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Antibacterial activity of pure flavonoids isolated from mosses

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Abstract

Seven pure flavonoids were isolated and identified from five moss species. The flavonoids were the flavones apigenin, apigenin-7-*O*-triglycoside, lucenin-2, luteolin-7-*O*-neohesperidoside, saponarine and vitexin; and the biflavonoid bartramiaflavone. Some of these flavonoids were shown to have pronounced antibacterial effects against *Enterobacter cloaceae, E. aerogenes* and *Pseudomonas aeruginosa* (minimal bacteriostatic concentration MIC in the range of 4–2048 μg/ml). Because of their antibacterial spectrum mainly active against Gram negative bacterial strains, responsible for severe opportunistic infections and resistant to common antibacterial therapy, these flavonoids may be important tools in antibacterial strategies.

Keywords: Mosses; Flavonoids; Biflavonoids; Antibacterial activity; MIC

1. Introduction

In recent years there has been an intensive search for substances with considerable antimicrobial properties. Plants are known to produce certain chemicals which are naturally toxic to bacteria and fungi.

In bryophytes, which are the simplest land plants, anatomical barriers are less effective and, as a consequence, the synthesis of particular molecules, secondary metabolites with antimicrobial activity: the so-called 'chemical barrier' (Harborne, 1988), is the most effective defence mechanism. Defence substances belong to a wide range of different chemical classes including flavonoids and isoflavonoids (Smith, 1996). Biflavonoids in mosses are also reported as possible chemical barriers against micro-organisms (López-Sáez, 1996; Geiger & Quinn 1988).

Extracts of various medicinal plants containing flavonoids have been reported to possess antimicrobial activity (Waage & Hedin, 1985; Vaughn, 1995; Shutz, Wright, Rali & Stucher 1995; Rhoca et al., 1995; Colombo & Bosisio, 1996; Li, Cai & Wu, 1997; Tereschuk, Riera, Castro & Abdala, 1997). The antibacterial activities of isoflavonoids and flavonoids and glycosides of luteolin and apigenin have been reported (Gnanamanichan & Mansfield, 1981; Miski, Ulubelen, Jobanson & Mabry, 1983). In this respect, the most investigated taxa are the angiosperms while few data are currently available about other groups of plants, including bryophytes (Asakawa, 1982; Asakawa, 1995, Markham, 1988; Markham, 1990). In this group of cryptogams, the production of antibacterial substances is a widespread phenomenon as much of the literature shows (Madson & Pates, 1952; McCleary, Sypherd & Walkington, 1960; McCleary & Walkington, 1966; Asakawa, 1981; Van Hoof, Vanden Berghe, Petit & Vlietinck, 1981; Ando & Matsuo, 1984; Castaldo Cobianchi, Giordano, Basile & Violante, 1988; Asakawa, 1990; Zinsmeister, Becker & Eicher, 1991; López-Sáez, 1994; Basile, Spagnuolo, 1997; Basile, Vuoto, et al., 1997). The antibiotically active substances of *Atrichum, Dicranum, Mnium, Polytrichum,*

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Table 1
MIC values expressed as μg/ml for the different substances considered and for reference (R) antibiotics

	Vitexin	Apigenin	Apigenin-7-*O*-triglucoside	Saponarine	Lucenin-2	Bartramia-flavone	Luteolin-7-*O*-neohesperidoside	CTAX	TET	PENG
S. aureus ATCC 13709	R	R	R	R	R	R	R	2	2	0.03
E. faecalis ATCC 14428	R	R	R	2048	R	R	R	R	2	8
P. vulgaris ATCC 12454	R	R	R	R	R	R	R	2	R	4
P. mirabilis ATCC 7002	128	16	R	8	R	8	R	0.03	32	4
P. aeruginosa ATCC 27853	R	8	256	8	8	256	R	16	32	R
S. typhi ATCC 19430	R	128	R	64	R	R	R	0.5	1	4
E. coli ATCC 11229	128	128	R	128	64	R	R	0.1	4	64
E. aerogenes ATCC 13048	R	4	256	4	8	8	256	R	R	4
E. cloaceae ATCC 10699	256	4	256	4	16	32	256	R	R	4
K. pneumoniae ATCC 27736	R	128	R	64	64	64	R	0.1	16	R

and *Sphagnum* spp. are considered to be polyphenolic compounds (McCleary & Walkington, 1966). In particular, flavonoids, including phenolic acids, are the main group of phenols from mosses and many new compounds have been detected in the last few years (Markham & Porter, 1978; Zinsmeister & Mües, 1980; Geiger & Quinn, 1988; Geiger, 1990; Markham, 1990; López-Sáez, Pérez Alonso & Velasco, 1996b). Flavones from bryophytes can be subdivided into derivatives of apigenin, luteolin, scutellarein, isoscutellarein, hypolaetin and tricetin (Huneck, 1983). Among the monoflavonoids apigenin, luteolin, kaempferol and orobol derivatives are the usual ones found in mosses (Zinsmeister & Mües, 1980; Markham, 1988; Mües & Zinsmeister, 1988; López-Sáez et al., 1996b). Biflavonoids from apigenin, luteolin and eryodictiol are also an important source of secondary metabolites from mosses (Geiger & Quinn, 1988; Geiger, 1990; Markham, 1990; López-Sáez, 1994; López-Sáez, Pérez Alonso & Velasco, 1996a; López-Sáez et al., 1996b).

The aim of this work was to determine the antibiotic activity of seven flavonoids (apigenin, apigenin-7-*O*-triglycoside, bartramiaflavone, lucenin-2, luteolin-7-*O*-neohesperidoside, saponarine and vitexin), isolated from five moss species, on Gram-positive and Gram-negative bacterial strains.

2. Results and discussion

The bacteria with the highest sensitivity are *Enterobacter cloacae, E. aerogenes* and *Pseudomonas aeruginosa*. In particular, the Enterobacteriaceae showed the greatest sensitivity to the flavonoids. Neither *Staphylococcus aureus* nor *Proteus vulgaris* showed any sensitivity to the substances tested (Table 1). Of the molecules tested, apigenin 7-*O*-triglycoside, vitexin and luteolin 7-*O*-neohesperidoside are the least active while saponarin isolated from *Plagiomnium cuspidatum* shows the highest antibacterial activity with MIC between 4 and 2048 μg/ml. Comparison of the activity of apigenin and vitexin both isolated from *Plagiomnium affine* showed that the former inhibits *S. typhi, P. mirabilis* and *P. aeruginosa* while vitexin is active only on *P. mirabilis*. These results demonstrate that in the same moss species there can co-exist antibacterial substances with different spectra of action and intensity. Vitexin (=8-C-glucopyranoside of apigenin) has been identified only and for the first time in the moss species *P. affine* (Melchert & Alston, 1965). Comparing the antibacterial activity of apigenin and apigenin 7-*O*-triglycoside (isolated from *Dicranum scoparium*) it is evident that the first has the highest activity. It is possible that the glycosilation of apigenin causes a reduction in antibacterial power due to the reduction in lipophilia and the consequent diminished ability to penetrate bacterial membrane.

The flavonoids tested are more active on Gram-negative becteria contrary to literature reports, indicating that Gram-positive bacteria are selectively inhibited by flavonoids and isoflavonoids derived from plants (Waage & Hedin, 1985). The pattern of selectivity of chemicals towards Gram-positive bacteria is not restricted to compounds from plants, but is a general phenomenon observed among most antibiotics.

The antibacterial activity of mosses against some Gram-negative bacteria has been shown in other studies: in particular, *Leptodictyum riparium* extract is able to inhibit Gram-negative more than Gram-positive bacteria; its extract is also particularly active against conventional antibiotic-resistant species (*Pseudomonas aeruginosa*) (Castaldo-Cobianchi et al., 1988). This is of considerable interest since conventional antibiotics are generally more active against Gram-positive bacteria.

From the ecological viewpoint, the presence in mosses of substances active prevalently on Gram-negative bacteria could be partly explained by the strong competition existing between bryophytes, lacking anatomical barriers, and soil bacteria that are mostly Gram-negative.

The importance of the activity of such substances for medical application lies in its selective activity against specific Gram-negative bacteria without affecting other bacteria.

3. Experimental

Air-dried plant material from moss species (*Bartramia pomiformis*, 250 g; *D. scoparium*, 285 g; *P. affine* and *P. cuspidatum*, 150 g each; *Hedwigia ciliata*, 210 g, of which voucher specimens are deposited in the MACB Herbarium, Madrid, Spain) were extracted with methanol and acetone (8:2) at room temperature (Geiger, 1990). The combined extracts were subjected to a four step Craig distribution (Geiger Anhut & Zinsmeister, 1988; Geiger, 1990; López-Sáez, 1994). The combined lower phases were reduced in vacuo to a thin syrup. The flavonoids apigenin (71 mg) and vitexin (86 mg) from *P. affine*, saponarine (95 mg) from *P. cuspidatum*, bartramiaflavone (92 mg) from *B. pomiformis*, lucenin-2 (53 mg) from *Hedwigia ciliata*, apigenin-7-*O*-triglycoside (43 mg) and luteolin-7-*O*-neohesperidoside (61 mg) from *D. scoparium* were isolated by column chromatography on polyamide-6 and purified on sephadex LH-20 (Geiger, 1990). They were identified by co-chromatography (TLC, HPLC), MS and NMR (Geiger et al., 1988; Geiger & Quinn, 1988; Geiger, 1990; López-Sáez, 1994). Only for *Bartramia* species and *D. scoparium* NMR and MS data are available from our previous researches that have been published elsewere (Geiger et al., 1993; López-Sáez, Pérez & Velasco, 1995a; López-Sáez, Pérez & Velasco, 1995b; López-Sáez, 1996).

3.1. Microorganisms

Ten bacterial strains obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) were employed. They included Gram-positive (G+) bacteria: *S. aureus* (ATCC 13709) and *Enterococcus faecalis* (ATCC 14428), and the following Gram-negative (G−) bacteria: *P. mirabilis* (ATCC 7002), *P. vulgaris* (ATCC 12454), *P. aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 11229), *Salmonella typhi* (ATCC 19430), *Enterobacter aerogenes* (ATCC 13048), *Enterobacter cloacae* (ATCC 10699), and *Klebsiella pneumoniae* (ATCC 27736).

3.2. Determination of MIC

Bacterial strains were grown on MH agar plates (DIFCO, Detroit, MI, USA) and suspended in MH broth (DIFCO). The MIC values against bacterial strains were performed using the Ericcson and Sherris (1971) broth-dilution method (MH broth). Inoculum suspensions were prepared from 6 h broth cultures and adjusted to 0.5 McFarland turbidity equivalents. The substances were sterilized by millipore filtration (0.45 μm) and added to MH broth medium. MIC determination was performed as reported previously (Basile, Spagnuolo et al., 1997; Basile, Vuotto et al., 1997). The substances were tested in triplicate, the experiment was performed four times and the results are shown as mean values of all experiments in Table 1.

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