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Antimicrobial activity of *Rubus imperialis* (Rosaceae)

Alexandre BELLA CRUZ *[1], Rosana Cé BELLA CRUZ [1], Márcia KANEGUSUKU [1], Valdir CECHINEL FILHO [1], Rosendo A. YUNES [2], Franco DELLE MONACHE [3] & Rivaldo NIERO [1]

[1] *Núcleo de Investigações Químico - Farmacêuticas (NIQFAR) and Programa de Mestrado em Ciências Farmacêuticas (PMCF) / CCS, Universidade do Vale do Itajaí (UNIVALI), 88.302-202, Itajaí, SC, Brazil*
[2] *Departamento de Química, Universidade Federal de Santa Catarina (UFSC), 88.040-900, Florianópolis, SC, Brazil*
[3] *Centro Chimica Recettori (CNR), Largo F. Vito 1, Rome, Italy*

SUMMARY. In this work the possible antimicrobial activity of crude methanol extract (CE), hexane, ethyl acetate and butanol fractions, as well as four pure compounds obtained from *Rubus imperialis* roots was evaluated. The minimal inhibitory concentrations (MIC) were determined using the agar dilution method. The experiments showed that the extract and some fractions exhibited antimicrobial action, particularly against the Gram-positive bacteria tested, with MIC values between 0.5 and 2.0 mg/mL.

RESUMEN. "Actividad antimicrobiana de *Rubus imperialis* (Rosaceae)". En este trabajo se evaluó la posible actividad antimicrobiana del extracto metanólico crudo (CE), de algunas fracciones, tales como hexano, acetato de etilo y butanol, así como de cuatro compuestos puros obtenidos de raíces de *Rubus imperialis.* Las concentraciones inhibitorias mínimas (MIC) fueron determinadas usando el método de la dilución en agar. Los experimentos demostraron que el extracto y algunas fracciones exhibieron acción antimicrobiana, particularmente contra las bacterias Gram-positivas probadas, con valores de MIC entre 0,5 y 2,0 mg/mL.

INTRODUCTION

Rosaceae is a large family of plants, represented by fruits as plums, cherries, damson plums, quinces, strawberries, pears and peaches [1]. The plants belonging to the genus *Rubus* have been used traditionally for the treatment of diarrhoea, burns, and as antioxidant and anti-diabetic agents [2-5]. Besides these activities, it has been reported that extracts of some species of this genus are potential antimicrobial sources [6].

The antimicrobial properties of several naturally occurring compounds have been known for decades. Recently, many plants have received special attention as sources of new antimicrobial agents [4,7]. Despite the absence of experimental studies concerning the anti-infective properties of *R. imperialis,* other plants of the genus *Rubus* have exhibited important antimicrobial effects [6, 8-11].

Phytochemical studies carried out with these plants indicated the presence of steroids, triterpene and ellagic acid derivatives [6,12,13]. *Rubus imperialis,* known popularly as "amora-do-mato" or "amora-branca" in Brazil, is a shrub measuring between 4 and 5 meters, being well-distributed in South of Brazil [14,15].

Recently, we have shown that *R. imperialis* presents hypoglycaemic, cytotoxic and antinociceptive actions [12,13,16,17]. The current study extends our previous work about the biological properties of *R. imperialis* and describes the *in vitro* antimicrobial activity against some pathogenic bacteria of extracts, fractions and pure compounds isolated from ethyl acetate and butanol fraction from roots, using the agar dilution method.

MATERIAL AND METHODS

Plant material

Rubus imperialis (Rosaceae) was collected at Florianópolis, Brazil, in June 1997 and identified by Dr. Ademir Reis (Department of Botany, UFSC). A voucher specimen was deposited at Barbosa Rodrigues Herbarium (Itajaí - SC) under number V.C. Filho 012.

KEY WORDS: Antimicrobial activity, Rosaceae, *Rubus imperialis.*
PALABRAS CLAVE: Actividad antimicrobiana, Rosaceae, *Rubus imperialis.*

* Author to whom correspondence should be addressed. *E-mail:* bellacruz@univali.br

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Phytochemical analysis

Air-dried material from different parts of *R. imperialis* (root, stem and leaves; 400 g each) were cut into small pieces and macerated separately with methanol at room temperature for 7 days. After filtration, the solvent was removed by rotary evaporation under reduced pressure, giving the respective crude extracts (CE). The extract was then suspended in MeOH-Water mixture (9:1) and successively partitioned with n-hexane, ethyl acetate and n-butanol, given the respective fractions. The ethyl acetate and butanol fractions from roots (4.1 and 3.2 g respectively) were chromatographed on silica gel column eluted with a mixture of $CHCl_3$:MeOH with increasing polarity. Similar fractions, which showed positive reaction with $FeCl_3$ or anisaldehyde sulfuric reagents, were combined and rechromatographed as in previous case, giving four pure colorless solids. They were identified on basis of their spectral data as niga-ichigoside F1 [**1**] (10 mg), 23-hydroxytormentic acid [**2**] (14 mg), 4'-methyl-3-O-methylellagic acid [**3**] (45 mg), and 3-O-methylellagic-4'-O-(rhamnose acid [**4**] (12 mg) (Fig. 1). The purity of all isolated substances was examined by thin layer chromatography (TLC) using Merck silica gel precoated aluminum plates 200 µm layer thickness, with several solvent systems of different polarity. Spots were visualized by short-wave UV light, anisaldehyde sulphuric and $FeCl_3$ reagents.

Microorganisms and Medium

To determine the antimicrobial activity, the following microorganisms were used: *Escherichia coli* (ATCC 11775), *Salmonella enterica* serovar Typhimurium (ATCC 14028), *Staphylococcus aureus* (ATCC 6538P), and *Streptococcus agalactiae* (ATCC 13813), *Candida albicans* (ATCC 10231), *Aspergillus fumigatus* (ATCC 26934), and *Rhizopus* sp. They were purchased from the tropical culture collection of "André Tosello Technology and Research Tropical Foundation", Campinas, SP, Brazil.

The bacteria were cultivated on Mueller-Hinton agar medium (Merck, 5437) for 18 to 24 h at 37 °C. Cell suspension in saline solution (0.86%) was adjusted to give a final concentration of approximately 1,5 x 10^8 cells/mL, standardized with 0.5 McFarland scale (λ = 530 nm) [18]. The yeast was cultivated on Sabouraud-dextrose agar (Merck, 5438) for 48 h at 37 °C. Cell suspension in sterile distilled water was adjusted to give a final concentration between 1 x 10^6 and 5 x 10^6 yeast cells/mL, standardized with 0.5 McFarland scale (λ = 530 nm). The filamentous fungi were maintained on Sabouraud-dextrose agar and sub-cultured every 15 days to prevent pleomorphic transformations. The inoculate were prepared by removing the sporulated fungi from the agar slant with a loop and suspending them in 10 mL of sterile water. The fungal suspensions were filtered to remove hypha. The resulting suspensions of conidia were vigorously vortexed and adjusted by adding sterile distilled water to a concentration of 1.4 x 10^6 cells/mL by using a hemacytometer cells counting chamber [19].

Quantitative antimicrobial evaluation

Minimum inhibitory concentrations (MICs) of the extract, fractions and pure compounds were

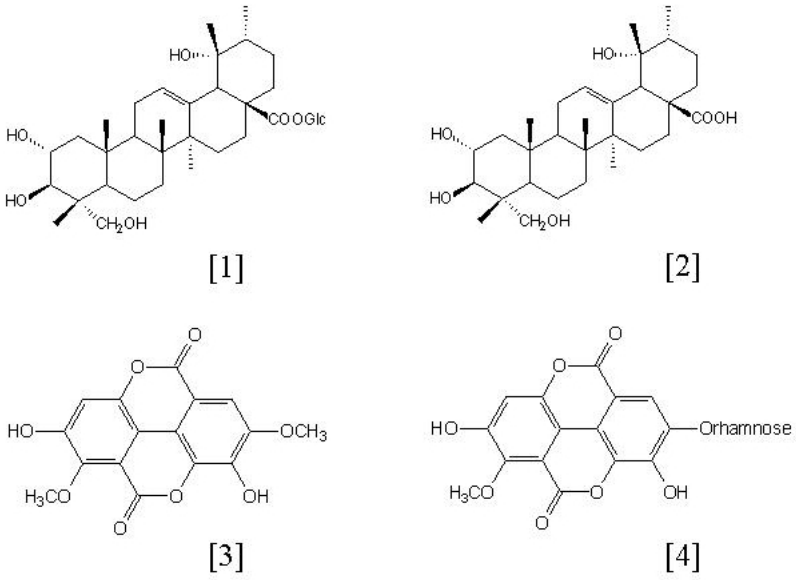

Figure 1.
Molecular structure of the isolated compounds from *R. imperialis*

1 = niga-ichigoside F1,

2 = 23-hidroxitormentic acid,

3 = 4'-methyl-3-O-methylellagic acid and

4 = 3-O-methylellagic-4'-O-α–rhamnose acid.

evaluated by the agar dilution. The assay was carried out in the macro dilution tubes. Stock solutions of extracts, fractions or compound in dimethylsulfoxide (DMSO) were diluted to give serial twofold dilutions that were added to each medium, resulting in concentrations ranging from 2.0 to 0.1 mg/mL. Final concentration of DMSO in the assay did not exceed 2%. Inocula of 1 µL having the bacteria, yeast cells or spore suspensions were added to the respective media. Drug-free solution was also used as a blank control. Tubes were incubated at 37 °C to 24 h for bacteria and 48 h for yeast and at 25 °C for 5 and 15 days (up to 15 days for dermatophyte strain) according to the control fungus growth. MIC was defined as the lowest concentration of extract, fraction or compound, showing no visible bacterial or fungal growth after the incubation period [18,19].

RESULTS AND DISCUSSION

The antimicrobial activities of the extract, fractions and isolated compounds from *R. imperialis* are shown in Table 1. Extract and compounds with MICs ≥ 2.0 mg/mL, were considered active.

The crude methanol extract obtained from roots and stem parts as well as the ethyl acetate and butanol fractions from roots caused the highest antimicrobial activity, with MIC values of 1.2 and 0.9; 1.0 and 0.9; 0.8 and 1.0; 0.8 and 0.5 mg/mL, against *Staphylococcus aureus* and *Streptococcus agalactiae,* respectively.

Results indicate that the Gram-positive bacteria tested are selectively inhibited particularly by the more polar components of *R. imperialis*, with MIC values between 0.5 and 1.8 mg/mL. Although the pattern of selectivity towards Gram-positive bacteria is common for plants in general, this phenomenon is also observed among several antibiotics [18,20]. No significant activity was observed against Gram-negative bacteria *(Escherichia coli* and *Salmonella enterica* serovar Typhimurium), with exception to that of methanol extracts from roots and stems which inhibited the grown of *E. coli* strain, with MIC values of 1.8 and 1.9 mg/mL, respectively. No activity was evidenced against some fungi tested

Component	MIC (mg/mL)						
	Gram-positive bacteria		Gram-negative bacteria		Fungi		
	S.a.	S.ag.	E.c	S.e.	C.a.	A.f.	R.sp.
Root							
CE	1.2	0.9	1.8	>2.0	>2.0	>2.0	>2.0
Hexane Fr.	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
EtOAc Fr.	0.8	1.0	>2.0	>2.0	>2.0	>2.0	>2.0
Butanol Fr.	0.8	0.5	>2.0	>2.0	>2.0	>2.0	>2.0
Stem							
CE	1.0	0.9	1.9	>2.0	>2.0	>2.0	>2.0
Hexane Fr.	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
EtOAc Fr.	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
Butanol Fr.	1.0	1.2	>2.0	>2.0	>2.0	>2.0	>2.0
Leaves							
CE	>2.0	1.8	>2.0	>2.0	>2.0	>2.0	>2.0
Hexane Fr.	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
EtOAc Fr.	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
Butanol Fr.	0.9	1.0	>2.0	>2.0	>2.0	>2.0	>2.0
Compounds							
1	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
2	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
3	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
4	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0

Table 1. Antimicrobial activity of extract, fractions and compounds of *R. imperialis* against bacteria and fungi, expressed as minimal inhibitory concentration (MIC). CE = Crude Methanol Extract; Hexane Fr. = Hexane fraction; EtOAc Fr. = Ethyl acetate fraction; Butanol Fr. = Butanolic fraction; **1** = niga-ichigoside F1, **2** = 23-hidroxitormentic acid, **3** = 4'-methyl-3-O-methylellagic acid and **4** = 3-O-methylellagic-4'-O-(rhamnose acid; *S.a. = Staphylococcus aureus; S.ag. = Streptococcus agalactiae; E.c. = Escherichia coli; S.e. = Salmonella enterica serovar* Typhimurium; *C.a. = Candida albicans; A.f. = Aspergillus fumigatus; R. sp. = Rhizopus* sp.

(Candida albicans, *Aspergillus fumigatus* and *Rhizopus* sp). This could be explained because both Gram-negative bacteria and some fungi have evolved significant permeability barriers, as well as pump mechanisms [18,21-23].

Considering that the ethyl acetate and butanol fractions from roots demonstrated the best antibacterial action, they were chromatographed on silica gel column eluted with $CHCl_3$:MeOH gradient, monitored by TLC given the following compounds: niga-ichigoside F1 [**1**], 23-hidroxitormentic acid [**2**], 4'-methyl-3-O-methylellagic acid [**3**], and 3-O-methylellagic-4'-O-α rhamnose acid [**4**], which were directly compared with authentic samples and spectroscopic data (IR, ^{1}H- and ^{13}C-NMR) (Fig.1). Table 1 shows that the compounds isolated were inactive in this experimental model, suggesting that the active principles are in minor concentration or the existence of a possible synergic effect.

In summary, this study had shown that despite the high values of MICs, *R. imperialis* could be used for treatment of the skin infection (topical) but would not be indicated for the systemic treatment of infection.

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