compounds 1-4 (Figure 1) were purified from the resinous exudate of *Heliotropium filifolium* (Miers) Reiche (Filifolinol) and of *Heliotropium sinuatum* (Miers) (naringenin, 3-O-methylgalangin and pinocembrin) as has been described (Torres et al., 1994; Torres et al., 1996). Briefly, the resinous exudate was extracted by immersion of the fresh plant material in CH₂Cl₂ for 30 s at room temperature. The extracts were concentrated to yield solid residues (6.3 and 6.0 % w/w, respectively). The extracts were purified by CC (silica gel) using a hexane-ethyl acetate step gradient. *H. filifolium* and *H. sinuatum* were recollected in III region, Chile. Voucher specimens were deposited in the Herbarium of the Faculty of Biological Science of the Catholic University of Chile (ST-2214 SSUC and ST-2563 SSUC).

**Figure 1****Structure of test compounds**

1 Filifolinol, 2 Naringenin, 3 3-O-methylgalangin and 4 Pinocembrin.

Bacterial strains and culture conditions

S. typhimurium (ATCC 14028) was grown in Luria-Bertani (LB) at 37° C, with an agitation of 200 rpm for 24 h and *S. aureus* (ATCC 8325-4) was grown in Tryptic soy broth medium (TSB) at 37° C, with an agitation of 200 rpm for 24 h.

Bactericidal activity

To determine the antibacterial activity, serial dilutions of each compound, were performed in LB and TSB medium to *S. typhimurium* and *S. aureus*, respectively. The concentrations tested for each compound were 10 µg/mL, 20 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL and 1000 µg/mL, in a final volumen of 990 µL with 10 µL of inoculum of *S. typhimurium* at a final concentration of $3,8 \times 10^8$ UFC/mL and 10 µL of inoculum of *S. aureus* at a final concentration $6,3 \times 10^6$ CFU/mL. Samples were incubated for 24 h at 37° C with agitation at 200 rpm. The bacteria were centrifuged at

5000 rpm for 5 min, the supernatant was discarded and the pellet was suspended in 300 µL of phosphate buffered saline (1 X PBS) containing 2 µL of propidium iodide (PI, 1 mg/mL). Viable cells (PI negative cells) were quantitated by flow cytometry using flow cytometer FACSCanto II (BD Biosciences). Bacteria incubated for 1 h at 95° C were used as positive control. Untreated bacteria were used as negative control.

Bacterial Quantitation

To determine the amount of bacteria, the quantification in plate method was used by counting CFU/mL. Serial dilutions from 10^{-1} to 10^{-12} were performed, taking from the bacterial culture to quantify 100 µL in 900 µL of sterile distilled water. 10 µL of the dilutions were seeded in 24-well plates previously prepared with 1 mL of Luria-Bertani agar (LB) and incubated for 16 hours at 30° C. CFUs/mL were calculated using the following equation:

$$\text{UFC/mL} = (n^\circ \text{ colonies/mL}) \times \text{dilution factor}$$

Statistical analysis

All data were analyzed using GraphPad software. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

The effect of the compounds (1-4) against *Salmonella typhimurium* and *Staphylococcus aureus* was measured by flow cytometry. The distribution graphs of the bacterial population delivered by the flow cytometer between the side scatter (SSC-A) and forward scatter (FSC-A) axis showed that the compounds (1) and (2) were not active at any of the tested concentration. These results are different from those of Urzúa *et al.* (2008) where filifolinol (1) showed a MIC of 1024 $\mu\text{g}/\text{mL}$ against *S. aureus*. This difference may be due to the different techniques that were employed in the evaluation of the activity. Flavonoids (3) and (4) showed important changes in the bacterial populations.

Treatment with pinocembrin (4) at 500 $\mu\text{g}/\text{mL}$ (Figure 2C), showed a decrease in size and shape of the cells and the total disappearance of the bacterial populations was observed at 1000 $\mu\text{g}/\text{mL}$ (Figure 2D and 3D). The disappearance of a bacterial population indicates cells lysis, by effect of treatment with the compounds (Saint-Ruf *et al.*, 2016) and it is the result of the loss of integrity of the population. The results obtained with the flavonoid were compared with the positive controls that consist in the treatment of the bacterial populations with temperature. In this experiment it was observed the disappearance of populations in the graphs (SSC-A/FSC-A). Similar results were observed for 3-O-methylgalangine (3), where the total disappearance of the bacterial population was observed to 1000 $\mu\text{g}/\text{mL}$ in both species; while the decrease of cell surface and complexity were observed at 200 $\mu\text{g}/\text{mL}$ for *S. typhimurium* (Figure 2) and 500 $\mu\text{g}/\text{mL}$ for *S. aureus* relative to the control (untreated bacteria) (Figures 3).

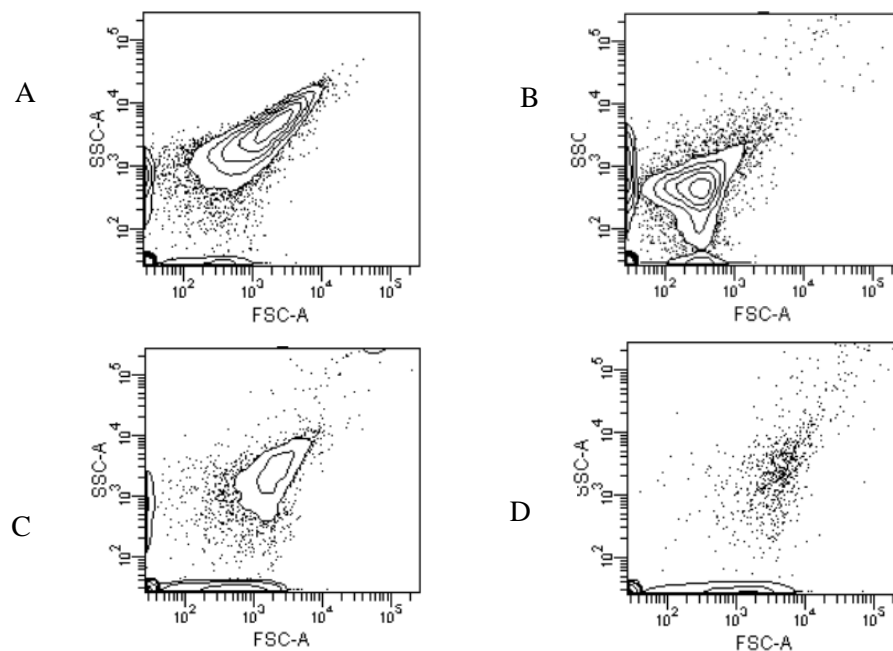


Figure 2

Flow cytometry obtained for *Salmonella typhimurium* incubated with compound 3 for 24 hours at 30° C

- A) Negative Control. B) Concentration 200 $\mu\text{g}/\text{mL}$.
C) Concentration 500 $\mu\text{g}/\text{mL}$. D) Concentration 1000 $\mu\text{g}/\text{mL}$

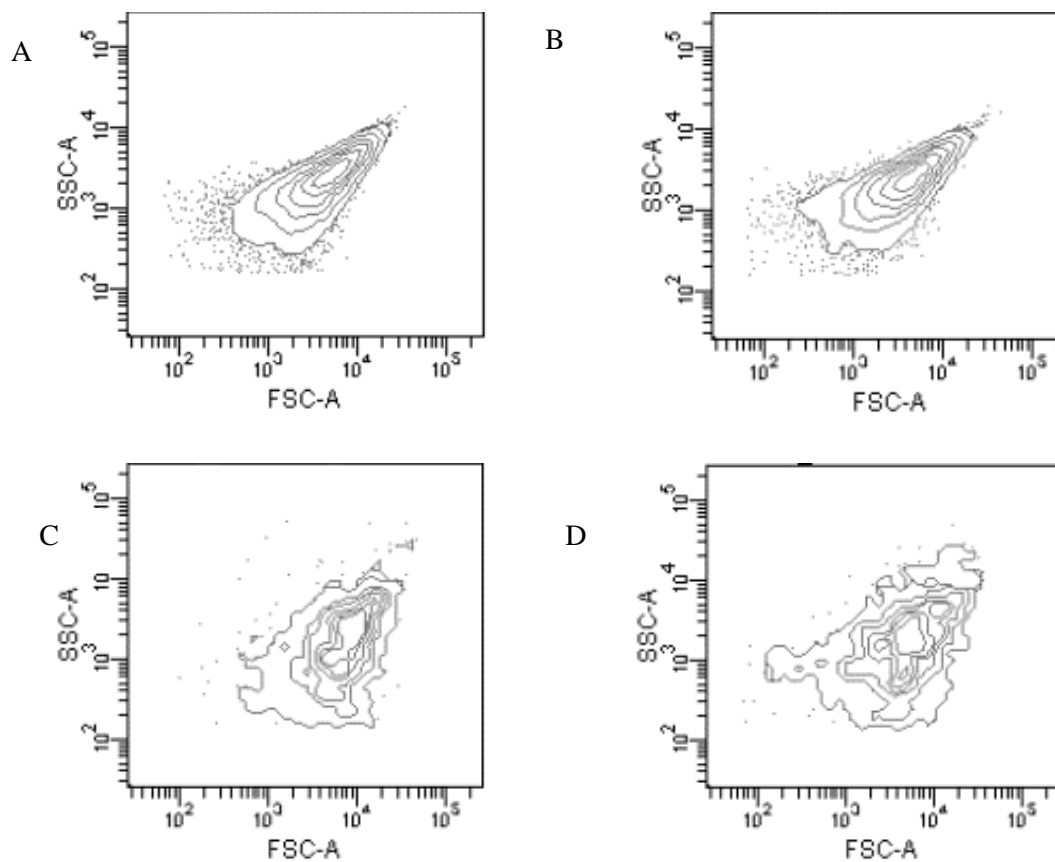


Figure 3

Flow cytometry obtained for *Staphylococcus aureus* incubated with compound 3 for 24 hours at 30° C
 A) Negative Control. B) Concentration 10 µg/mL. C) Concentration 500 µg/mL. D) Concentration 1000 µg/mL.

To check if the change in the bacterial populations was related to the antimicrobial capacity of the compounds, a titration was performed by counting colony forming units (CFU/mL) for compounds (3) and (4) that had generated a change in populations of *Salmonella typhimurium* and *Staphylococcus aureus*. The results showed that for *S. aureus*, the two compounds at concentrations which resulted a change in the bacterial population, led to a decrease in the number of bacterial (Figure 4A), reaching decrease by up to five orders of magnitude, as was observed for 3-O- methylgalangin (3) to 1000 µg/mL. In the case of *S. typhimurium*, the same concentrations of compound (3) showed a decrease by up to two orders of magnitude in the number of bacterial population relative to the control (Figure 4B).

The results obtained with compounds (3) and (4) are significant, considering that in other studies, reduction of *S. aureus* population required 800 µg/mL of vancomycin after 24 hours of exposure,

resulting in 2-log-unit reduction in CFU respect to control (Harriott & Noverr, 2009). Other study shows that with amoxicillin (1024 µg/mL), a reduction of 90% of *S. typhimurium*, was observed after 6 hours of exposure (Junod *et al.*, 2013).

Several factors explain the capacity of *S. aureus* and *S. typhimurium* to avoid the action of antibiotics, where the biofilm formation might be the main reason for a deficient antibiotic effect, when foreign bodies are involved in the infections (Chuard *et al.*, 1991; Saramago *et al.*, 2014).

On the other hand, flow cytometry has proved to be a good tool to determine the antibacterial activity of compounds that have low solubility to determine the MIC.

Finally, based on the results obtained, the flavonoids 3-O-methylgalangin (3) and pinocembrin (4) are good candidates to test their antibacterial activity *in vivo*.

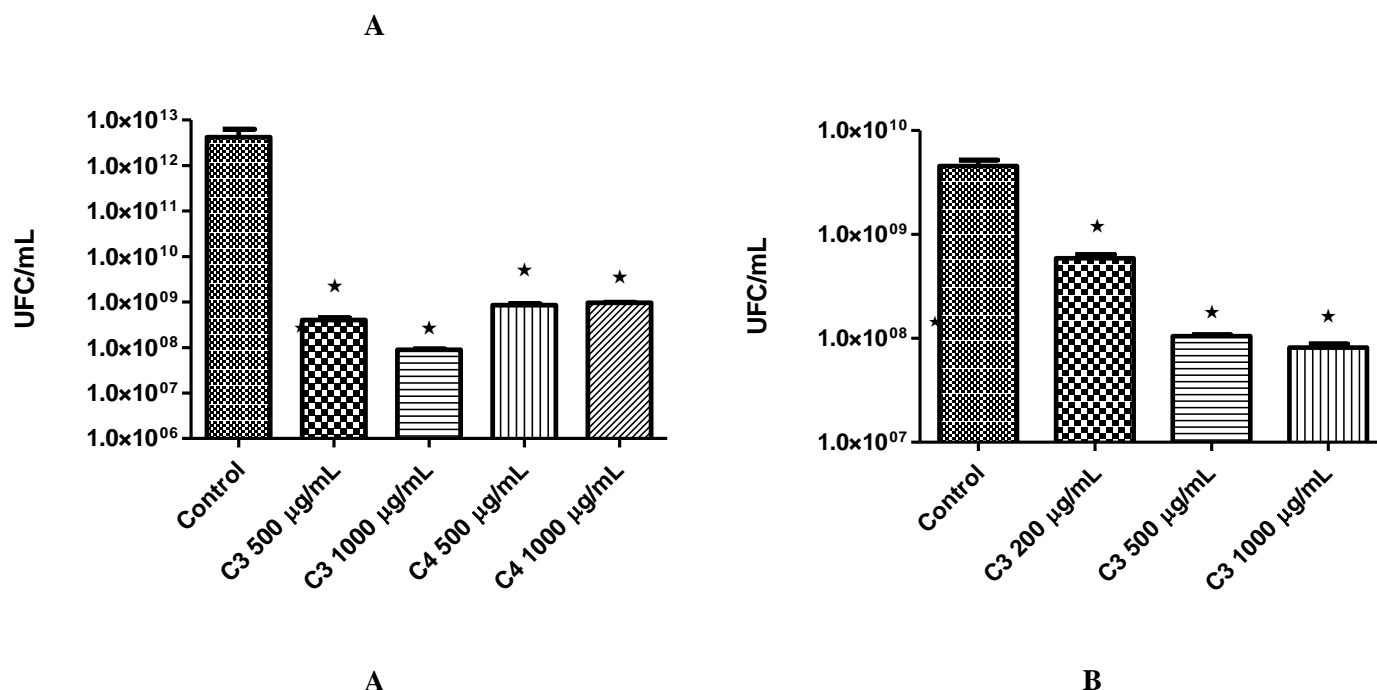


Figure 4

Titration of *Staphylococcus aureus* and *Salmonella typhimurium* incubated at 30° C for 24 h with compound (3) and (4). A) *Staphylococcus aureus* incubated with compound (3) (500 µg/mL y 1000 µg/mL) and compound (4) (500 µg/mL and 1000 µg/mL). The significant difference was established between the control (*S. aureus*) and the different compounds, based on the Mann-Whitney test ($p \leq 0.05$). B) *Salmonella typhimurium* incubated with compound (3) (200 µg/mL, 500 µg/mL and 1000 µg/mL). The significant difference was established between the control (*S. typhimurium*) and the different concentrations, based on the Mann-Whitney test ($p \leq 0.05$).

ACKNOWLEDGMENTS

This work was supported by FONDECYT N°1140261.

REFERENCE

- Camporese A, Balick MJ, Arvigo R, Esposito RG, Morsellino N, De Simone F, Tubaro AJ. 2003. Screening of anti-bacterial activity of medicinal plants from Belize (Central America). **J Ethnopharmacol** 87: 103 - 107.
- Chambers HF, Deleo FR. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. **Nat Rev Microbiol** 7: 629 - 641.
- Chuard C, Lucet JC, Rohner P, Herrmann M, Auckenthaler R, Waldvogel F, Lew DP. 1991. Resistance of *Staphylococcus aureus* recovered from infected foreign body in vivo to killing by antimicrobials. **J Infect Dis** 163: 1369 - 1373.
- Demain AL, Sanchez S. 2009. Microbial drug discovery: 80 years of progress. **J Antibiot** 62: 5 - 16.
- Ding Q, Yang LX, Yang HW, Jiang C, Wang YF, Wang S. 2009. Cytotoxic and antibacterial triterpenoids derivatives from *Clematis ganpiniana*. **J Ethnopharmacol** 126: 382 - 385.
- Dubey D, Sahu MC, Rath S, Paty BP, Debata NK, Padhy RN. 2012. Antimicrobial activity of medicinal plants used by aborigines of Kalahandi, Orissa, India against multidrug resistant bacteria. **Asian Pac J Trop Biomed** 2: S846 - S854.
- Harriott M, Noverr M. 2009. *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: Effects on antimicrobial resistance. **Antimicrob Agents Chemother** 53: 3914 - 3922.
- Henzler DJ, Opitz HM. 1992. The role of mice in the epizootiology of *Salmonella enteritidis* infection on chicken layer farms. **Avian Dis** 36: 625 - 631.
- Junod T, López-Martin J, Gädicke P. 2013. Estudio de susceptibilidad antimicrobiana de *Salmonella enterica* en muestras de origen

- animal y alimentario. **Rev Med Chile** 141: 298 - 304.
- Kendall P, Medeiros LC, Hillers V, Chen G, DiMascola S. 2003. Food handling behaviors of special importance for pregnant women, infants and young children, the elderly, and immune-compromised people. **J Am Diet Assoc** 103: 1646 - 1649.
- Modak B, Torres R, Wilkens M, Urzúa A. 2004. Antibacterial activity of compounds isolated of the resinous exudates from *Heliotropium sinuatum* on phytopathogenic bacteria. **J Chil Chem Soc** 49: 1 - 3.
- Molinari G. 2009. Natural products in drug discovery: present status and perspectives. **Adv Exp Med Biol** 655: 13 - 27.
- Mun SH, Joung DK, Kim YS, Kang OH, Kim SB, Seo YS, Kim YC, Lee DS, Shin DW, Kwon KT, Kwon DY. 2013. Synergistic antibacterial effect of curcumin against methicillin-resistant *Staphylococcus aureus*. **Phytomedicine** 20: 714 - 718.
- O'Neill AJ. 2010. *Staphylococcus aureus* SH1000 and 8325-4: comparative genome sequences of key laboratory strains in staphylococcal research. **Lett Appl Microbiol** 51: 358 - 361.
- Otto M. 2010. *Staphylococcus* colonization of the skin and antimicrobial peptides. **Expert Rev Dermatol** 5: 183 - 195.
- Saint-Ruf C, Crussard S, Franceschi C, Orenge S, Ouattara J, Ramjeet M, Surre J, Matic I. 2016. Antibiotic susceptibility testing of the Gram-Negative bacteria based on flow cytometry. **Front Microbiol** 7: 1121 - 1133.
- Saramago M, Domingues S, Viegas SC, Arraiano CM. 2014. Biofilm formation and antibiotic resistance in *Salmonella typhimurium* are affected by different ribonucleases. **J Microbiol Biotechnol** 24: 8 - 12.
- Torres R, Villarroel L, Urzúa A, Delle-Monache F, Delle-Monache G, Gacs-Baitz E. 1994. Filifolinol, a rearranged geranyl aromatic derivative from the resinous exudate of *Heliotropium filifolium*. **Phytochemistry** 36: 249 - 256.
- Torres R, Modak B, Urzúa A, Villarroel L, Delle Monache F, Sánchez-Ferrando F. 1996. Flavonoides del exudado resinoso de *Heliotropium sinuatum*. **Bol Soc Chil Quim** 41: 195 - 197.
- Torres R, Modak B, Arrieta A, Urzúa A. 2002. Actividad antibacteriana de flavonoides aislados del exudado resinoso de *Heliotropium sinuatum*: efecto del tipo de estructura. **J Chil Chem Soc** 47: 19 - 23.
- Urzúa A, Echeverría J, Rezende M, Wilkens M. 2008. Antibacterial Properties of 3*H*-Spiro[1-benzofuran-2,1'-cyclohexane] Derivatives from *Heliotropium filifolium*. **Molecules** 13: 2385 - 2393.
- Youn SY, Kwon YK, Song CS, Lee HJ, Jeong OM, Choi BK, Jung SC, Kang MS. 2016. Increased efficacy of inactivated vaccine candidates prepared with *Salmonella enterica* serovar *Typhimurium* strains of predominant genotypes in ducks. **Poult Sci** 95: 1764 - 1773.