Evaluation of the Antibacterial Activity of Four Ethanolic Extracts of Bryophytes and Ten Fruit Juices of Commercial Interest in Colombia against Four Pathogenic Bacteria

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Abstract

Diversity of pathogenic microorganisms overcomes the defenses of animals, plants, and humans, causing severe diseases. The use of traditional antibiotics may have negative secondary effects on organisms and the environment. Therefore, we must search for new alternatives in plants that contain antimicrobial compounds, such as flavonoids, bioflavonoids, terpenes, fatty acids derivates, amongst others, which can be used as functional foods or phytotherapeutic products. Through the agar diffusion method (50 µl per well), the antibacterial activity (against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*) of commercial fruit juices (pineapple, blueberry, pink pear, sweet aji, corozo, starfruit, Santander's medlar, sour grape, Isabella grape, and wild blackberry) and the ethanolic extracts of bryophytes, two mosses (*Sphagnum magellanicum* and *Hypnum amabile*) and two liverworts (*Metzgeria decipiens* and *Trichocolea tomentosa*) was evaluated and compared with ampicillin and clindamycin. The juices of blueberry, sour grape, Isabella grape and wild blackberry and all ethanolic extracts of bryophytes were active against at least two of the evaluated bacteria with different magnitudes of inhibition. This study opens the door to the use of an unexplored part of Colombian flora with the first report of antibacterial activity of these Colombian bryophytes (especially *H. amabile* and *T. tomentosa*), and confirms the potential use of fruit juices (mainly blackberry and blueberry) for the future development of natural products against pathogenic bacteria.

INTRODUCTION

Pathogenic microorganisms negatively affect the economy of agricultural sectors and the health of human beings (Sati and Joshi, 2011). Synthetic products against pathogens can harm the individual's health and the environment (Rawani et al., 2011). The chronic use of drugs is associated with the development of multi-resistance species, which generates a potential increase in the number of pathogenic diseases that are difficult to treat (Rawani et al., 2011; Tenover, 2006). Thus, the development of natural products with the desired medicinal properties is a valuable alternative. This is because the chemical metabolism of the plant has an evolutionary composition that offers a variety of secondary metabolites as terpenes, flavonoids, biflavonoids, fatty acids derivatives, diterpenoids, aromatic compounds and so on (Asakawa, 2007; Krzaczkowski et al., 2008), with a natural balance (minimum amount necessary in the defense) and synergistic activity between compounds (Cseke et al., 2006; Liu, 2004; Xie and Lou, 2009).

Among the huge diversity of plants species in the world, the angiosperms (the largest group of plants) (APWeb, 2008) contain fruits species with great nourishing effects and possible use in prevention and treatment of diseases (Liu, 2004; Lock et al., 2005). Bryophytes are the second largest group (divided into mosses, liverworts, and anthoceros) with most of the species having great ecological importance in the water and nutrient cycle regulation (Hallingbäck and Hodgetts, 2000). In addition, while vascular plants have cuticle and cuticular projections (i.e., spines, thorns, prickles) as defense

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systems against herbivores, bryophytes do not have these anatomical barriers. Nonetheless, bryophytes are rarely attacked by pathogens, which suggests that they have a special chemical metabolism with antimicrobial compounds (Xie and Lou, 2009).

The vast variety of Colombian ecosystems have a diversity of plants species and cultivars exposed to changing environmental conditions that provide them with resistances and different molecules with economic potential (Pérez, 1996; Xie and Lou, 2009). This study evaluated the antibacterial activity of ten fruit juices (pineapple, blueberry, pink pear, sweet ají, corozo, startfruit, Santander's medlar, sour grape, Isabella grape, wild blackberry) and four bryophyte ethanolic extracts of two mosses (*Sphagnum magellanicum* and *Hypnum amabile*) and two liverworts (*Trichocolea tomentosa* and *Metzgeria decipiens*) against four bacteria indicators of activity (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*). This study was done to generate basic knowledge for their bioprospection, by the screening and selection of vegetable species with antibacterial activity.

MATERIALS AND METHODS

Collection and Processing of Plant Material

Plant material was obtained from farmers of commercial fruits or was collected from the paramo wild environment between June and August in 2011 (Table 1). The fruits were selected mature, turgid, with consistent texture and strong and bright colors, while bryophytes were selected by chemotaxonomy and abundance criteria. The specimens were deposited in the Herbarium of the Pontificia Universidad Javeriana (HPUJ) in Bogotá, for their taxonomic identification of the species and cultivars. The fruits (stored at -70°C) were triturated, filtered, and centrifuged (10 min in 300 rpm), total solids, water content, and density were measured based on the official AOAC methods (Table 2). The rotary evaporator, water bath, vacuum chamber, and oven used in the processing of plant-derived material were within the metrology program (periodic review). This program ensures the proper functioning of equipment. Calibration of this equipment was performed according to official AOAC methods and phytochemical methods (Harborne, 1998).

The bryophytes were dried at room temperature (±18°C), manually triturated, and their compounds were extracted in cold maceration with 95% ethanol for eight days. The extracts were concentrated using a rotary evaporator under reduced pressure (175 mbar/hPa) in a water bath at 40°C and in a vacuum chamber (Bilbao, 1997; Hostettmann, 2008). During processing of the bryophytes, we calculated the weight (wet, dry, and final extract) to estimate the moisture content, mass-mass percentage, and the sample yield; based on phytochemical methods (Harborne, 1998) and AOAC methods (Table 3). Finally, 100 mg of each extract was diluted in 1 ml of pure dimethyl sulfoxide (DMSO) for bioassay (Sabovljević et al., 2011).

Antibacterial Activity Test

By the well agar diffusion method (5 mm of diameter and 50 µl from each sample) (Lalitha, 2005; Bodade et al., 2008) all the samples were evaluated against four pathogenic bacteria obtained from Ceparium of Microorganisms at the Pontificia Universidad Javeriana (CMPUJ) and selected based on BioScreen Testing (2007). The strains were cultivated in nutrient broth and were compared with 0.5 McFarland scale (3×10^8 CFU/ml), subsequent 0.5 ml were added in each bacterial suspension for each 25 ml of Müller-Hinton agar in different glass petri dishes. Positive controls were ampicillin (200 mg/ml, Binotal®, Bayer S.A.) and clindamycin (150 mg/ml, Vitalis S.A.C.I., Vitrofarma S.A.) and the negative controls were distilled water (for fruits) and DMSO (for bryophytes). All the dishes were incubated at 35°C, and after 24 h, the inhibitions zones were measured around the wells in mm and the results were expressed in mm of inhibition for each mg of sample. This was done in order to do comparisons between the results of all the evaluated samples (Table 4) (Cona, 2002; Bodade et al., 2008).

RESULTS AND DISCUSSION

The antibacterial property of ten fruit juices and the ethanolic extracts of four bryophytes were tested using Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative (*P. aeruginosa* and *E. coli*) bacteria in the agar well diffusion method (Lalitha, 2005; Bodade et al., 2008). Positive results were obtained with the mosses (*S. magellanicum* and *H. amabile*), liverworts (*T. tomentosa* and *M. decipiens*), and four fruits (blueberry, sour grape, Isabella grape and wild blackberry). The rest of the fruits (pineapple, pink pear, sweet ají, corozo, startfruit, Santander's medlar), however, did not inhibit the bacterial growth (Table 4).

The more active samples (in mm/mg of extract) were: wild blackberry, specifically against *E. coli* (2.6 mm/mg), *S. aureus* (2.3 mm/mg), *P. aeruginosa* (2.9 mm/mg) and *B. subtilis* (1.9 mm/mg) in relation with the other evaluated fruits; the moss *H. amabile*, particularly against *B. subtilis* (3.3 mm/mg), *P. aeruginosa* (3.1 mm/mg) and *E. coli*. (2.3 mm/mg) compared with other bryophytes; blueberry, with the highest inhibition against *E. coli* (7.2 mm/mg) and *S. aureus* (4.1 mm/mg); and the liverwort *T. tomentosa* against *S. aureus* (2.1 mm/mg), *P. aeureginosa* (2.9 mm/mg) and *B. subtilis* (2.0 mm/mg). The best values of inhibition of the ampicillin were 4.2 mm/mg and 3.6 mm/mg against *S. aureus* and *B. subtilis*, respectively; for the clindamycin were 3.3 and 3.1 mm/mg against *S. aureus* and *B. subtilis*, respectively.

The potential use of the eight samples with antibacterial activity is evidenced by: (1) the wide range of the sample's action against Gram-positive and Gram-negative bacteria (Gram-negative commonly are more resistant by their physiology) (Nohynek et al., 2006); (2) the greater effect of the extracts of Isabella grape (1.4 mm/mg), wild blackberry (2.9 mm/mg), *H. amabile* (3.1 mm/mg), *T. tomentosa* (2.9 mm/mg), and *M. decipiens* (1.5 mm/mg) against *P. aeruginosa* compared with traditionally used antibiotics such as ampicillin (1.3 mm/mg) and clindamycin without inhibition; and (3) the greatest extraction yields of the extracts of *H. amabile* and *T. tomentosa* (Table 3), species of bryophytes with higher antibacterial activity. These results demonstrate the important features of the samples for the future development of herbal products.

Coloration patterns of blueberry, sour grape, Isabella grape, and wild blackberry were from red to blue-violet presumably given by anthocyanins. Extracts of these berries have been studied for antimicrobial activity and possible synergistic effect between compounds (especially their phenolic compounds) has been reported (Nohynek et al., 2006; Puupponen-Pimiä et al., 2005). This study confirms the antibacterial activity of four berries juices grown in the country by analyzing their juices (a common form of consumption of fruits) using a simple process and preserving the synergy and natural balance of the compounds, which are useful features in the development of functional foods, nutritional supplements, and natural additives in food preservation.

Compared to vascular plants, the phytochemical study of bryophytes has been neglected due to the difficulty of obtaining and identifying them (Sabovjević et al., 2011). This is the first report of antibacterial activity of these Colombian bryophytes; the studies in the country have more taxonomic and ecologic than phytochemical approaches. Positive results of antioxidant activity effects in the central nervous system and against cobra venom had been found (López et al., 2007; Morantes et al., 2007; Pereañez et al., 2010), but no positive results in antimicrobial activity had been reported. Many other studies, however, have reported the antimicrobial activity (Bodade et al., 2008; Sabovljević et al., 2011; Sati and Joshi, 2011) and biological activity as antioxidant, antipyretic, antidotal, antiseptic, cytotoxic, anti-HIV, antifeedant, nematocidal, neurothophic, and so on, especially in the United States, Japan, Germany, India, and Turkey (Glime, 2007; Asakawa, 2007; Krzaczkowski et al., 2008). Here we report the potential antibacterial activity of Colombian bryophytes. Our results warrant further investigation on biological activities of these species, as well as the development and application of mass production techniques (i.e., in vitro or in bioreactors) (Hohe and

Reski, 2005) that may provide future economic benefits.

CONCLUSIONS

The berry and bryophyte species, especially the juices of blackberry (2.9 mm/mg against *P. aeruginosa*) and blueberry (7.2 mm/mg against *E. coli*) and the ethanol extracts of the moss *H. amabile* (3.3 mm/mg against *B. subtilis*), and the liverwort *T. tomentosa* (2.9 mm/mg against *P. aeruginosa*) have antibacterial activity and are candidates for the development of natural antibacterial products and food preservatives. Furthermore, this study allows us to observe that nonvascular plants (bryophytes) also have antibacterial activity. This knowledge is very important for the development of ex situ propagation techniques and mass production in artificial conditions for their sustainable use (Hohe and Reski, 2005). For future studies, we suggest the evaluation of species in different concentrations against pathogenic microorganisms and the species that did not show any activity in this study with other methods of antimicrobial tests. In the second phase of this project with promising species of this work, we will study the biological activity as antioxidant and antitumoral and we will identify the active compounds.

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Tables

Table 1. Source and parameters of selection of fruits and bryophytes species.

	Common name	Scientific name	Origin	Evaluated parts[2]
Fruit	Pineapple	*Ananas comosus (*L.) Merr.	Cultivated	Flesh
	Corozo	*Bactris minor* Jacq.		Flesh, peel
	Santander medlar	*Eriobotrya japónica* (Thunb.) Lindl.		
	Isabella grape	*Vitis labrusca* L.		
	Blueberry	*Vaccinium myrtillus* L.		Flesh, peel, seed
	Pink pear	*In identification process*[1]		
	Sweet aji	*Capsicum* L.		
	Starfruit	*Averrhoa carambola* L.		
	Sour grape	*Vaccinium meridionale* Sw.		
	Wild blackberry	*Rubus megalococcus* Focke	Wild	
Moss		*Sphagnum magellanicum* Brid.	Wild	Gametophyte[3]
		Hypnum amabile (Mitt.) Hampe		
Liverwort		*Trichocolea tomentosa* (Sw.) Gottsche		
		Metzgeria decipiens (C. Massal.) Schiffner		

[1] In process identification by specialists at HPUJ.
[2] In the antimicrobial test.
[3] Vegetative part of bryophytes.

Table 2. Physicochemical parameters of fruit juices.

Fruit	Crucible mass (g)			% (m/m)[1]		Density	Mass
	Initial	With juice	Final	Solids[2]	Humidity[3]	(g/ml)[4]	(mg)[5]
Pineapple	13.2472	14.3329	13.8383	54.4	45.6	1.3186	35.9
Bilberry	13.3879	14.4596	13.4383	4.7	95.3	1.2881	3.0
Pink pear	13.2536	14.1081	13.3706	13.7	86.3	1.2805	8.8
Sweet ají	12.1863	13.6246	12.2767	6.3	93.7	1.2803	4.0
Corozo	13.6235	14.2414	13.7309	17.4	82.6	1.0871	9.4
Starfruit	13.5595	15.8380	14.1110	24.2	75.8	1.2713	15.4
Santander's medlar	13.2568	15.8819	13.5115	9.7	90.3	1.3023	6.3
Sour grape	13.1208	14.6222	13.4478	21.8	78.2	1.3779	15.0
Isabella grape	13.6777	15.7810	13.9603	13.4	86.6	1.3211	8.9
Wild blackberry	13.1216	14.9984	13.3412	11.7	88.3	1.0622	6.2

[1] Mass-mass percentage.
[2] AOAC method 920.151.
[3] Calculate by difference.
[4] AOAC method 950.28.
[5] 50 µl per well.

Table 3. Yield and concentrations of extracts of bryophytes evaluated.

Bryophyte	% m/m[1]	Dry base extraction			Concentration [2]	
		Initial (g)	Final (g)	%	mg/ml[3]	mg/well[4]
S. magellanicum	11.11	129.9	3.94	3.03	100	5
H. amabile	18.53	100.8	5.74	5.7	100	5
T. tomentosa	11.39	28.2	1.84	6.52	100	5
M. decipiens	12.97	31.8	1.21	3.8	100	5

[1] Percentage mass-mass (% m/m) for the total solids content. Moisture (AOAC method 930.04) and solids calculated by difference.
[2] Amount of extract tested in the bioassay.
[3] Initial dilution of each extract.
[4] 50 µl per well.

Table 4. Antibacterial activity measured as inhibition[1] in fruits juices and ethanolic extracts of bryophytes.

Item		Inhibition zone[2] (mm/mg) [2]			
		B. subtilis	*S. auerus*	*P. aeruginosa*	*E. coli*
Fruits	Pineapple	-	-	-	-
	Blueberry	-	4,07±0,19	-	7,23±0,72
	Pink pear	-	-	-	-
	Sweet ají	-	-	-	-
	Corozo	-	-	-	-
	Starfruit	-	-	-	-
	Santander's medlar	-	-	-	-
	Sour grape	0,67±0,07	0,89±0,08	0,69±0,02	0,82±0,04
	Isabella grape	1,12±0,02	1,50±0,13	1,40±0,32	1,23±0,10
	Wild blackberry	1,91±0,08	2,30±0,14	2,93±0,27	2,56±0,28
Bryophytes	*S. magellanicum*	1,89±0,10	-	1,27±0,92	1,87±0,23
	H. amabile	3,27±0,23	1,67±0,12	3,13±0,31	2,27±0,31
	T. tomentosa	2,04±0,03	2,13±0,23	2,87±0,12	-
	M. decipiens	1,80±0,00	-	1,53±0,12	-
Antibiotics	Ampicillin	3,62±0,10	4,22±0,08	1,25±0,07	2,33±0,14
	Clindamicyn	3,09±0,07	3,26±0,02	-	1,76±0,30

[1] Values are: average (n=3 determinations) ± SD (Standard deviation).
[2] Antibacterial activity of each sample (50 µl per well), measured in millimeters of inhibition per milligram of extract (mm/mg). Negative controls were DMSO and water (both without inhibition).