Phytomedicine 20 (2013) 258–261

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Short communication

Understanding the interactions between metabolites isolated from *Achyrocline satureioides* in relation to its antibacterial activity

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ARTICLE INFO

Keywords:
Achyrocline satureioides
Synergism
Natural antibiotics

ABSTRACT

As part of our ongoing research on the antibacterial activity of *Achyrocline satureioides*, this study seeks to better understand the interactions between the metabolites isolated from this plant. For this purpose, the combined effect of 23-methyl-6-*O*-desmethylauricepyrone (**1**), quercetin (**2**) and 3-*O*-methylquercetin (**3**), obtained through bioguided fractionation from *A. satureioides* ethanol extract, was evaluated against *Staphylococcus aureus* and *Escherichia coli*. In first place, the antibacterial effect of the combination of flavonols **2** and **3** was assessed, as these showed individual effectiveness lower than or equal to that of the fraction from which they were obtained. When the flavonols were applied together at concentrations below their minimum inhibitory concentration (MIC) values, a synergistic effect (FICI < 0.30) against *S. aureus* was observed. In addition, compounds **2** and **3** in combination reduced 1000 times the MIC of compound **1**, showing a clear synergistic interaction (FICI < 0.15) in treatments against the Gram (+) bacterium. The most active combination against *E. coli* showed an additive interaction (FICI < 0.62) between the three assayed compounds **1**–**3**. These results indicated the existence of concerted action between these metabolites, evidence of the importance of the synergistic interactions between the components of plant-derived extracts for the control of pathogenic bacteria.

Introduction

Plants produce a wide range of metabolites with an evidence-based inhibitory effect against pathogenic microorganisms, including bacteria (Carpinella et al. 2011). These plant-derived compounds are gaining increasing interest, since the extensive and indiscriminate use of conventional antibiotics has led to the relentless emergence of resistant microorganisms (Flamm et al. 2012).

Responding to market and industrial requirements, the search for new effective antibacterials from plants has aimed to find a single active compound (Ulrich-Merzenich et al. 2010) and this is currently known as a "reductionist approach" (Verpoorte et al. 2006). Many efforts have been made in recent times to isolate and identify molecules that could be considered really successful. However, in most cases, plant secondary metabolites have shown a weak effect against bacteria (Hemaiswarya et al. 2008). Even then, plants themselves can successfully deal with bacterial invasions, which may be due to their adopting another approach to combat these: the strategy of "synergy" (Hemaiswarya et al. 2008).

Among the plants popularly used as remedies, *Achyrocline satureioides* (Lam.) DC. (Asteraceae) is an aromatic annual herb native to South America (Hnatyszyn et al. 2004), infusions of which have been used for the treatment of different disorders (Joray et al. 2011a,b). Continuing our investigation of the antibacterial activity of *A. satureioides*, the present work takes a step forward and studies the interactions between metabolites isolated from this.

Materials and methods

Plant material

A. satureioides was collected in the hills of Córdoba Province, Argentina. A voucher specimen (UCCOR 46) has been deposited in the "Marcelino Sayago" Herbarium, Catholic University of Córdoba. Crushed aerial plant material was extracted by 48 h maceration with ethanol.

Microorganisms

Escherichia coli (Migula) Castellani and Chalmers (ATCC 25922) and *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 6538) were purchased from Medicatec S.R.L (Buenos Aires, Argentina). Bacterial suspensions were prepared on sterile saline from each organism grown overnight. Turbidity was spectrophotometrically

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http://dx.doi.org/10.1016/j.phymed.2012.10.015

Table 1
Antibacterial activity of 23-methyl-6-*O*-desmethylauricepyrone (**1**), quercetin (**2**) and 3-*O*-methylquercetin (**3**) against *Escherichia coli* and *Staphylococcus aureus*.

Sample	MIC (MBC) (mg/ml)	
	Escherichia coli	*Staphylococcus aureus*
1	0.250 (0.500)[a]	0.002 (0.008)[a]
2	>4 (>4)	>4 (>4)
3	>4 (>4)	0.125 (>4)
F2	2 (2)	0.125 (1)
Erythromycin	0.125 (4)	0.001 (0.060)
Gentamicin	0.004 (0.008)	0.008 (0.010)

[a] Results obtained from Joray et al. (2011a,b).

adjusted to a 0.5 McFarland standard. Dilution was performed with sterile saline to give an adjusted concentration of 1.5×10^7 CFU/ml.

Chemicals, equipment and reagents

^{1}H- and ^{13}C NMR spectra were recorded with a Bruker AVANCE II 400 spectrometer (Bruker Corporation, Ettlingen, Germany). HPLC was performed on a Shimadzu LC-10 AS (Shimadzu Corp., Tokyo, Japan), equipped with a Phenomenex Prodigy 5μ ODS (4.6 mm i.d. × 250 mm) reversed-phase column. The mobile phase was methanol/phosphoric acid 0.16 M 53:47 and UV detection was at 210 or 340 nm.

Gentamicin sulfate (potency: 550–590 μg/mg) and erythromycin (potency: 863 μg/mg) were purchased from Laboratorio Fabra, Buenos Aires, Argentina, and Unifarma, Buenos Aires, Argentina, respectively.

Bioguided isolation of the active principles from A. satureioides

The ethanol extract from *A. satureioides* (8.2 g) was chromatographed using vacuum liquid chromatography on silica gel with hexane–ethyl ether–methanol gradient, obtaining five fractions (F1–F5). F2 and F3, which showed bactericidal activity at 2 mg/ml, were further purified using column chromatography, yielding 12 fractions (F1–F12). Two groups of antibacterial fractions were observed, F5–F7 and F9–F11. From the latter, the active compound 23-methyl-6-*O*-desmethylauricepyrone (**1**) was obtained as previously described (Joray et al. 2011a). F5–F7 were further submitted to column chromatography, finally obtaining F1–F4. Of these, F2 showed bactericidal activity against both Gram (−) and (+) strains (Table 1). This fraction was submitted to two-dimensional ascending paper chromatography with tert-butanol/glacial acetic acid/water 3:1:1 as the first phase and glacial acetic acid 25% as the second one. Two spots that presented UV absorption at 365 nm were cut and eluted with methanol, yielding two compounds. These were identified as quercetin (**2**) (95% purity; 0.17 g/100 g of crushed plant material, by HPLC) and 3-*O*-methylquercetin (**3**) (90% purity; 0.47 g/100 g of crushed plant material, by HPLC) by comparison of their spectral data (^{1}H NMR and ^{13}C NMR) with previously reported values (Rizk et al. 1993; Zheng et al. 2008). The HPLC profiles of F2 showed that **2** and **3** were by far the major components of this active fraction. The minor compounds present in the fraction did not show antibacterial activity (data not shown).

Determination of MICs and MBCs

MICs and MBCs were determined as previously described (Joray et al. 2011a).

Diagonal constant ratio combination assay

The interactions between the isolated compounds were tested using a fixed diagonal ratio scheme (Berenbaum 1977). In the assays

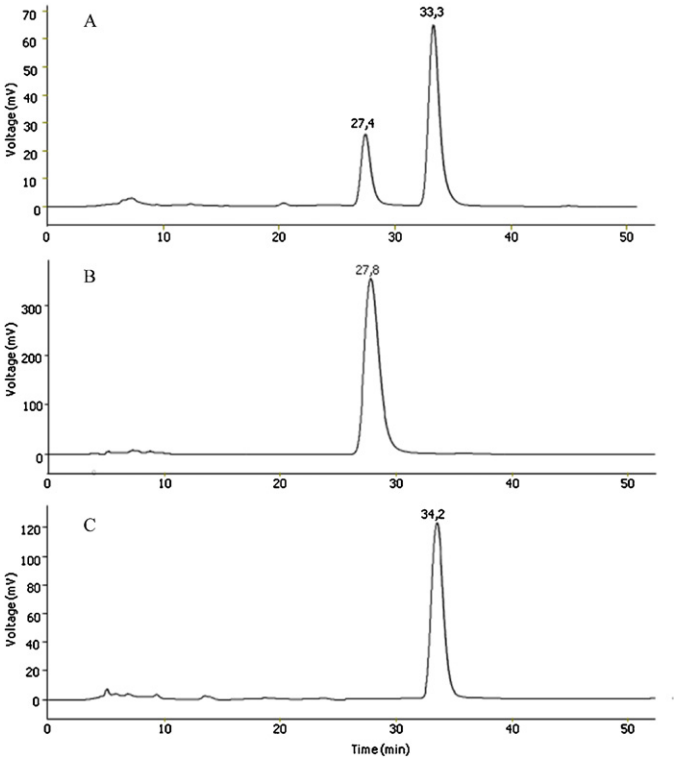

Fig. 1. Chromatographic fingerprints of *Achyrocline satureioides* active fraction F2 (A), quercetin (**2**) (B) and 3-*O*-methylquercetin (**3**) (C) obtained by HPLC at 340 nm. Each chromatographic peak appears with its corresponding retention time.

against *S. aureus*, the initial concentration of the mixture of both flavonols corresponded to the MIC value observed in treatments with F2 or **3** (0.125 mg/ml). The proportion at which each flavonoid was paired was decided according to the ratio of these in the original fraction F2, determined by HPLC, which was approximately quercetin/3-*O*-methylquercetin 40:60 (Fig. 1). The same proportion was also used in studies against *E. coli*, with a maximum initial concentration of 2 mg/ml.

The fractional inhibitory concentration (FIC) was calculated using the MIC from the diagonal constant ratio combination assay and the MIC of each compound alone, obtained in parallel in the same assay according to the following formula:

$$\text{FIC} = \frac{\text{MIC of antimicrobial agent in combination}}{\text{MIC of antimicrobial agent alone}}$$

Then, the synergistic effect was evaluated by calculating the $\text{FIC}_{\text{index}}$ (FICI) for each combination by adding the individual FIC values. Since at least one of the compounds included in the combination has no effect, an imprecise FICI was calculated from the results obtained with the fixed ratio scheme, following Berenbaum (1977). An imprecise FICI was also reported by other authors, who performed tests for antibacterial activity by the checkerboard method (Eumkeb et al. 2010).

A FICI < 0.5 indicated synergy for the combination. When it fell between 0.5 and 1, it was defined as an additive effect and between 1 and 4.0, it was classified as "no interaction". Finally, a FICI > 4.0 would indicate antagonism between the components in the combination.

Results and discussion

Determination of MICs and MBCs

In a previous work we reported the isolation of the antibacterial principle 23-methyl-6-*O*-desmethylauricepyrone (**1**) from *A. satureioides* extract (Joray et al. 2011a). This time, further bioguided fractionation in this plant resulted in the isolation of quercetin (**2**) and 3-*O*-methylquercetin (**3**). Although both compounds had been previously reported in *A. satureioides* (Hnatyszyn et al. 2004), this is the first time that their role in the antibacterial activity of this plant is investigated. As seen in Table 1, compounds **2** and **3** showed null or moderate bacteriostatic activity, with no killing effect on either bacterium. Even though there are precedents in the literature about the antibacterial activity exerted by compound **2** against *E. coli* and against some methicillin-susceptible and methicillin-resistant *S. aureus* (Hirai et al. 2010; Kuete et al. 2011), other authors reported a lack of effectiveness (Hossion et al. 2010; Li et al. 2009) matching that obtained in this work. The level of activity shown by compound **3** against *E. coli* and *S. aureus*, does not match that found by Van Puyvelde et al. (1989), but it does agree with the results obtained by Talib et al. (2012) in a microtiter plate dilution test performed against *E. coli*. Discrepancies in the effectiveness of flavonoids have been attributed to differences in the solubility of these compounds, in the conditions of the assays performed and in the judgment of the MICs (Cushnie and Lamb 2005; Wu et al. 2008).

Diagonal constant ratio combination assay

The absence of or the low inhibitory effect observed in treatments with compounds **2** or **3** is discordant with the effectiveness observed in the active fraction F2 from which these compounds were obtained (Table 1). This led us to suspect the presence of a concerted action between them, and therefore to evaluate the antibacterial activity of combinations of both flavonols.

As expected, a considerable synergistic effect (FICI < 0.30) was observed between compounds **2** and **3** against *S. aureus* when they were jointly applied at >160 and 3 times below their MICs, respectively (Table 2). This may explain why F2 showed higher or the same level of activity as **2** or **3**. However, no interaction was observed in assays with *E. coli*, even at 0.8 and 1.2 mg/ml of **2** and **3**, respectively (data not shown). Since F2 showed inhibition on this bacterium, this result would suggest that the presence of the other minority compounds may contribute to the inhibitory effect demonstrated by this fraction. This agrees with Wagner and Ulrich-Merzenich (2009), who reported that interactions between all the components of a plant extract or fraction, including minor metabolites and fibers, may contribute to synergy effects.

In a second phase of the study, we decided to include compound **1** with the paired combination of flavonols. The results obtained following *S. aureus* challenge by this ternary combination were very encouraging. Compounds **2** and **3**, combined at 0.012 and 0.018 mg/ml, reduced the MIC of **1** 1000 times, with a FICI value of <0.15 (Table 3), thus showing a clear synergistic interaction between them. Moreover, the most active ternary combination against *E. coli* showed an additive interaction with a FICI value of <0.62, with **1**, **2** and **3** combined at concentrations of 2 and at least 20 and 13 times lower than their MIC values, respectively (Table 4).

In order to check whether the effectiveness observed was due to the action of each flavonoid in paired combination with **1**, treatments were carried out with combinations of **1** and **2** or **1** and **3,** no inhibition was observed against *E. coli* or *S. aureus*.

Table 2
Minimum inhibitory concentrations (MICs) in mg/ml and fractional inhibitory concentration indices (FICIs) of combinations of quercetin (**2**) and 3-*O*-methylquercetin (**3**) against *Staphylococcus aureus*.

Compound **2** (MIC > 4) MIC_c	Compound **3** (MIC = 0.125) MIC_c	Bacterial growth	FIC	FICI
0.050	0.075	–	FIC_2 < 0.012 FIC_3 = 0.600	<0.612
0.025	0.037	–	FIC_2 < 0.006 FIC_3 = 0.296	<0.302
0.012	0.018	+	–	–
0.006	0.009	+	–	–

MIC: minimum inhibitory concentration of the compound alone; MIC_c: minimum inhibitory concentration of the combination.
+: presence of bacterial growth; –: absence of bacterial growth.
FIC: fractional inhibitory concentration; FICI: fractional inhibitory concentration index.

Table 3
Minimum inhibitory concentrations (MICs) in mg/ml and fractional inhibitory concentration indices (FICIs) of combinations of 23-methyl-6-*O*-desmethylauricepyrone (**1**), quercetin (**2**) and 3-*O*-methylquercetin (**3**) against *Staphylococcus aureus*.

Compound **1** (MIC = 0.002) MIC_c	Compound **2** (MIC > 4) MIC_c	Compound **3** (MIC = 0.125) MIC_c	Bacterial growth	FIC	FICI
0.00003	0.012	0.018	–	FIC_1 = 0.015 FIC_2 < 0.003 FIC_3 = 0.144	<0.162
0.000015	0.012	0.018	–	FIC_1 = 0.007 FIC_2 < 0.003 FIC_3 = 0.144	<0.154
0.0000078	0.012	0.018	–	FIC_1 = 0.004 FIC_2 < 0.003 FIC_3 = 0.144	<0.151
0.0000019	0.012	0.018	–	FIC_1 = 0.001 FIC_2 < 0.003 FIC_3 = 0.144	<0.148
0.00000097	0.012	0.018	+	–	–
0.001	0.006	0.009	+	–	–

MIC: minimum inhibitory concentration of the compound alone; MIC_c: minimum inhibitory concentration of the combination.
+: presence of bacterial growth; –: absence of bacterial growth.
FIC: fractional inhibitory concentration; FICI: fractional inhibitory concentration index.

Table 4
Minimum inhibitory concentrations (MICs) in mg/ml and fractional inhibitory concentration indices (FICIs) of combinations of 23-methyl-6-*O*-desmethylauricepyrone (**1**), quercetin (**2**) and 3-*O*-methylquercetin (**3**) against *Escherichia coli*.

Compound **1** (MIC = 0.250) MIC_c	Compound **2** (MIC > 4) MIC_c	Compound **3** (MIC > 4) MIC_c	Bacterial growth	FIC	FICI
0.125	0.8	1.2	–	$FIC_1 = 0.500$ $FIC_2 < 0.200$ $FIC_3 < 0.300$	<1.000
0.125	0.4	0.6	–	$FIC_1 = 0.500$ $FIC_2 < 0.100$ $FIC_3 < 0.150$	<0.750
0.125	0.2	0.3	–	$FIC_1 = 0.500$ $FIC_2 < 0.050$ $FIC_3 < 0.075$	<0.625
0.125	0.1	0.15	+/–	–	–
0.125	0.05	0.075	+	–	–
0.062	0.2	0.3	+	–	–

MIC: minimum inhibitory concentration of the compound alone; MIC_c: minimum inhibitory concentration of the combination.
+: presence of bacterial growth; +/–: moderate bacterial growth; –: absence of bacterial growth.
FIC: fractional inhibitory concentration; FICI: fractional inhibitory concentration index.

The present results highlight the presence of synergistic interactions against bacteria between some of the constituents of *A. satureioides* extract.

These results support the suggestion that, when studying the pharmacological mechanism of a plant-derived product, interactions between components may explain biological actions that are often absent when the isolated substance is tested individually (Spinella 2002).

Conflict of interest

There was no conflict of interest.

Acknowledgments

This work was supported by FONCYT BID 1728 33593 and PICTO CRUP 6-31396, MINCyT GRF 2008, CONICET PIP 11220100100236 and UCC.

MBJ acknowledges receipt of a fellowship from CONICET. We thank Joss Heywood for revising the English language.

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