UNIVERSIDAD DE SANTIAGO DE CHILE

BOLETÍN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
17 (3): 270 - 285 (2018)
© / ISSN 0717 7917 / www.blacpma.usach.cl

Blacpma

Artículo Original | Original Article

Antimicrobial, antioxidant and phytochemical assessment of wild medicinal plants from Cordillera Blanca (Ancash, Peru)

[Evaluación antimicrobiana, antioxidante y fitoquímico de plantas medicinales silvestres de la Cordillera Blanca (Ancash, Perú)]

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Abstract: Twenty-eight native plants mainly used to cure diseases related to microbial infection and stress oxidative disorders were selected to test the antimicrobial activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *C. albicans* using diffusion and microdilution methods. The antioxidant activity was determined by scavenging DPPH free-radical and phytochemical evaluation was performed for plants with promising activities. Twenty-seven plants showed antibacterial activity, four had anti-Candida activity, and four showed antioxidant activity. It was found that *Oreocallis grandiflora*, *Gentianella weberbaueri*, *Gamochaeta americana*, *Hypericum laricifolium*, *Loricaria ferruginea*, *Muehlenbeckia volcanica*, and *Oenothera multicaulis*, showed promising biological activity and contained alkaloids, phenolic compounds, flavonoids, catecholic or gallic tannins. This study leaves evidence about the medicinal potential of wild high-Andean plants; thus, further pharmacological, phytochemical, ecological and biotechnological studies will contribute to promote their conservation and sustainable use; especially since they are highly vulnerable and risk extinction.

Keywords: Cordillera Blanca; Andean plant; antibacterial; anti-Candida; antioxidant; phytochemical assay.

Resumen: Se seleccionó veintiocho plantas nativas usadas principalmente para tratarcurar enfermedades relacionadas principalmente con infecciones microbianas y desordenes oxidativos. A estas plantas se para ser evaluóados en su actividad antimicrobiana sobre *E. coli*, *P. auriginosa*, *S. aureus*, *B. subtilis*, y *C. albicans* usando métodos de difusión y microdilución. Se determinó la actividad antioxidante mediante el ensayo del libre radical DPPH y se realizó la evaluación fitoquímica de las plantas con actividades promisorias. Veinte siete plantas mostraron actividad antibacteriana, cuatro mostraron actividad anti-Candida, y cuatro actividad antioxidante. *Oreocallis grandiflora*, *Gentianella weberbaueri*, *Gamochaeta americana*, *Hypericum laricifolium*, *Loricaria ferruginea*, *Muehlenbeckia volcanica*, y *Oenothera multicaulis* mostraron actividad biológica promisoria, y se encontró que contienen alcaloides, compuestos fenólicos, flavonoides, taninos gálicos y catecólicos. Este estudio deja evidencia del potencial medicinal de las plantas silvestres alto andinas; por lo tanto, los estudios farmacológicos, fitoquímicos, ecológicos y biotecnológicos contribuirían en la promoción de su conservación y uso sustentable debido a su alta vulnerabilidad y riesgo de extinción.

Palabras clave: Cordillera Blanca; planta Andina; antibacteriano; anti-Candida; antioxidante; ensayo fitoquímico.

Recibido | Received: August 6, 2017
Aceptado | Accepted: January 14, 2018
Aceptado en versión corregida | Accepted in revised form: April 12, 2018
Publicado en línea | Published online: May 31, 2018
Declaración de intereses | Declaration of interests: this research was financially supported by the Dirección General de Investigación (DGI) of the Universidad Nacional Santiago Antúnez de Mayolo (UNASAM-Perú) as a component of the project "Caracterización biológica, fitoquímica y molecular de plantas Peruanas altoandinas con potencial en la industria farmacológica"
Este artículo puede ser citado como / This article must be cited as: C Tamariz-Angeles, P Olivera-Gonzales, M Santillán-Torres2. 2018. Antimicrobial, antioxidant and phytochemical assessment of wild medicinal plants from Cordillera Blanca (Ancash, Peru). **Bol Latinoam Caribe Plant Med Aromat** 17 (3): 270 – 285.

INTRODUCTION

Facing an increased demand for new bioactive principles to cure new and emergent diseases secondary metabolites from plants are being explored continuously. Nowadays pathogen microorganisms have the ability to develop resistance to conventional antibiotics (Abedini *et al*., 2014; Guil-Guerrero *et al*., 2016), therefore, new antimicrobial compounds become necessary to fight the prominent microbial infections (Aumeeruddy-Elalfi *et al.*, 2016); which can be achieved by focusing in plants used in traditional medicine (Aumeeruddy-Elalfi *et al*., 2016; Gupta *et al*., 2016; Chander *et al*., 2016). It seems that plants have different mechanisms for microbial growth inhibition compared to microbial or synthetic origin antibiotics, furthermore it seems that pathogen microorganisms do not develop resistance to antimicrobial compounds from plants (Abedini *et al.*, 2014).

Likewise, within the cells oxidative stress generates free radicals (ROS) that degrade proteins, lipids and DNA (Jayathilake *et al.* 2016); this damage is associated with neurodegenerative and coronary diseases, diabetes, atherosclerosis, inflammation, digestive system disorders, viral infections, and cancer (Tauchen *et al.*, 2016; Al-Jaber *et al*., 2011; Jayathilake *et al*., 2016). Cells produce antioxidant substances for its self-protection, but when this activity is low or the oxidative stress increases, it is recommended the consumption of antioxidant substances commonly provided by food and medicinal plants (Jayathilake *et al.*, 2016); usually these plant antioxidant metabolites are polyphenols (Al-Jaber *et al.*, 2011).

Therefore, the knowledge of medicinal plants as a source of bioactive principles provides great opportunities for the development of new medicines (Barboza *et al*., 2009; Rehecho *et al.*, 2011; Chander *et al*., 2016). Ethnopharmacology studies in Andean zones report a wide diversity of plants used to treat different ailments (Rojas *et al*., 2003; De-la-Cruz *et al.*, 2007; Bussmann *et. al.*, 2010; Rehecho *et al*., 2011; Gonzales De La Cruz *et al.*, 2014), including some diseases related to microbial infections and oxidative stress.

The Cordillera Blanca in Ancash has a representative biodiversity of high-Andean Peruvian ecosystems from which wild medicinal plants are extracted and marketed. Most of them are herbaceous and are collected with roots and flowers generating risks of extinction by over exploitation such as *Muehlenbeckia volcanica*, *Perezia coerulescens*, *Perezia multiflora*, *Perezia pinnatifida*, *Senecio canescens*, *Senecio rhizomatus*, *Stangea sp.* (De-la-Cruz *et al*., 2007; Gonzales De La Cruz *et al*., 2014). In addition, the climate change is making the high-Andean biodiversity conservation problem bigger, because it increases the vulnerability and irreplaceability of the biodiversity of the high-Andean ecosystem represented by small populations of endemic species (Ramirez-Villegas *et al.*, 2014); therefore conservation effort should take place urgently in order to protect most vulnerable species (Gonzales De la Cruz *et al.*, 2014). Some studies to perform *in vitro* culture techniques to protect these genetic resources were described only to *Perezia coerulescens* (Olivera *et al.*, 2010), *O. grandiflora* (Olivera-Gonzales *et al.*, 2017a), and *P. pinnatifida* (Olivera-Gonzales *et al.*, 2017b); but in this panorama, studies of their pharmacological activities, phytochemical composition, and even less genetic authentification are not considered as a urgent topic to study.

Hence, this research is focused in medicinal wild plants from the Ancash high Andean zone. The antimicrobial activity was evaluated against four bacteria and one yeast involved in common infections illnesses such as respiratory, skin, urinary, stomach, etc.. Their antioxidant activity was also screened, and phytochemical studies began. The results support other studies, which aim to promote biotechnological applications for the conservation and sustainable use of these threatened plants.

MATERIALS AND METHODS

Plant material

Some wild plants mostly used to treat diseases related to microbial infections were selected. Most were collected from or around the Huascaran National Park located in Cordillera Blanca (Ancash-Peru); and a few were bought at popular market from Huaraz. Other criteria of selection were vulnerability, risk of extinction and/or endemism. The taxonomic identification and storage of herbarium specimens (samples with flowers) were made at the David Smith Herbarium of the Universidad Nacional Santiago Antúnez de Mayolo **Http://grbio.org/institution/uiversidad-nacional-santiago-antunez-de-mayolo)** In addition, little piece of dried leaves were stored at -70° C for future DNA barcoding preparation.

Plant extracts preparation

Plant extract preparation was performed according

Martin *et al*. (1965) and Cáceres (1996). The plants were washed with tap water and dried under fresh air and shade. The dried samples were milled and sieved up to 2 mm. After that, they were macerated with ethanol 50º (10:90 w/v) under darkness and room temperature (18-22° C) for 12 days to attain maximum extraction. The samples were filtered on filter paper followed by sterilization using 0.2 µm millipore filter, and the extracts were concentrated using rotavapor (Buchi, Switzerland) up to 40° C, and left at 4° C for 10 days to achieve complete dryness.

Dry extracts were dissolved at 100 mg·mL^{-1} using DMSO and water in the relation 1:24. These stock extract dilution were used in antimicrobial and antioxidant assays immediately or were stored at 4 °C for 14 days as a maximum. These stocks were diluted in culture medium (antimicrobial microdilution assay) or methanol (antioxidant assay). For the antimicrobial diffusion method, in order to standardize amount (mg) of extract by filter paper disk and considering that they are crude extract. Filter paper disks impregnated with 2 mg of plant extract were prepared in the following way: 20 µL (4 µL x 5 times) of extracts were soaked in sterile filter paper disks of 5 mm and dried in a sterile chamber for 1 hour. The amount of tetracycline, streptomycin or nystatin by each filter paper disk was 0.2 or 1.6 mg respectively, since they showed good inhibition halos between 10 - 25 mm against the strains tested.

Antibacterial activity

E. coli ATTC25922, *S. aureus* ATCC25923, *B. subtilis* ATCC11774, and *P. aeruginosa* ATCC27853 were tested by diffusion and microdilution methods according to M02-A7 and M07-A8 protocols of the Clinical and Laboratory Standards Institute (CLSI).

In both methods, the initial inoculum (IIB) was made with two colonies from a 14 hours grown plate diluted in NaCl 0.8% until 0.08 - 0.1 OD_{620}. Streptomycin (Sigma) and tetracycline (Sigma) were used as standard antibiotics. Three replicates per test were performed, and the reproducibility was evaluated two times.

Antibacterial diffusion assay

The IIB was done by swabbing it twice onto 12 x 12 cm Petri dishes (Sigma) containing 40 mL of Müller and Hinton Agar (MHA, Merck), and immediately filter paper disks soaked with a sample or standard antimicrobial solutions were placed on the inoculated Petri dishes. The sample disk contained 2 mg of sample or 0.2 mg of standard antibiotic. Plates were incubated for 24 hours at 35° C. The inhibition halos were measured subtracting the disk diameter; then values ≥ 2 were considered as inhibition.

Antibacterial minimum inhibitory concentration (MIC) using tetrazolium salt

Extracts were serial diluted in Müller and Hinton Broth II (MHBII, Difco) at 0.10, 0.25, 0.50, 0.75, 1.00 to 10.00 mg·mL^{-1} (increasing 0.50). The serial dilutions were dispensed into 96-well plates (100 µL per well) and were inoculated with 10µL of IIBd (IIB diluted in MHBII 1:20). Controls of growth (MHBII with inoculum) and contamination (MHBII or extracts without inoculum) were prepared. Plates were incubated for 24 hours at 35° C. Tetrazolium salt was used as growth indicator *(Ncube et al*., 2008; Klančnik *et al*., 2010); adding 10 µL of tetrazolium violet 0.1% (TV, Sigma T0138) to each well and incubated for 4 hours at 35° C under dark. TV is reduced to violet precipitated, in presence of respiratory activity. MICs were considered like the lowest concentrations of extracts that inhibited the bacterial growth that was noticed by the absence of violet precipitated.

Anti-Candida activity

The susceptibility of *C. albicans* ATCC90028 was evaluated by diffusion and microdilution methods according to M44-A and M27-2A (CLSI) protocols with few modifications. Nystatin (commercial suspension) was used as an antifungal standard. Three replicates per test were performed and the reproducibility was evaluated two times. Preparation of initial inoculum (IIY) was similar to antibacterial assay.

Anti-Candida diffusion assay

IIY was done by swabbing it twice onto 12 x 12 cm Petri dishes with TSA and continued as described previously for the Antibacterial diffusion assay. Incubation was for 48 hours. The sample disk contained 2.0 mg, and the nystatin disk had 1.6 mg.

Anti-Candida MIC using tetrazolium salt

Extracts were serial diluted in TSB at 0.2, 0.5, 1.0, 1.5, 2.0 to 20.0 mg·mL^{-1} (increasing 1.0). The serial dilutions were dispensed into 96-well plates (100 µL per well) and inoculated with 100 µL of IIYd (IIY diluted in TSB 1:5). Controls of growth and contamination were prepared. Plates were incubated

at 35° C for 48 hours, and MICs were obtained as described in Antibacterial minimum inhibitory concentration (MIC) assay.

Antioxidant activity tested by DPPH scavenging method

Microdilution method described by Guaratini *et al.* (2012) was used, and ascorbic acid was used as a standard. Extracts (100 $mg \cdot L^{-1}$) were serially diluted with methanol at 25 to 2000 $\mu g \cdot mL^{-1}$. 100 µl of each dilution was dispensed into six wells; immediately 10 µL of fresh DPPH (10 $mg \cdot L^{-1}$ dissolved in MeOH) was added into three well, and 10 µL of MeOH into the other three. Plates were incubated at 25° C in darkness for 30 min. After, the absorbance at 517 nm was measured twice in a microplate spectrophotometer (BioTek, USA). The percentage of radical inhibition per sample was calculated as *% I = [DPPHA - (MA-BMA)/DPPHA] x 100*. Where *DPPHA* is the DPPH absorbance, *MA* is the absorbance of the sample plus DPPH, *BMA* is the absorbance of the sample without DPPH.

The antioxidant activity was expressed as IC_{50}, which represent the extract concentration to inhibit 50% of DPPH radical (Lee *et al.*, 2003), and it is calculated from the regression curve I% *versus* extract concentration.

Phytochemical evaluation

Thin layer chromatography (TLC) and method proposed by Lock (1994) were used for phytochemical evaluation of samples leaf extracts that gave the best results. The evaluation was repeated twice and 250 mL of ethanol extracts prepared as previously described, were used.

In the first method, 20 mL of ethanol extract were separated (fraction A), and 230 mL were dried and fractionated in 4 fractions (B, C, D, E) considering pH, solubility, and polarity (Lock, 1994). The presence of phenols, alkaloids, amino acids, tannins, triterpenes and/or steroids, leucoanthocyanidins, and quinones was evaluated with color reactions.

TLC was developed on silica gel 60 F_{254}, Aluminum sheets (Merck), with BAW (n-butanol-acetic acid-water) at ratios (3:1:3), (4:1:1), (4:1:3) and (4:1:5). For the phenolic groups, the chromate plates were evaluated without or with $FeCl_3$ (1%) or H_2SO_4 (1%) and were observed using white light, UV_{254}, and UV_{365}; the retention factor (Rf) of the separated compounds were calculated, color and fluorescence were observed and compared with literature (Wagner & Bladt, 1966; Harborne, 1984; Lock, 1994; Galand *et al.*, 2002). Similarly, alkaloids were studied using Dragendorff reagent.

Statistical analysis

The antimicrobial and antioxidant assays were performed using 6 replicates, and phytochemical screening had 2 replicates. The results were expressed as values, and standard deviations were calculated. ANOVA and Duncan's test were used to compare the values of antimicrobial diffusion assay and antioxidant DPPH scavenging method.

Table No. 1
Taxonomic, ethnopharmacology, market and endemism information of selected plants

N°	Scientific name, family, common names*/used part in popular medicine*	Traditional medicinal use*	CS**		E*
			M	P	
1	*Alonsoa linearis* (Jacq.) Ruiz & Pav. Scrophulariacea rirkacock, shoqumpa wëta, aturash, flor de muerto, pillwi pillwi, rinriqora, chanllillí ϕ/whole plant	To cure stomach pain. It is used with other plants to take baths against cold. (5, 6)	x	a	
2	*Baccharis genistelloides* Pers. Asteraceae carqueja, kima esquina, cuchu-cuchu/stem	To cure urinary, liver, and kidney problems; to treat malaria, rheumatism, diabetes, and cholesterol. Diuretic, antidiarrheal, and blood purification.(5, 6, 7)	x	a	
3	*Berberis lutea* Ruiz & Pavon Berberidaceae chekci, qarwash casha, qontsi, qarwa quinche, carhuascasa/fruit, root, leaf, stems	As anti-inflammatory during respiratory tract, and muscle pains. It is used as dye: the fruit (color blue) and roots (color yellow). (4,6)			
4	*Buddleja incana* Ruiz & Pav. Scrophulariacea	To treat the Carrion disease (an endemic disease caused by *Bartonella bacilliformis*), it is an astringent	x	a	

	quisuar, quishuar/leaf	to treat wart and to wash open sore. Leaf and barks are rubbed on genitals in order to prevent postpartum infections. It is used to take baths against cold. (4,5,6)			
5	*Gamochaeta americana* (Mill) Wedd. Asteraceae alkupa allung, lengua de perro/leaf, stem, flowers	Against cold, conjunctivitis, and diarrhea. (5, 10)	x	w	
6	*Gentianella weberbaueri* (Gild) Fabris Gentianacea puca makashqa, puca shaqwa, puca shaqapa, antenaria /whole plant	To prevent cavities. The flowers are collected to decorate religious statues and the boiled extract is used as red coloring. (6)		f$^{\Psi}$	y
7	*Hypericum laricifolium* Juss. Hypericaceae chinchango, cypres, romero de la altura/stem, leaf, flower	To cure warts, cold, chills, tiredness of all the body. Antidepressant. (5,6)	x	a	
8	*Jungia paniculata* (DC.) A. Gray Asteraceae matico, qaramati, caramate/leaf	For wound healing, injuries, stomach ulcers, nephritis, hemorrhoids, vaginitis, urinary tract illness and hurts. Antiseptic or antibiotic, and anti-inflammatory. (4, 6, 8, 9)	x	a	
9	*Loricaria ferruginea* (Ruiz & Pav.) Wedd. Asteraceae wallpapa chaquin, pata de gallo/stem	To alleviate menstrual delay, and for blood circulation. Steam mixed up with other plants is used for bath to cold. (2, 5, 6)	x	a	
10	*Lupinus weberbaueri* Lubr. Fabacea taulli macho, tararwa /leaf	For stomach problems or ulcers, diabetes. The flowers are collected to decorate religious statues. (5)		f$^{\Psi}$	
11	*Muehlenbeckia volcanica* (Benth) Endl. Polygonaceae mullaca , mullak´a, viruta, mullaka/whole plant, fruit	For diarrhea, flu, asthma, other bronchia disorders, stomach pain and "internal wounds" with hemorrhage. Antipyretic, antitussive, analgesic and antiseptic to treat throat infections. (4,5, 8)	x	w	
12	*Notrotice obtusa* A. W. Hill Malvacea raíz de alte, qori waqaq, shiric-yalckoy, tupucpa-ashuan/leaves, stem and flower	Leaves and flowers of *Notrotiche sp.* are used to make a tea to treat colic. (6) To kidney problems$^{\Phi}$.	x	w	y
13	*Oenothera multicaulis* Ruiz & Pavon Onagraceae chupasangre,tullpish$^{\Phi}$/leaf	Wound healing, and antiseptic. (9)	x	w	
14	*Oreocallis grandiflora* (Lam.) R. Br. Proteacea tzaq'pa, chacpá, cucharillo/flowers, leaf, stem	To alleviate flu and strong sensation of cold, cough, pain after hard work in the sun, headache, diabetes, and fever. To cure liver and kidney problems. (5,10)	x	f	
15	*Peperomia hartwegiana* Miq. Piperacea congona redonda $^{\Phi}$, congona, winayquilla, congona /stem, leaf	To cure gingivitis and otitis; lung, liver and kidney diseases, stomach ulcers, bruises. (5, 6)	x	a	
16	*Perezia coerulescens* Wedd. Asteraceae valeriana, patza maki/root, rhizome	To alleviate heart pain. Sedative, diuretic, and diaphoretic. (4, 5)	x	w	
17	*Perezia multiflora* (Bonpl.) Less Asteraceae escorzunera, chancoruma/leaf	Diuretic, febrifuge, sudorific, expectorant, antitussive, analgesic, anti-inflammatory. To treat back ache, stomach ache, flu, cough, bronchitis, tuberculosis, and diarrhea. To cicatrize teeth and throat injuries. (1, 4, 5, 6, 7)	x	w	
18	*Perezia pinnatifida* (Bonpl.) Wedd. Asteraceae valeriana, contrahierba, valeriana fina/leaf, root, rhizome	To treat asthmatic cough, headache, and heart disorders. Central nervous system stimulant, antitussive, sedative, tonic, tranquilizer. (4, 6)	x	w	
19	*Senecio calvus* Cuatrec. Asteraceae Huamamrripa, vira vira/leaf	For cough, respiratory sickness, and it is used in bath against cold. (4, 5)	x	w	y
20	*Senecio canescens* (Bonpl.) Cuatr.	Sudorific, expectorant, antipyretic. To treat bronchia	x	w	

	Asteraceae ancosh, anqush, wila-wila, oreja de venado, utco, oreja de conejo/leaf	disorders, flu, asthma, cough and cold. (2, 4, 6, 7, 8)			
21	*Senecio leucophorbius* Cuatr. Asteraceae	Used as *S. canescens* because it have similar morphology. (1)			y
22	*Senecio rhizomatus* Rusby Asteraceae llancahuasΦ, llancahuasa/leaf	For liver colic, and kidney infection. (4)	x	w	
23	*Senecio serratifolius* (Meyen & Wapl.) Cuatrec. Asteraceae wamanripa /leaf, flowers	For pneumonia. (6)			
24	*Valeriana henrici* (Graebn.) B. Eriksen Caprifoliaceae corehuajay, qallu-qallu, llingli-llingli, rosetón/whole plant	To alleviate heart pain, "mal aire", and nauseas. (5,6)	x	w	
25	*Valeriana aff. pycnantha* A. Gray. Caprifoliaceae Siete sabiosΦ/whole plant	To alleviate heart pain Φ	x	w	
26	*Valeriana globularis* A. Gray. Caprifoliaceae Siete sabios/whole plant	Its roots are eaten. (6) To alleviate heart painΦ.	x	w	
27	*Werneria nubigena* Kunth. Asteraceae yekishkoda, patzamaki/leaf, root	For cold, and diarrhea. (5)			
28	*Xenophyllum dactylophyllum* (Sch. Bip.) V.A. Funk Asteraceae cuncush, peqa-peqa, botón-botón/stem	For head ache. It is used with other plants. (6)	x	a	

***, it was done with literature references: (1) Beltrán *et al*. (2006), (2) Bussman *et al*. (2010), (3) Ccana-Ccapatinta *et al*. (2014), (4) De-la-Cruz *et al*. (2007), (5) Gonzales De La Cruz *et al*. (2014), (6) Kolff & Kolff (2005), (7) Monigatti *et al*. (2013), (8) Rehecho *et al*. (2011), (9) Rojas *et al*. (2003), (10) Tene *et al*. (2007).**
ΦAuthor observation from Huaraz traditional markets.
****, commercial status (CS) observed in traditional markets from Huaraz. M, observed in traditional markets from Huaraz: x, presence in traditional markets. P, part of the plant collected and marketed to diverse use: a, aerial part containing stem, leaves and flowers; f, only flower; w, whole plant; Ψ, collected but not marketed. E, endemic from Peru: y, positive answer.**

Table No. 2
Collection data of specimen plants selected

N°	Species	Sample ID	Collection Date	Collection place			
				Sector	Latitude	Longitude	Elevation
1	*Alonsoa linearis*	UNASAM-HDS-127	14/06/14	Qda. Quillcayhuanca	-9.50447	-77.44772	3850m
2	*Baccharis genistelloides*	UNASAM-HDS-104	10/06/13	Qda. Huaripampa	-8.95068	-77.56283	3751 m
3	*Berberis lutea*	UNASAM-HDS-118	29/09/13	Qda. Llanganuco	-9.04832	-77.60837	3953 m
4	*Buddleja incana*	UNASAM-HDS-128	22/09/13	Qda. Shallap	-9.50106	-77.36886	4137 m
5	*Gamochaeta americana*	UNASAM-HDS-105	14/05/15	Marketed			
6	*Gentianella weberbaueri*	UNASAM-HDS-121	23/01/13	Qda. Churup	-9.47707	-77.42478	4570 m
7	*Hypericum laricifolium*	UNASAM-HDS-119	15/06/14	Qda. Quillcayhuanca	-9.50604	-77.44159	3861m
8	*Jungia paniculata*	UNASAM-HDS-106	24/06/13	Road to Pitec, Huascaran National Park	-9.51771	-77.48164	3392m

9	*Loricaria ferruginea*	UNASAM -HDS-107	29/09/13	Portachuelo from Llanganuco	-9.04041	-77.57577	4403 m
10	*Lupinus weberbaueri*	UNASAM -HDS-120	22/09/13	Qda. Shallap	-9.49495	-77.36011	4246 m
11	*Muehlenbeckia volcanica*	UNASAM -HDS-125	29/09/13	Portachuelo from Llanganuco	-9.04492	-77.59792	4443 m
12	*Nototriche obtusa*	UNASAM -HDS-122	10/06/13	Punta Unión, between Qda. Huaripampa-Santa Cruz	-8.91228	-77.58159	4735 m
13	*Oenothera multicaulis*	UNASAM -HDS-123	12/01/15	Aquia	-10.0759	-77.14399	3409m
14	*Oreocallis grandiflora*	UNASAM -HDS-126	30/09/13	Qda. Llanganuco	-9.10854	-77.68562	3420m
15	*Peperomia hartwegiana*	UNASAM -HDS-124	22/09/13	Qda. Shallap	-9.50209	-77.37635	4128 m
16	*Perezia coerulescens*	UNASAM -HDS-108	13/12/13	Qda. Churup	-9.47619	-77.42422	4695 m
17	*Perezia multiflora*	UNASAM -HDS-109	11/06/13	Path to Alpamayo, Qda. Santa Cruz	-8.90928	-77.62427	4201 m
18	*Perezia pinnatifida*	UNASAM -HDS-110	24/04/14	Marketed			
19	*Senecio calvus*	UNASAM -HDS-111	29/09/13	Portachuelo from Llanganuco	-9.04641	-77.59298	4597 m
20	*Senecio canescens*	UNASAM -HDS-112	10/06/13	Punta Unión, between Qda. Huaripampa-Santa Cruz	-8.91228	-77.58159	4735 m
21	*Senecio leucophorbius*	UNASAM -HDS-113	11/06/13	Path to Alpamayo, Qda. Santa Cruz	-8.89591	-77.63219	4293 m
22	*Senecio rhizomatus*	UNASAM -HDS-114	29/09/13	Portachuelo from Llanganuco	-9.04041	-77.57577	4403 m
23	*Senecio serratifolius*	UNASAM -HDS-115	05/01/13	Qda. Ulta	-9.12989	-77.51445	4864 m
24	*Valeriana aff. pycnantha*	UNASAM -HDS-130	13/12/13	Qda. Churup	-9.47650	-77.42436	4680 m
25	*Valeriana globularis*	UNASAM -HDS-131	27/05/16	Marketed			
26	*Valeriana henrici*	UNASAM -HDS-129	27/01/16	Marketed			
27	*Werneria nubigena*	UNASAM -HDS-117	10/06/13	Qda. Huaripampa	-8.95067	-77.56283	3751 m
28	*Xenophyllum dactylophyllum*	UNASAM -HDS-116	10/06/13	Punta Unión, between Qda. Huaripampa-Santa Cruz	-8.91228	-77.58159	4735 m

Qda. quebrada. Collectors were C.Tamariz & P. Olivera

RESULTS

Plant material

According to the ethnopharmacology information twenty-eight plants of twelve families were selected. Then, twenty four were collected and four were bought at Challhua, a popular market from Huaraz (Tables No. 1 & 2 and Figure No. 1). Most species are used for flu, some for urinary diseases, dental cleaning, wounds or stomach pain. A few are used for diseases related to oxidative stress such as diabetes, heart pain or nerves. From observation in traditional markets from Huaraz, thirteen species are marketed as whole plant, from which *P. coerulescens*, *P. pinnatifida*, *N. obtusa*, *V. henrici*, *V. aff. pycnantha* are considered highly vulnerable because they are collected with lots of flowers and sailwomen refer that they are collected from places near the glaciers. About endemisms, four plants are endemic (Table No. 1). Additional, the flowers of *G. weberbaueri*, *L. weberbaueri*, and *O. grandiflora* are used as ornamental. This last one is marketed, and other two are used to decorate statues in Holy week.

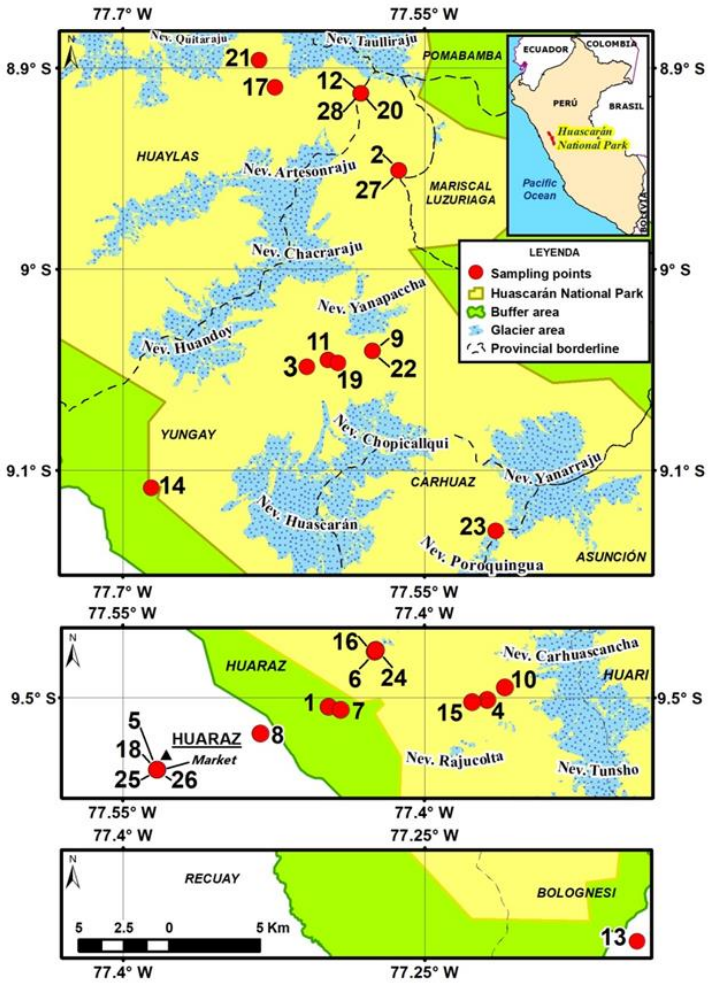

Figure No. 1
Map of the collection area. Numbers are the samples are in the Table No. 2

Antibacterial activity

The size of the clear zone of growth inhibition (halo) and MICs are shown in Table No. 3. Two plants (*M. volcanica* and *O. grandiflora*) showed activity against the four bacteria tested, three against three bacteria (*H. laricifolium*, *L. ferruginea*, and *O. multicaulis*), 17 plants against two bacteria, three against one bacterium tested, and only one plant did not show activity.

In the diffusion test against *S. aureus*, the statistic test showed that 2 mg·L^{-1} ethanolic extracts activity of *B. incana*, *G. americana*, *A. linearis*, *O. multicaulis*, *O. grandiflora* did not have a significant difference with 0.2 mg·L^{-1} streptomycin, it means that these extracts have the same activity that streptomicin at the condition descripted. In this way, the activity of *P. hartweguiana* (2 mg·L^{-1}) against *B. subtilis* did not show a statistic difference with tetracycline (0.2 mg·L^{-1}), this activity was followed by *S. rhizomatus*, *O. multicaulis*, *L. ferruginea*, and others.

It was found that positive results in the diffusion test were also positive in the dilution method with exception of a few extracts (7, 11, 14), and this could be related to the bioactive metabolite solubility involved in the antibacterial effect. Then, considering the two methods, three (9, 11, 14) and four (7, 11, 13, 14) plants had antibacterial activities against *E. coli*, and *P. aeruginosa* respectively. In the disk diffusion assay against *E. coli* only two (9, 14) extracts were positive, but their activities was low compared to standard antibiotics used. Interestingly, 2 mg·L^{-1} of *O. grandiflora* extract showed better inhibition halo than 0.2 mg·L^{-1} tetracycline against *P. aeruginosa*.

Finally, according to diffusion assay, low MICs and/or a broad spectrum of antibacterial activity were found for *H. laricifolium*, *L. ferruginea*, *O. grandiflora*, *M. volcanica*, and *O. multicaulis*.

Anti-Candida activity

The antifungal activity against *C. albicans* was uncommon in the plants tested; only *G. weberbaueri*, *L. ferruginea*, and *O. multicaulis* showed anti-*Candida* activities using the dilution and diffusion methods, while *X. dactylophyllum* had a slightly positive activity with the diffusion method (Table No. 3).

Antioxidant activity

Table No. 3 shows the IC_{50} of antioxidant activity using DPPH scavenging assay, values and the statistic test was made only to samples which showed IC_{50} less than 300 $\mu g \cdot L^{-1}$; values $\geq$ 300 $\mu g \cdot L^{-1}$ are not reported. The lowest IC_{50} values were found in leaves and stems of *H. laricifolium* (55.6 ± 2.4 and 62.3 ± 2.0 $\mu g \cdot L^{-1}$), and leaves of *G. americana* (65.4 ± 12.1 $\mu g \cdot L^{-1}$); followed for *M. volcanica* (98.7 ± 3.1 $\mu g \cdot L^{-1}$) and leaves of *O. grandiflora* (113.5 ± 4.4 $\mu g \cdot L^{-1}$). These plants are considered promissory to continue research.

Phytochemical evaluation

In concordance to the antimicrobial and antioxidant results, *O. grandiflora, H. laricifolium, G. weberbaueri, G. americana, L. ferruginea, M. volcanica, and O. multicaulis* were selected for phytochemical evaluation. Following Lock (1994) method, the tannins were present in the majority of plants, flavonoids and leucoanthocyanidins were also abundant. Alkaloids were found in five plants, triterpenes and/or steroids in four, amino acids in three, and quinones only in one (Table No. 4). In the TLC method, BAW (4:1:5) was the best mobile phase and was selected to all assays. According to the different retention factor (Rf), color and fluorescence of the different TLC treatments with or without $FeCl_3$ or H_2SO_4 for phenolic compounds and Dragendorf for alkaloids; it was possible to differentiate some types of tannins, flavonoids, alkaloids and other phenolic compounds for each sample (Table No. 5). Both methods assayed were complementary, and did not show contradictory results.

DISCUSION

Most of plants selected (96%) showed antibacterial activity at least against one bacterial strain tested supporting the importance and usefulness of the ethnobotanical information about plants that cure infection during research of new antimicrobial compounds. The anti-Candida activity is consider as an urgent need to explore in active constituents of traditional medicine, because candidiasis of the skin, nails, oral cavity, esophagus and vagina is the most prevalent fungal infection; (Tangarife-Castaño *et al.*, 2011; Zida *et al.*, 2017) and four plants (14%) showed this activity; correlation with their traditional uses are discussed below. On the other hand, four species (5, 7, 11, 14) showed promising antioxidant activity; this activity could be helpful in the case of diseases caused by oxidative stress such as cancer, diabetes, and neurodegenerative problems (Tauchen *et al.*, 2016) but other assays to confirm this activity could be necessary. Although most of the plants showed antimicrobial activities, the discussion is focusing mainly in the plants with promising results.

Table No. 3
Antimicrobial and antioxidant activities of ethanolic extracts of medicinal plants

N°	Species	PE	Antimicrobial activity Diameter inhibition halo* mm, (MIC mg·mL^{-1})					DPPH activity IC_{50} $\mu g \cdot L^{-1}$
			Sa	*Bs*	*Ec*	*Pa*	*Ca*	
1	*A. linearis*	l	15.5 ± 2.1^{bcd} (2.5)	5.5±0.7^{ef} (2.5)				274.2 ± 17.0^{g}
		s	8.5±0.7^{gh} (3.0)	4.5±2.1^{efgh} (3.0)				
2	*B. genistelloides*	s	4.5±0.7^{ijkl} (4.0)	3.5±0.7^{efgh} (4.0)				
3	*B. lutea*	l	7.0±1.4^{hi} (7.5)	3.5±0.7^{efgh} (5.0)				202.2 ± 8.5^{d}
4	*B. incana*	l	16.5±0.7^{bc} (3.0)					

5	*G. americana*	l	15.5±0.7^{bcd} (2.5)	2.5±0.7^{gh} (3.5)				65.4 ± 12.1^{b}
6	*G. weberbaueri*	l	6.0±1.4^{hijk} (3.0)	4.0±1.4^{efgh} (3.0)			3.0±0.0^{d} (8.0)	236.4 ± 1.9f^{g}
		r	6.5±0.7^{hij} (2,5)	4.5±2.1^{efgh} (3.0)			8.0±0.0^{bc} (5.0)	282.6 ± 15.9^{e}
7	*H. laricifolium*	l	8.5±0.7^{gh} (0.5)	4.0±1.4^{efgh} (0.5)		0.0 (8.0)		55.6 ± 2.4^{b}
		s	7.0±1.4^{hi} (0.4)	5.0±1.4^{efg} (1.0)		0.0 (8.0)		62.3 ± 2.0^{b}
8	*J. paniculata*	l	6.0±2.8^{hijk} (3.0)	2.5±0.7^{gh} (9.0)				
9	*L. ferruginea*	l+s	13.5±0.7^{cdef} (2.5)	6.0±1.4^{e} (3.0)	3.5±0.7^{d} (>10)		8.0±0.0^{bc} (8.0)	
10	*L. weberbaueri*	l						
11	*M. volcanica*	l+s	3.0±0.0^{ijkl} (2.0)	0.0 (3.0)	0.0(8.0)	7.0±0.0^{d} (8.0)		98.7 ± 3.1^{c}
12	*N. obtusa*	l	3.5±0.7$_{ijkl}$ (7.5)	4.5±2.1^{efgh} (3.0)				
		s	1.5±0.7^{l} (>10.0)	3.5±0.7^{efgh} (5.0)				
13	*O. multicaulis*	l	15.0±2.8^{bcde} (2.0)	9.5±0.7^{d} (1.5)		0.0 (8.50)	8.0±1^{bc} (1.0)	
		r	4.0±1.4^{ijkl} (2.0)	9.5±0.7^{d} (5.5)		4.0±0.7^{d} (9.0)	7.0±1^{c} (1.5)	
14	*O. grandiflora*	l	15.0±1.4^{bcde} (1.5)	2.5±0.7^{gh} (2.5)	5.0±0.0^{c} (8.0)	15.0±1.4^{b} (1.5)		113.5 ± 4.4^{c}
15	*P. hartwegiana*	l+s	7.0±0.0^{hi} (2.0)	16.5±0.7^{bc} (3.0)				
16	*P. coerulescens*	l	2.5±0.7^{kl} (>10)	4.5±0.7^{efjh} (5.5)				
		rh+ r	6.5±0.7^{hij} (>10)	4.5±0.7^{efgh} (3.0)				
17	*P. multiflora*	l		2.5±0.7^{gh} (7.0)				
18	*P. pinnatifida*	rh+ r	2.5±0.7^{kl} (>10)	4.5±0.7^{efgh} (3.0)				
19	*S. calvus*	l	12.0±2.8^{ef} (5.0)	2.0±0.0^{h} (>10)				
20	*S. canescens*	l	5.5±2.1^{hijk} (5.0)	0.0 (8.0)				
21	*S. leucophorbius*	l	11.0±2.8^{fg} (>10)					
22	*S. rhizomatus*	l	4.5±0.7^{ijkl} (>5.0)	14.5±0.7^{c} (3.0)				
23	*S. serratifolius*	l	4.5±2.1^{ijkl} (8.5)	0.0 (8.0)				
24	*V. henrici*	l+r		0.0 (8.0)				
25	*V. aff. pycnantha*	l+r	6.5±2.1^{hij} (3.0)	3.0±0.0^{fgh} (1.5)				
26	*V. globularis*	l	6.0±1.4^{hijk} (5.5)	0.0 (3.0)				
27	*W. nubigena*	l+r	3.0±0.0^{jkl} (4.0)	3.0±0.0^{fgh} (6.0)				
28	*X. dactylophyllum*	l+s	12.5±2.1^{def} (2.0)	4.5±0.7^{efgh} (2.0)			9.0±1.0^{b} (>10)	
	Tetracycline		20,0±1,4^{ab}	18.5±2.1^{b}	15.5±0.7^{b}	13.0±0.0^{c}		

	(<0.02)	(<0.02)	(<0.02)	(<0.02)			
Streptomycin	17,5±0,7[a] (<0.02)	25.5±0.7[a] (<0.02)	20.0±0.0[a] (<0.02)	17.5±0.7[a] (<0.02)			
Nystatin					11.0±1.4[a] (>1.0)		
Ascorbic acid							22.6 ± 0.3[a]

PE, part of plant used to prepare the extract; l, leaf; s, stem; r, root; rh, rhizome; f, flower; w, whole plant. Sa, *S. aureus*; Bs, *B. subtilis*; Ec, *E. coli*; Pa, *P. aueriginosa*; Ca, *C. albicans*.
***, inhibition halo was calculated minus 5 mm (filter paper disk).**
Values ± standard deviation, and letters as super index correspond to statistic group according to Duncan's test ($p<0.05$).

Table No. 4
Phytochemical composition using Lock (1994) method

Type of secondary metabolite	Color reagent	Plants						
		Og[l]	Hl[l]	Gw[l]	Ga[l]	Lf[a]	Mv[a]	Om[l]
Alkaloid	Dragendorff Mayer	x	x	x	x	x		
Tannin	Gelatin	x	x		x	x	x	x
Phenolic compound	$FeCl_3$	x	x	x	x	x	x	x
Flavonoid	Shinoda	x	x	x	x			x
Leucoanthocyanin	Rosenheim				x	x	x	
Triterpen and/or steroid	Lieberman-Buchard		x	x			x	x
Quinone	Bortranger							x

Og, *O. grandiflora*; Hl, *H. laricifolium*; Gw, *G. weberbaueri*; Ga, *G. americana*; Lf, *L. ferruginea*; Mv, *M. volcanica*; and Om, *O. multicaulis*. l, leaves; a, whole aerial part."x" represent the presence of the secondary metabolite

H. laricifolium is a shrub that grows in the high Andean areas from Venezuela, Ecuador, and Peru (Crockett *et al.*, 2010). Some *Hypericum* species are well known for their medicinal properties (Cirak *et al.*, 2017), and *H. laricifolium* is used mainly to treat cold, wart, and as an antidepressant (Table No. 1). Their leaves and stems showed activities against *P. aeruginosa*, *B. subtilis*, and *S. aureus*. The latter activity was also reported for ethanolic, methanolic and chloroformic extracts of samples from other regions (Bussmann *et. al.*, 2010; Jerves-Andrade *et al.*, 2014). Antibacterial compounds of *H. laricifolium* have not still been determined; but tetraketone and hyperforin were isolated from *H. perfolatum* had antibacterial and antidepressant activities (Saddiqe *et al.*, 2010). Some *Hypericum* species are known for their antioxidant property (Kızıl *et al.*, 2008; Boga *et al.*, 2016), and *H. laricifolium* showed antioxidant activity with DPPH assay. In their phytochemistry, *Hypericum* species contain phenolic, flavonoids and tannins compounds, which would be responsible for their cytotoxic, antitumor, and anti-inflammatory properties (Boga *et al.*, 2016); in concordance, *H. laricifolium* has phenolic compounds especially flavonoids and gallic tannins. Likewise, its leaves contain caffeic acid (El-Seedi *et al.*, 2003) which is a potent antioxidant (Gülçin, 2006); acylphloroglucinol derivatives (Ccana-Ccapatinta & von Poser, 2015) which are associated with antitumor, antibacterial, and anti-HIV activities of *H. sampsonii* (Zhu *et al.*, 2015). Also, *H. laricifolium* inhibited a proliferative growth of Hep3 cells (Carraz *et al.*, 2015), but the compounds involved in this effect are not described yet.

O. grandiflora is a tree that grows in the Peruvian inter-Andean valleys between 1500-4000 m.a.s.l. (Reynel & Marcelo, 2009), and some areas from Ecuador (Alejandro-Espinosa *et al.*, 2013). It is used to treat cold and fever (Tene *et al.*, 2007; Gonzales De La Cruz *et al.*, 2014); and it has shown a broad spectrum of antibacterial activity, where the halos size against *S. aureus* and *P. aeruginosa* were similar to reference antibiotic halos. Likewise, *O. grandiflora* has shown antioxidant activity, possibly by the presence of phenolic compounds such as catecholic tannins and flavonoids found in its

phytochemical evaluation. It is used to treat diabetes (Gonzales De La Cruz *et al.*, 2014), which could be related to its antioxidant activity; however, Alejandro-Espinosa *et al*. (2013) have found that *O. grandiflora* leaves inhibited α-amylase and β-glucosidase as a hypoglycemic activity mechanisms, and showed antioxidant activity using DPPH assay. About its conservation status, Pretell *et al*. (1985) consider that *O. grandiflora* is an endangered species in Peru due to its irrational use; besides it is being displaced from its habit by exotic and agricultural forest species; thus, Olivera-Gonzales *et al.* (2017a) have performed a methodology for *O. grandiflora in vitro* propagation such as an alternative for its conservation.

Table No. 5
Phytochemical evaluation using TLC with reagent and detection method

Plant	Alkaloid	Type of tannins	Type of flavonoid (number)	Other phenolic compound
Og[l]	2	catechol (1)	flavanonol, flavone, flavonone or isoflavone (3); anthocyanin (1)	2
Hl[l]	3	catechol (3)	flavone, isoflavone or flavonol (6)	4
Gw[l]	2	-	flavone, isoflavone or flavonol (5)	2
Ga[l]	2	catechol (2)	flavanone or flavone (2)	4
Lf[a]	3	catechol (1)	flavanone or flavone (2)	6
Mv[a]	3	catechol (1)	flavone, flavonol or flavanone (3)	4
Om[l]	1	gallic acid (1)	flavonol, flavone or chalcone (2)	1

Og, *O. grandiflora*; Hl, *H. laricifolium*; Gw, *G. weberbaueri*; Ga, *G. americana*; Lf, *L. ferruginea*; Mv, *M. volcanica*, and Om, *O. multicaulis*. [l], leaves; [a], whole aerial part.

G. weberbaueri is a herb that is an endemic plant from Ancash (Peru) that grows between 3900 to 5100 m a. s. l., and it is in vulnerable status (Castillo *et al.*, 2006). It is used to avoid cavities (Kolff & Kolff, 2005), and we observed antimicrobial activity against *S. aureus*, *B. subtilis*, and *C. albicans*, remarking that *C. albicans* is a yeast involved in dental infections (de Oliveira *et al*., 2013). No other medicinal uses have been reported, interestingly *G. weberbaueri* has shown antioxidant activity. Other *Gentianella* species are used to treat diabetic, liver and depurative disorders (Li *et al*., 2010; Gonzales de la Cruz *et al*., 2014); and according to the phytochemical and ethnopharmacology review of *Gentianella* this genus is considered as a great source of medicinal plants characterized by the presence of xanthones, *C*-glucoflavonoids, and terpenoids, which are probably responsible for their biological activity (Li *et al.*, 2010). The phytochemical evaluation has found alkaloids, phenolic compounds, flavonoids, and triterpenes and/or steroids in its leaves, but studies about this plant are scarce.

G. americana is a herb with a wide distribution in Central and South America; in Peru, it grows in different habitats (Dillon & Sagástegui, 1991) mainly as weed. In traditional medicine, it is used as an antiseptic and for this reason is named commonly "alkupa allung", which means "dog's tongue"; it is also used to alleviate diarrhea (Gonzales De La Cruz *et al.*, 2014). In agreement with previous ethnopharmacology studies, *G. americana* showed good antibacterial activity against *B. subtilis* and *S. aureus*, being the last a common pathogen of purulent infections (Lima *et al.*, 2016). Both bacteria could be involved in diarrheal processes. Although *G. americana* is not used for chronic diseases, it showed good antioxidant activity (IC_{50} 65.4 ± 12.1 $\mu g \cdot L^{-1}$). The phytochemical evaluation revealed the presence of alkaloids, tannins, catechol, flavonoids and phenolic compounds.

M. volcanica grows between 1500 - 4500 m.a.s.l. from Mexico to Bolivia (Heim, 2014). It showed broad activity against gram positive and negative bacteria, which could be in agreement with its traditional use as antiseptic and anti-diarrheal (Rehecho *et al.*, 2011). It also showed good antioxidant activity and could be due to the presence of phenolic compounds such as catechol. Despite its wide distribution, it has been categorized as endangered species in Canta - a Peruvian Andean

province - because it is highly collected without agro-ecological study for its conservation and sustainable use (De-la-Cruz *et al.*, 2007).

O. multicualis inhibited the growth of *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *C. albicans*, such as reported Rojas *et al*. (2003). Furthermore, the last three bacteria are related to skin infections (Lima *et al.*, 2016), which could explain its usefulness as an antiseptic (Table No 1). *O. multicaulis* has shown the presence of tannins, phenolic compounds, flavonoids, triterpenes and/or steroids, quinones and amino acids; with exception of the two last compounds all were described in other *Oenothera* species (Festa *et al.*, 2015). Likewise Srivastava *et al*. (2007) isolated oenosyticin - a potent metabolite against *S. aureus* and *S. epidermidis* - from *O. biennis*; but no more phytochemical research was found for the family. *O. multicualis* is widely distributed from Ecuador to Bolivia; however, the whole plant is collected identifying it as a probably threatened species.

L. ferruginea is a shrub that grows from Central Ecuador - Central Peru between 3300-4800 masl, and is sold as a medicinal plant in popular markets (Dillon & Sagastegui, 1991). It is used together to others plants for bathing (Gonzales de la Cruz *et al.*, 2014). It showed antibacterial activity against *S. aureus*, *B. subtilis*, and *E. coli*; as well as anti-*Candida* activity. Concerning its phytochemical composition, phenolic compounds and catechol-type tannins were found. Malca *et al*. (2016), reported the presence of 5,7-dimethoxycoumarin that inhibit cancer cells and 5,7,8-trimethoxycoumarin with anti-HIV activity, being the only other publication describing the phytochemical content of this plant

In addition, ethanolic extracts from others species such as *A. linearis*, *B. incana*, *J. paniculata*, *P. hartwegiana*, *V.* aff. *pycnantha*, and *X. dactylophyllum* showed MICs up to 3.0 $mg \cdot mL^{-1}$ against *S. aureus*; this activity could be important because *S. aureus* is one of the main opportunistic pathogens with high capacity to acquire resistance to chemotherapy (Mendem *et al*., 2016); likewise, other plants are still important because it is possible that they have other medicinal properties, e.i. *P. coerulescens* and *P. muliflora* have shown an antiproliferative activity of Hep3B hepatocarcinoma cells (Carraz *et al.*, 2015).

CONCLUSION

This is the first study that focuses on completely wild native plants from the Peruvian high Andes, a fragile ecosystem facing global warming; and according to these results the most promising species are *G. americana*, *G. weberbaueri*, *H. laricifolium*, *L. ferruginea*, *M. volcanica*, *O. multicaulis* and *O. grandiflora*. There is enough evidence about their pharmacological potential as an important source for new drugs. Most of the plants do not have phytochemical and bioactive activity information and in many cases, the whole plants are collected without management plans and are over exploited. For this reason, their promising biological activities studies and biotechnological applications could contribute to improve their conservation and sustainable use.

ACKNOWLEDGEMENTS

The authors want to thank Cecilia Vásquez-Robinet and Nikolai Hidalgo who revised and corrected the English grammar in the draft manuscript. Too, thank Alberto Castañeda who prepared the map. Likewise, this research was financially supported by the Dirección General de Investigación (DGI) of the Universidad Nacional Santiago Antúnez de Mayolo (UNASAM-Perú) as a component of the project "Caracterización biológica, fitoquímica y molecular de plantas Peruanas altoandinas con potencial en la industria farmacológica".

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