Iranian Journal of Microbiology

Volume 13 Number 1 (February 2021) 98-103

ORIGINAL ARTICLE

Antimicrobial activity of bioactive compounds of *Haplopappus multifolius* and *Haplopappus taeda* against human pathogenic microorganisms

Carlos Padilla, Olga Lobos, Patricia Poblete-Tapia, Verónica Carrasco-Sánchez*

Department of Microbiology, Faculty of Health Sciences, University of Talca, Talca, Chile

Received: September 2020**, Accepted:** January 2021

ABSTRACT

Background and Objectives: *Haplopappus multifolius Phil. Ex Reiche* and *Haplopappus taeda Reiche* are medicinal shrubs native to Chile and are popularly known as "Bailahuén". Regularly, this plant is used for liver, digestive and renal affections, as well as colds and the cleaning of infected wounds. The aim of the study was to identify the responsible compounds for the antimicrobial activity of *H. multifolius* and *H. taeda.*

Materials and Methods: Infusions and ethanolic extracts of *H. taeda* and *H. multifolius* were analysed by thin-layer chromatography bioautography (TLC-B) to determine the compounds responsible for the antimicrobial activity against Gram-positive and Gram-negative bacterial strains and yeasts of Bailahuén. Finally, the minimum inhibitory concentration (MIC) of pure compounds isolated was determinate.

Results: Extract of Bailahuén had activity only against Gram-positive bacterial strains and this activity was associated with aesculetin, 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid and aromadendrin-7-methyl ether compounds.

Conclusion: *H. multifolius* and *H. taeda* have antibacterial capacity on different species of Gram-positive bacteria pathogenic for humans.

Keywords: *Haplopappus taeda; Haplopappus multifolius;* Flavonoids; Terpenoids; Sesculetin

INTRODUCTION

The expanding bacterial resistance to antimicrobial substances has become a growing concern worldwide (1). Increasing bacterial resistance is prompting resurgence in research of the antimicrobial role of herbs against resistant bacterial strains (2), taking into account that throughout history the therapeutic properties of native plants have been used to treat diseases in humans (3).

Haplopappus multifolius Phil. ex Reiche and *Haplopappus taeda Reiche* are two species of the genus *Haplopappus* (Asteraceae) and they are known by the common name of “Bailahuén” (4). This plant is endemic to Chile and distributed in the central valley of this country. The infusions of the resinous leaves of these shrubs are popularly used as a digestive stimulant, antiseptic, relief of liver ailments and intestinal and urinary disorders (4, 5). The aborigines also employed the leaves for healing wounds in horses. In Chile, several studies have been carried out in native plants in order to show their bioactive capacity on different types of microorganisms; thus, species of the family *Asteraceae* have been studied with respect to its antimicrobial potential (6, 7). However, the detection of the pure bioactive compound and the MIC has not been addressed in previous studies. For this reason, the goal of this work was to determine the active compounds with their respective MIC of

*Corresponding author: Verónica Carrasco-Sánchez, Ph.D, Department of Microbiology, Faculty of Health Sciences, University of De Talca, Talca, Chile.
Tel: +56-71-2200489
Fax: +56-71-2200489
Email: vecarrasco@utalca.cl

H. multifolius and *H. taeda*.

MATERIALS AND METHODS

***Haplopappus* plant material.** 12 kg of fresh plant without flowers were collected from *H. multifolius* (Hm) (M R, 31°S, Chile) and then dried at room temperature in the shade and circulation of air, obtaining 2 kg of dry plant. From *H. taeda* (Ht), 13 kg of fresh plant were collected with floral buds (VI R, 33ºS, Chile) and after drying, 3.9 kg of dry plant were obtained.

Extracts and sub-extracts of *Haplopappus*. Ethanolic extracts of *H. multifolius* (Hm-E) and *H. taeda* (Ht-E) were obtained by maceration of the dry plant (1.5 and 2.0 Kg respectively) in ethanol (>95%, Sigma-Aldrich, St Louis, MO, USA) (20°C, 18 h) and then concentrated to dryness to give 173 g of Hm-E and 320 g of Ht-E.

The infusions of *H. multifolius* (Hm-I) and *H. taeda* (Ht-I) (5 g of dry leaves and 100 mL of distilled boiling water, 10 min.) were filtered and then lyophilized. The yields were 0.06 g and 0.04 g, respectively.

Then, to get sub-extracts, ethanolic extracts (Hm-E and Ht-E; 30 g for each) were submitted to column chromatography (Sephadex LH-20 and MeOH, >98%) obtaining fractions with their major compounds as a coumarins (C), flavonoids (F) and terpenoids (T). From the ethanolic extracts of *H. multifolius* and *H. taeda*, two fractions, from each one, were obtained by column chromatography. For *H. multifolius*, a fraction enriched in coumarins (Hm-E-C) (yield of 66.7%) and another in flavonoids (Hm-E-F) (yield of 32.4%) were obtained. For *H. taeda*, a fraction rich in terpenoids (Ht-E-T) and another in flavonoids (Ht-E-F) were obtained with a yield of 45.5% and 44.9%, respectively.

Antimicrobial activity screening of ethanolic extracts, infusions and sub-extracts. The bacterial strains studied were *Acinetobacter baumannii* ATCC 17978, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 35667, *Escherichia coli* ATCC 35421, *Listeria monocytogenes* ATCC 7646, *Morganella morganii* ATCC 25830, *Proteus mirabilis* 43071, *Proteus vulgaris* 8427, *Providencia stuartii* ATCC 33672, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhi ATCC 6539, *Shigella sonnei* ATCC 9290, *Staphylococcus aureus* ATCC 43300, *Staphylococcus epidermidis* ATCC 29887, *Streptococcus agalactiae* ATCC 12386, and *Streptococcus pyogenes* ATCC 19615. *Candida albicans* and *C. tropicalis* were obtained from the microbiology laboratory of the Universidad de Talca.

The susceptibility of the microorganism to extracts, sub-extracts and infusions were determined using diffusion assay on Müeller-Hinton (MH) agar dishes. For this, the microorganisms were grown in Müeller-Hinton broth over night at 37°C. Then, the microorganisms in broth were adjusted to 0.5 Mc Farland turbidity (≈1.5×10^8 CFU mL^{-1}) and immediately were sowed on lawn, on the agar plates. Paper discs (6 mm) were impregnated with 5 mg of Hm-E and its sub-extracts (Hm-E-C and Hm-E-F), Hm-I, Ht-E and its sub-extracts (Ht-E-T and Ht-E-F) and Ht-I. Then, the discs were placed on the agar plate. Chloramphenicol (C, >98%, Sigma-Aldrich, USA) was used as a positive control. The tests were carried out in triplicate for each extract.

Thin-layer chromatography bioautography (TLC-B), determination of pure compounds and their antimicrobial activity. The sub-extracts that were active on some bacterial strains were subjected to an autobiography analysis to determine the possible compounds responsible for the activity. For this, sub-extracts were dissolved in dimethyl sulfoxide (>99.9%, Sigma-Aldrich, St Louis, MO, USA) to a concentration of 10 mg mL^{-1}.

Identification of possible active compounds was achieved by TLC-B method. Briefly, 30 µL of sub-extract solutions were put in TLC plate (silica gel 60 F-254, Merck, Germany) and were separated using dichloromethane/methanol (97:3 v/v) and dichloromethane/ethyl acetate (90:10 v/v) as solvent systems for two-dimensional chromatography. Then, TLC plates were covered with 12 mL of Müeller-Hinton agar layer mixed with bacterial concentration (≈1.5×10^8 CFU mL^{-1}). The plates were left incubating at 37°C for 12 hours and then the areas that exhibited microbial growth inhibition were visually identified.

Then, the compounds belonging to the identified zones of inhibition were isolated by repeated flash column chromatography using silica gel and different solvent mixtures of increasing polarity (hexane/ethyl acetate; dichloromethane/ethyl acetate; dichlorometh-

ane/methanol) and the pure compounds were identified by their NMR spectroscopic data and by direct comparison with samples previously identified in other studies, esculetin (10), 9-p-coumaroyloxy-α-terpineol, 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid, 19-hidroxy-cis-cleroda-3,13E-dien-15-oic acid, aromadendrin-7- methyl ether and eriodictyol-7-methyl ether (11, 12).

Finally, the antimicrobial activity to the compounds isolated was determined. All assays were performed in triplicate. The values of the antimicrobial activity assay were expressed as mean ± standard deviation using the software OriginPro 8.

Minimum inhibitory concentration (MIC) of pure compounds. The minimum inhibitory concentration (MIC) of aesculetin, 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid and aromadendrin-7-methyl ether was determined using the standard microdilution method (CLSI M100- S25) (13).

The MIC was determined in MH broth using dilutions of compounds in concentrations ranging from 5 µg mL^{-1} to 2560 µg mL^{-1}. The bacterial concentration was standardized to an ≈ 1 × 10^8 CFU mL^{-1} using the McFarland's standard (optical density of 0.1 at 625 nm). The positive control used in this study contained only MH broth medium with tested bacterial and the negative control was MH broth without molecules and without bacterial suspension. Finally, the plates were put in incubation during 24 h at 37°C. The MIC is the lowest concentration of antimicrobial agents that visually inhibits 99% growth of microorganisms. The MIC was noted by the visual turbidity of the tubes both before and after incubation and it was repeated 3 times for each bacterium.

RESULTS

Antimicrobial activity screening of ethanolic extracts, infusions and sub-extracts. Antimicrobial activity studies of extracts and sub-extracts and infusions showed high activity on Gram-positive bacteria (Table 1) compared with a weak activity on some Gram-negative and antimicrobial activity was not observed on *Candida* species and *A. baumanii*, *E. faecium*, *E. coli*, *L. monocytogenes*, *M. morganii*, *P. stuartii*, *P. aeruginosa*, *S.* Enteritidis, *S.* Typhi, and *S. sonnei*.

Thin-layer chromatography bioautography (TLC-B), determination of pure compounds and their antimicrobial activity. To identify of possible active compounds, bioautography method was performed on *B. cereus* (Figs. 1 and 2), *B. subtillis*, *S. aureus* (Fig. 3), *S. epidermidis* and *S. pyogenes.* And the antimicrobial activity of these determined pure compounds (esculetin, 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid, 19-hidroxy-cis-cleroda-3,13E-dien-15-oic acid, 9-p-coumaroyloxy-α-terpineol, aromadendrin-7- methyl ether and eriodictyol-7-methyl ether) is reported in Table 2 and the antimicrobial activity of esculetin, 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid and aromadendrin-7-methyl ether on *S. aureus* is show in the Fig. 4.

Minimum inhibitory concentration (MIC) of pure compounds. The MIC of the active compounds was determined using 2560, 1280, 640, 320, 160, 80, 40, 20, 10 and 5 µg mL^{-1} of esculetin, 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid and aromadendrin-7- methyl ether. The highest MIC was observed

Table 1. Activity of extract and sub-extract of "Bailahuén" on susceptible microorganisms.

Extracts/sub-extract	Susceptible microorganisms Diameter of inhibition zone (mm) ± SD					
	B. cereus	***B. subtilis***	***S. aureus***	***S. epidermidis***	***S. agalactiae***	***S. pyogenes***
Hm-E	8.6 ± 0.1	7.8 ± 0.2	9.3 ± 0.2	8.7 ± 0.2	-	-
Hm-E-C	8.1 ± 0.3	7.4 ± 0.2	12.1 ± 0.2	11.7 ± 0.2	-	-
Hm-E-F	9.2 ± 0.1	7.6 ± 0.2	18.5 ± 0.2	12.4 ± 0.5	-	7.8 ± 0.1
Hm-I	-	-	12.4 ± 0.1	6.8 ± 0.4	-	10.9 ± 0.5
Ht-E	15.2 ± 0.4	12.6 ± 0.1	7.9 ± 0.2	6.8 ± 0.1	7.4 ± 0.1	7.4 ± 0.2
Ht-E-T	13.1 ± 0.3	12.0 ± 0.5	-	-	-	-
Ht-E-F	7.9 ± 0.2	12.7 ± 0.2	-	-	-	-
Ht-I	12.2 ± 0.3	8.5 ± 0.3	7.2 ± 0.1	-	8.5 ± 0.3	12.1 ± 0.1

Values are the means ± SD from n=3 cultures.

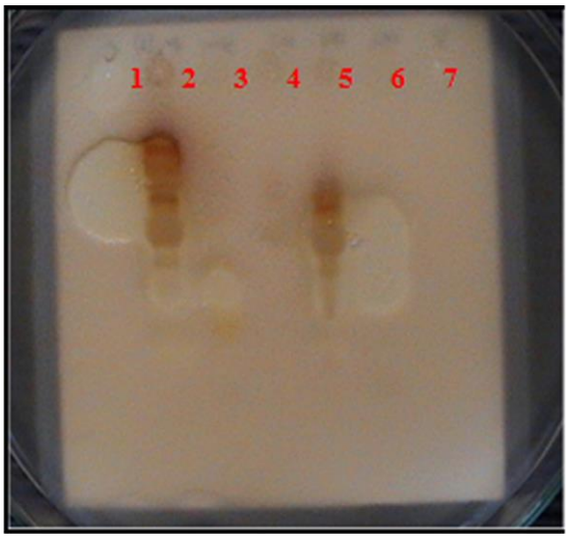

Fig. 1. Bioautography to *B. cereus* of all sub-extracts on a single plate: [1] C (Chloramphenicol); [2] Hm-E-F (Flavonoids fraction of ethanolic sub-extract of *H. taeda*; [3] Hm-E-C (Coumarins fraction of ethanolic sub-extract of *H. multifolius*); [4] Ht-I (Infusion of *H. taeda*); [5] Ht-E-F (Flavonoids fraction of ethanolic sub-extract of *H. taeda*); [6] Ht-E-T (Terpenoid fraction of ethanolic extract of *H. taeda*); [7] Negative control (consistent in solvent without sub-extract)

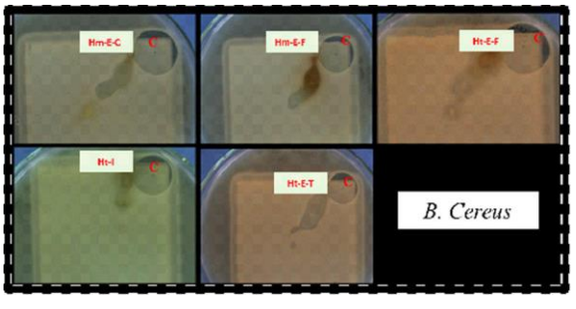

Fig. 2. Bioautography to *B. cereus* of all sub-extracts in different plates: C: Chloramphenicol; Hm-E-C: Coumarins fraction of ethanolic sub-extract of *H. multifolius*; Hm-E-F: Flavonoids fraction of ethanolic sub-extract of *H. taeda*; Ht-E-F: Flavonoids fraction of ethanolic sub-extract of *H. taeda*); Ht-I: Infusion of *H. taeda*; Ht-E-T: Terpenoid fraction of ethanolic extract of *H. taeda*)

with esculetin against *S. epidermidis.* The lowest concentrations were the clerodane and 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid on *B. cereus* and *S. aureus.* Of the three bacterial species, *B. cereus* was the most susceptible to the three bioactive compounds (Table 3).

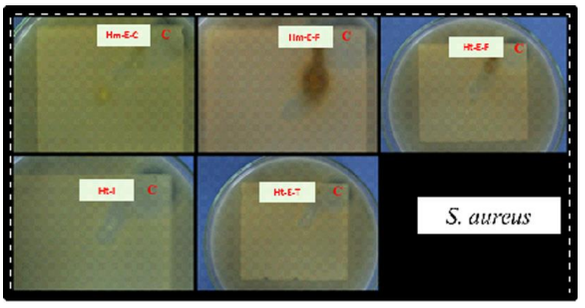

Fig. 3. Bioautography to *S. aureus* of all sub-extracts in different plates: C: Chloramphenicol; Hm-E-C: Coumarins fraction of ethanolic sub-extract of *H. multifolius*; Hm-E-F: Flavonoids fraction of ethanolic sub-extract of *H. taeda*; Ht-E-F: Flavonoids fraction of ethanolic sub-extract of *H. taeda*); Ht-I: Infusion of *H. taeda*; Ht-E-T: Terpenoid fraction of ethanolic extract of *H. taeda*)

DISCUSSION

Currently, there is a worldwide emergency regarding the high resistance of bacteria that are pathogenic to humans. The natural products are very important reservoir of compounds with antimicrobial activity. In Chile, several studies have been carried out in native plants in order to show their bioactive capacity on different types of microorganisms, thus, species of the family *Asteraceae* have been studied with respect to its antimicrobial potential (6, 7). However, the determination of the MICs of active pure compounds of *H. taeda* and *H. multifolius* had not been demonstrated.

In this work, extracts of *H. multifolius* and *H. taeda* were studied, from which their compounds were isolated according to the methodology described above. According to the results obtained it was determined that the greater antagonistic capacity was on Gram-positive bacteria, concordant with other studies (8, 9).

It was demonstrated that the Gram-negative bacteria present greater resistance, due to the complexity of their shell structures, particularly their double membrane. It is interesting to note that among the Gram-positives, *S. aureus* and *B. cereus* are two important contaminants of food and it can be considered that the products studied could have a potential role in the preservation of food products.

S. aureus causing various infections in humans is one of the bacteria with the highest levels of resistance worldwide. This study showed an interesting susceptibility response to the infusion and flavonoid

Table 2. Antimicrobial activity of sub-extracts and pure compounds

Sub-extracts	Pure compounds	Diameter of inhibition zone (mm) ± SD				
		B. cereus	*B. subtilis*	*S. aureus*	*S. epidermidis*	*S. pyogenes*
Hm-E-C		15.1 ± 0.3	11.5 ± 0.1	12.6 ± 0.3	9.3 ± 0.2	-
	Esculetin	11.4 ± 0.2	10.6 ± 0.2	7.5 ± 0.1	6.9 ± 0.1	6.2 ± 0.3
Hm-E-F		14.5 ± 0.1	8.2 ± 0.2	12.2 ± 0.9	22.4 ± 0.4	7.0 ± 0.2
Ht-E-T		14.7 ± 0.2	11.7 ± 0.1	10.1 ± 0.3	7.0 ± 0.1	-
	18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid	11.5 ± 0.1	8.5 ± 0.4	10.3 ± 0.3	7.2 ± 0.2	-
	19-hidroxy-cis-cleroda-3,13E-dien-15-oic acid	8.3 ± 0.2	-	7.3 ± 0.2	-	-
	9-p-coumaroyloxy-α-terpineol	17.3 ± 0.2	11.5 ± 0.5	13.0 ± 0.6	-	-
Ht-E-F		10.7 ± 0.2	10.7 ± 0.2	15.5 ± 0.1	12.3 ± 0.3	6.9 ± 0.1
	Aromadendrin-7- methyl ether	10.6 ± 0.2	9.9 ± 0.7	12.9 ± 0.2	7.7 ± 0.2	-
	Eriodictyol-7-methyl ether	8.7 ± 0.3	-	9.7 ± 0.1	6.1 ± 0.1	-
Control	Chloramphenicol	23.2 ± 0.2	23.1 ± 0.4	28.6 ± 0.1	23.4 ± 0.3	21.2 ± 0.5

Values are the means ± SD from n=3 cultures.

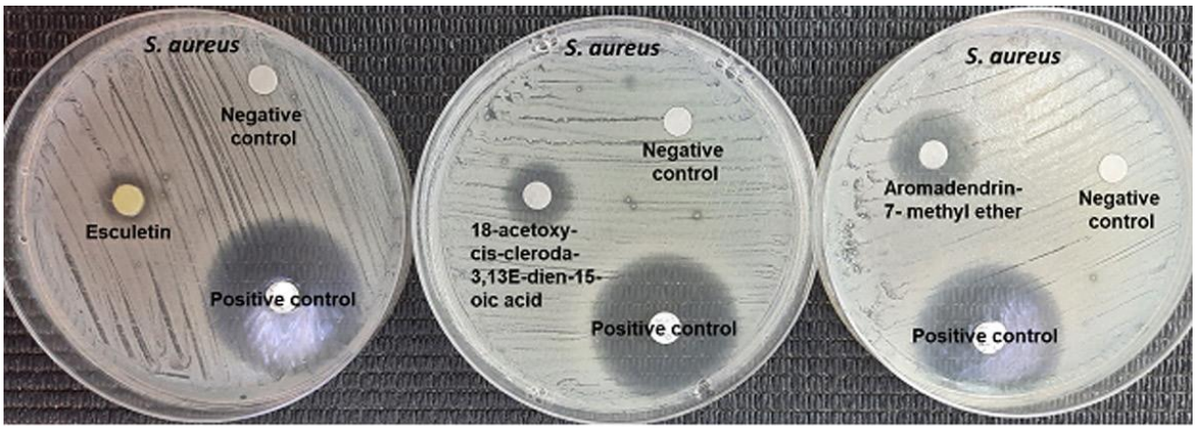

Fig. 4. Antimicrobial activity of pure compounds (esculetin, 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid and aromadendrin-7- methyl ether on *S. aureus*

Table 3. MIC of bioactive compounds of *H. multifolius* and *H. taeda*

Bioactive compounds	MIC, pure compounds (μg mL^{-1})		
	B. cereus	*S. aureus*	*S. epidermidis*
Esculetin	40	80	160
18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid	20	20	40
Aromadendrin-7- methyl ether	20	20	40

and coumarinic fractions of *H. multifolius* and the terpenoids and flavonoids fractions of *H. taeda*.

Regarding *H. multifolius*, it was determined that one of the major coumarin compounds, esculetin, has a high antimicrobial activity against *B. cereus, B. subtilis, S. aureus, S. epidermidis* and *S. pyogenes,* presenting a MIC of 40 and 80 μg mL^{-1} on *B. cereus* and *S. aureus* respectively.

The flavonoid, aromadendrin 7-methyl ether, presents a clear antagonistic activity against the studied bacteria, being its greater activity on *S. epidermidis* presenting a MIC of 40 μg mL^{-1}.

On the other hand, clerodane, 18-acetoxy-cis-cleroda-3,13-dien-15-oic acid, is the compound that has the greatest antagonistic activity on the studied bacteria, presenting a MIC of 20, 20 and 40 μg mL^{-1}

on *B. cereus, S. aureus* and *S. epidermidis.* As for the antimicrobial activity of this molecule, previous studies suggest that the activity of diterpenoids is due to their ability to cross or cause damage to cell membranes (14-16). Our results support the possible use of this plant for the treatment of infectious diseases in the traditional systems of medicines.

CONCLUSION

In this work it was possible to determine that certain natural products obtained from ethanolic extracts, infusions and sub-extracts of *H. multifolius* and *H. taeda* have antibacterial capacity on different species of Gram-positive pathogenic bacteria. The presence of bioactive compounds, esculetin in *H. multifolius,* 18-acetoxy-cis-cleroda-3,13E-dien-15-oic acid and aromadendrin-7- methyl ether in *H. taeda*, are some of the compounds responsible for their antimicrobial activity respectively.

In future studies it will be important to carry out similar studies but using a greater number of microorganisms of each bacterial species sensitive to the products studied, particularly on *Staphylococcus aureus* strains that currently have a high resistance capacity to different antimicrobials. The use of some of the products studied with lethal capacity on *S. aureus* would be a contribution to the global fight against antibacterial resistance.

ACKNOWLEDGEMENTS

V.C.S thanks ANID, Conicyt-Fondecyt project Nº 11181303. The authors are grateful for the collaboration of Dr. Francesca Faini in the chemical work.

REFERENCES

1. Gardam MA. Is methicillin-resistant *Staphylococcus aureus* an emerging community pathogen? A review of the literature. *Can J Infect Dis* 2000; 11: 202-211.
2. Alviano DS, Alviano CS. Plant extracts: search for new alternatives to treat microbial diseases. *Curr Pharm Biotechnol* 2009; 10: 106-121.
3. WHO (2002). Traditional Medicine Growing Needs and Potential - WHO Policy Perspectives on Medicines, No. 002, May, World Health Organization, Geneva, Switzerland.
4. Vogel H, González M, Faini F, Razmilic I, Rodríguez J, San Martín J, et al. Antioxidant propierties and TLC characterization of tour Chilean Haplopappus- species known as bailahuén. *J Ethnopharmacol* 2005; 97: 97-100.
5. Muñoz M, Barrera E, and Meza I (1981). El uso medicinal y alimenticio de plantas nativas y naturalizadas en Chile. Publicación Ocasional, 33, Museo Nacional de Historia Natural, Santiago.
6. Urzúa A, Torres R, Muñoz M, Palacios Y. Comparative antimicrobial study of the resinous exudates of some Chilean Haplopappus (Asteraceae). *J Ethnopharmacol* 1995; 45: 71-74.
7. Candan F, Unlu M, Tepe B, Daferera D, Polissiou M, Sokmen A, et al. Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium subsp. millefolium Afan*. (Asteraceae). *J Ethnopharmacol* 2003; 87: 215-220.
8. Murillo-Alvarez J, Franzblau S. Antimicrobial and cytotoxic activity of some medicinal plants from Baja California Sur (México). *Pharm. Biol* 2001; 39: 445-449.
9. Urzúa A, Torres R, Mendoza I, Delle Monache F. Antibacterial new clerodane diterpenes from the surface of *Haplopappus foliosus. Planta Med* 2003; 69: 675-677.
10. Chiang MT, Bittner M, Silva M, Mondaca A, Zemelman R, Sammes PG. A prenylated coumarin with antimicrobial activity from *Haplopappus multifolius. Phytochemistry* 1982; 21: 2753-2755.
11. Torres R, Faini F, Modak B, Urbina F, Labbe C, Guerrero J. Antioxidant activity of coumarins and flavonols from the resinous exudate of Haplopappus multifolius. *Phytochemistry* 2006; 67: 984-987.
12. Faini F, Labbé C, Torres R, Rodilla J, Silva L, Delle Monache F. New phenolic esters from the resinous exudate of *Haplopappus taeda*. *Fitoterapia* 2007; 78: 611-613.
13. CLSI (2015). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CLSI Document M100-S25. http://www.clsi.org/
14. Biswanath D, Reddy MR, Ramu R, Ravindranath N, Harish H, Ramakrishna KV, et al. Clerodane diterpenoids from *Pulicaria wightiana*. *Phytochemistry* 2005; 66:633-638.
15. Murthy MM, Subramanyam M, Bindu Hima M, Annapurna J. Antimicrobial activity of clerodane diterpenoids from *Polyathia longifolia* seeds. *Fitoterapia* 2005; 76: 336-339.
16. Urzúa A, Jara F, Tojo E, Wilkens M, Mendoza L, Rezende MC. A new antibacterial clerodane diterpenoid from the resinous exudates of *Haplopappus uncinatus. J Ethnopharmacol* 2006; 103: 297-301.