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Research Article

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The antioxidant and antibacterial activity of *Azorella multifida* on phytopathogenic bacteria

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ABSTRACT

The objective of this study was to investigate the inhibition of antibacterial and antioxidant activity by extracts of the aerial parts of Azorella multifida collected in Cuenca, Ecuador. Azorella multifida presented poor antioxidant activity in dichloromethane and petroleum ether extracts (DPPH) with IC_{50} values >300 ug/mL, while it showed moderate antioxidant activity in methanolic extracts. Total phenolic content was determined using Folin-Ciocalteu method and total flavonoid content by the formation of a flavonoid-aluminum complex. All extracts were evaluated as antibacterial agents on clinical isolates of human pathogens without demonstrating activity on the microorganisms. Antibacterial activity against phytopathogenic microorganisms was evaluated, with interesting results.

Key words: Antibacterial phytopathogens, antioxidants, phenolics, *Azorella multifida*.

INTRODUCTION

Microbial resistance to conventional antibiotics has increased significantly in recent times, and the study of the antibacterial properties of plant extracts and natural phytochemicals has attracted significant interest from specialists in the field. Latin American countries have a long tradition of applying plants in different fields, and their use is widespread in rural and semirural areas. In recent years considerable attention has been devoted to medicinal plants with antioxidant and antimicrobial properties. Many natural antioxidants, especially flavonoids, have a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory activity [1]. Antioxidant activity is a fundamentally important property for life, and it has often been argued that antioxidant properties play an important role in preventing diseases caused by oxidative stress, such as cancer, coronary arteriosclerosis, and the aging processes [2].

Phytopathogenic microorganisms are the main infectious agents in plants, causing alterations during developmental stages, including post-harvest. In fruit and vegetables, a wide variety of fungal and bacterial genera exist, causing quality problems in a number of aspects such as nutritional value, organoleptic characteristics, and leading to limited shelf life [3].

Bacterial plant diseases have always been major constraints in crop production causing severe losses every year. Control of bacterial plant pathogens is a well-known property of natural products, as microorganisms constantly

generate resistance against traditional treatments. It is therefore necessary to search for novel biologically active compounds to replace those with reduced effectiveness.

The plant kingdom has provided a variety of compounds of known therapeutic properties, for example, plants from the genus Pterocaulon, known as "quitoco", are commonly used in veterinary medicine in southern Brazil to treat animal problems popularly diagnosed as "mycoses". Several works have demonstrated in laboratory trials that different plant tissues, such as roots, leaves, seeds and flowers possess inhibitory properties against bacteria, fungi and insects. Currently, there is little evidence on the antimicrobial properties of plants under investigation against phytopathogen fungi [3].

A productive use for natural products may be in the control of phytopathogenic bacteria, such as *Erwinia carotovora, Pseudomonas syringae, Clavibacter michiganensis* and *Xanthomonas campestri. E. carotovora* subsp. Carotovora, *X. campestris* and *P. syringae* are Gram negative bacteria, and responsible for the disease in a wide range of species (for example: potatoes, tomatoes and olives). On the other hand, *C. michiganensis* is a Gram positive bacterium and produces ulcers and chancres in vegetables species, mainly in *Licopersicum* sp (*L.esculentum*).

Azorella *(*Apiaceae) is a genus of 70 species, is known by its vernacular name, "Yareta," and the genus is distributed among the high Andes Mountains, including Chile, Venezuela, Ecuador, Bolivia, Peru and Colombia. It is used in folk medicine as antitussive and expectorant, for treating asthma, colds and bronchitis, and as an antiseptic, antiparasitic, antirheumatic and hypoglycaemic [4]. This species produces unique structures with novel mulinane and azorellane skeletons and a wide variety of bioactivities, including antiprotozoal, antibacterial, antiviral, spermicidal, cytotoxic, antihyperglycemic, antiinflamatory, analgesic and antituberculosis activity [5]. Some Andean communities use aqueous extracts of this genus to spray crops and control pests. The determination of potential antioxidant and antibacterial activity against phytopatogenic microorganisms of *A. multifida* extracts could be useful for future use as natural antimicrobial agents for the control of phytopatogens.

EXPERIMENTAL SECTION

Plant Material

Plant materials were collected in provinces in Cuenca, Ecuador in 2012 (2°54′08″S 79°00′19″O). Plant material was botanically identified by the Azuay Herbarium at the University of Azuay, Ecuador. Aerial parts were air-dried for 10 days at room temperature. The dried material was coarsely powdered. The plant (1220.76 g) was then extracted by maceration using solvents of different polarity

Preparation of the Extracts

Powdered materials were extracted for maceration using organic solvents of different polarity. The solvents used for preparing the different extracts were methanol (MeOH), a high polarity solvent, dichloromethane (DCM) with intermediate polarities and petroleum ether (PE) a low polarity solvent. The extracts were filtered, successively concentrated under reduced pressure and kept in the dark at 4 °C until tested.

Microorganisms

Collection of bacterial isolated:

Bacterial cultures used in the present study were isolated from infections plant and were classificated in the phytopathology laboratory of Universidad de Talca.

Maintenance of bactertial cultures

All the bacterial isolates were cultured and maintained in LB(Luria Bertain) medium (1% tryptone, 1% sodium chloride, 0.5% yeast extract) during all the experiments of the study until mentioned. The bacterial cultures were refreshed fortnightly.

Antibiotic used:

The antibiotics used in this investigation were: Penicillin G and Streptomycin. The control strains were run simultaneously with the test organisms.

Preservation of Isolates:

Glycerol stocks were prepared and stored at -80^{o} C for long term preservation. Pure cultures strains were incubated at 37^{o}C for 48 h in isolation broth. Then 0.5 ml of each of the cultures was transferred into cryotubes and 0.5 ml broth containing 40% glycerol was added. The samples were mixed gently and stored at -80^{o}C.

Determination of Antibacterial Activity

Antibacterial assays against phytopatogenic bacterial *E. carotovora, P. Syringae, X. campestri* and *C. michiganensis* were carried out using the doubling dilution method following the procedure reported by Gutierrez *et al*, 2005 [6] in 96-well microtiter plates. Bacterial suspensions were obtained from overnight cultures in Luria Broth Base nutrient broth (Gibco BRL, Scotland) cultured at 25 °C and diluted to approximately 10^5 colony-forming units (CFU)/well in fresh medium. The compounds were dissolved to produce 5 mg/mL in MeOH as a stock solution. Stock solutions of compounds were diluted to give serial 2-fold dilutions that were added to each medium resulting in concentrations ranging from 1.25 to 0.078 mg/mL. The final concentration of MeOH in the assays did not exceed 2%. The plates were kept at 25 °C overnight (12 h). After incubation, 20 μL of 0.5 mg/mL aqueous 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) was added in each well and re-incubated for 30 min to detect living bacteria. The absorbance was read in a universal microplate reader (Multiskan EX Thermo, Finland) at 405 nm. The results were transformed to the control percentage, and median inhibitory concentration (IC_{50}) values were graphically obtained from the dose-response curves. Penicillin G (Sigma-Aldrich, St. Louis, MO) and streptomycin (Laboratorio Chile, Santiago, Chile) were used as standard antibacterials.

Total Phenolic Content (TPC) Estimation

The TPC of the extracts was determined according to the Folin-Ciocalteu method [7]. Briefly, 20 µL of extracts (100 µg/mL) or standard (gallic acid), were mixed with 1.58 mL of distilled water and 100 µL of Folin-Ciocalteu reagent, the reaction mixture was preincubated for 8 min and then 300 µL of sodium carbonate 20%, were added. Finally, each tube was incubated for 2h at room temperature and absorbance was obtained in a spectrophotometer (Thermo Spectronic Genesys 10 UV) at a wavelength of 765 nm. The TPC was determined by extrapolating the calibration curve created by preparing gallic acid solution. The TPC was expressed as gallic acid equivalents (GAE) in milligrams per g of extract.

Total Flavonoid Content (TFC)

The TFC was determined spectrophotometrically using the method of Zhishen *et al*., 1999 [8], based on the formation of a flavonoid-aluminum complex. Briefly, 0.5 mL of extracts (100 µg/mL) or standard (quercetin) were mixed with 2 mL of distilled water and 0.15 mL of sodium nitrate ($NaNO_3$, 5%). After 6 min of incubation, 0.15 mL of aluminum chloride ($AlCl_3$, 10%) were added and allowed to incubate for another 6 min, after which 2.0 mL of sodium hydroxide (NaOH, 4%) were added to the mixture. Water was added to achieve a final volume of 5 mL, and the mixture was incubated for another 15 min. Absorbance was obtained in a spectrophotometer (Thermo Spectronic Genesys 10 UV) at a wavelength of 510 nm. The standard TFC curve was produced using quercetin standard solution. The results are reported as quercetin equivalents (QE) in milligrams per g of extract.

Antioxidant assays

Each sample was dissolved in dimethylsulfoxide (DMSO) to create a concentration of 1mg/mL and then diluted to prepare series concentrations for antioxidant assays. In all assays the final concentration of DMSO was inferior to 2%. Reference chemicals were used for comparison in all assays.

Free radical scavenging assay (DPPH).

The scavenging activity of the extract was estimated using DPPH as the free radical model according to the method adapted from Brand-Williams *et al*., 1995 [9], and Molyneux, 2004 [10]. Briefly, an aliquot of 1 mL of extracts (10, 50 and 100 µg/mL) and control (2% DMSO final), respectively, were mixed with 2 mL of DPPH. The mixture was shaken vigorously and left to stand at room temperature for 5 minutes in the dark. The mixture was measured spectrophotometrically at 515 nm. Free radical scavenging activity was calculated as percentage of DPPH decoloration using the following equation:

% scavenging DPPH free radical = 100 × (1-AE/AD) where AE is the absorbance of the solution after adding the extract and AD is the absorbance of the blank DPPH solution. Quercetin was used as the reference compound.

RESULTS AND DISCUSSION

Extracts were obtained by macerating plant materials (1220.76 g) and different yields resulted depending on the solvent used. The solvent used for extraction, yield obtained, phenolic and flavonoid content and antioxidant activity are shown in table 1.

As described in the materials and methods section, the TPC for crude *A. multifida* extracts were determined by a regression equation of the calibration curve ($Ab= K_i xC + K_o$ where C is concentration of phenolic; $K_i= 8x10^{-2}$ and $K_0=5.32x10^{-3}$; $r^2=0.999$) and expressed in gallic acid equivalents (GAE). The TFC for crude *A. multifida* extracts was evaluated by aluminium chloride colorimetric assay, using quercetin as a standard and determined by a

regression equation of the calibration curve ($Abs=K_i xC + K_0$ where C is concentration of flavonoids; $Ki= 5.75x10^{-3}$ and $K_0= 0.0$; $r^2=0.998$). Amounts of phenolics and flavonoids are showed in table 1 and correlated with antioxidant activity.

Table 1. Plant materials, solvent used for extraction and yield obtained; total phenolics and flavonoids in crude extracts and antioxidant activity of crude extracts using the DPPH assay

Extracts	Yield (g)	CPT	CFT	DPPH decoloration % (IC50 µg/mL)
PE	6.56	1.45±0.14	0.07±0.02	>300
DCM	11.80	1.68±1.16	7.70±0.06	>300
MeOH	57.80	1.56±0.14	8.67±0.07	50.39
Quercetine	-	-	-	5.8±1.4

All extracts obtained were evaluated as antimicrobial agents in terms of their potential to inhibit clinical isolates of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Acinetobacter baumannii*, owever, not showed activity. All extracts were evaluated on phytopathogenic bacteria of agricultural interest. Results of antibacterial activity are shown in table 2 where values are expressed as IC_{50} in µg/mL.

Table 2. Antibacterial activity of *A. multifida* extracts, IC_{50} (µg/mL)

	P. syringae	*C. michiganensis*	*X. campestris*	*E. carotovora*
MeOH	97.22±6.3	>833.3	>833.3	>833.3
DCM	105.64±4.5	>833.3	305.07±7.2	>833.3
PE	82.12±3.8	98.15±2.7	65.35±4.8	>833.3
Penicillin	32.15±2.1	54.17±1.7	30.22±1.5	98.23±2.5
Streptomicin	18.54±1.2	23.14±1.5	28.72±2.0	47.15±2.5

At present, plant diseases are controlled using synthetic pesticides. However, indiscriminate application of these products causes health hazards in animals and humans due to their residual toxicity. Considering such deleterious effects of synthetic pesticides on life-supporting systems, an urgent need for alternative agents to manage phytopathogenic microorganisms arises [11]. Bacterial diseases are treated using fixed copper compounds, although the resulting control is generally inadequate due to the prevalence of cooper-resistant strains and weather conditions that often favor bacterial diseases in the field [12]. At present, natural products available for control phytopathogenic bacteria are limited. Hence, it is very important to detect natural products that may be active on them. Bactericides of plant origin are considered an interesting alternative to synthetic antimicrobials due to their lower negative impact, eco-friendly nature, availability and low cost.

The 2,2-diphenyl-2-picryl hydrazyl (DPPH) radical is used as a model for investigating the scavenging activities of chemical compounds. This radical is scavenged by antioxidants by meansof proton donation forming a reduced DPPH. Its color changes from purple to yellow after reduction, and may be quantified by a decrease of absorbance at wavelength 517nm [13]. The discoloration caused by a methanolic DPPH solution was studied for all *A. multifida* extracts. Antioxidant activity in *A. multifida* extracts was dependent on the concentration and solvent used, among which the methanolic extract demonstrated enhanced free radical entrapment as corroborated by its higher phenolic compound content.

Antibacterial activity against phytopathogenic strains was determined using colorimetric methods. This assay is based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a spectrophotometrically measured insoluble, colored formazan product. Since MTT reduction can only occur in metabolically active cells, the level of activity is a measure of cell viability. *Azorella multifida* showed marked activity over *P. syringae* without much distinction in terms of the solvent used for extraction. *Clavibacter michiganensis* was only controlled by petroleum ether extract; implying that control is restricted to non-polar metabolites similar to *X. campestris* present in this species. *Azorella multifida* does not affect the cell viability of *E. carotovora*. The results show the potential use of this species for controlling microorganisms pathogenic to plants.
Azorella multifida may be considered a source of natural antioxidant and antimicrobial compounds. Its extracts are effective against phytopathogenic microorganisms, and the application of *A. multifida* extracts may be promising, offering an economical and non-contaminant treatment alternative for controlling agronomic diseases in fruit and/or vegetables.

CONCLUSION

The results obtained from this work showed that extracts of *A. multifida* exhibit antimicrobial effects against phytopatogenic bacterial. Further studies are needed to determine the chemical identity of the bioactive compounds responsible for the observed antioxidant and antimicrobial activity. The biodegradation of natural compounds after

field application is another benefit which deals the environmental bioaccumulation troubles derived from some commercial antibiotics currently used.

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