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**Inhibition of efflux pumps in meticillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* resistant strains by triterpenoids from *Momordica balsamina***

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## ABSTRACT

Six cucurbitane-type triterpenoids (**1–6**) isolated from the aerial parts of *Momordica balsamina* were evaluated for their ability to inhibit the activity of bacterial efflux pumps of methicillin-resistant *Staphylococcus aureus* (MRSA) COL<sub>OXA</sub>, *Enterococcus faecalis* ATCC 29212, *Salmonella enterica* subsp. I serovar Typhimurium 5408 and *S. Typhimurium* 5408CIP strains. The latter strain overproduces the AcrB transporter of the AcrAB-TolC efflux pump six-fold compared with its parent. Compounds **4–6** were also tested for similar activity against *Escherichia coli* AG100 wild-type strain and *E. coli* AG100TET8 that overproduces the AcrAB-TolC efflux pump. Evaluation of efflux activity was performed using a semi-automated method that measures accumulation of the universal efflux pump substrate ethidium bromide (EtBr). Some of the compounds significantly inhibited efflux of EtBr by MRSA COL<sub>OXA</sub> and *E. faecalis* ATCC 29212. A correlation between activity and the topological polar surface area of the compounds was found for MRSA COL<sub>OXA</sub>.

## 1. Introduction

Among the mechanisms that may be involved in bacterial resistance, efflux pumps that extrude antibiotics thereby decreasing the intracellular concentration of the antibiotic are considered as one of the most important contributors to antibiotic resistance [1]. These efflux systems can be responsible for resistance to a specific class of antibiotics or to a large number of unrelated antimicrobial agents, thus conferring a multidrug-resistant (MDR) phenotype [2].

Development of compounds able to modulate efflux systems [efflux pump inhibitors (EPIs)] and thus restore the activity of antibiotics when used in combination has been considered a promising approach to overcome bacterial resistance [3].

In the search for biologically active compounds from *Momordica balsamina* L. (Cucurbitaceae), a plant that is widespread in tropical and subtropical regions and is largely used as food, mainly in sub-Saharan Africa, several cucurbitane-type triterpenes have been isolated and characterised [4–6]. *Momordica balsamina* has also been widely used traditionally in Africa as a medicinal plant for curing various diseases such as diabetes and malaria [4–6]. In this study, several compounds isolated from *M. balsamina* were evaluated for their efflux-modulating effects on Gram-positive and Gram-negative bacteria using a real-time fluorometric method that utilises the fluorochrome ethidium bromide (EtBr), a universal substrate of bacterial efflux pumps [7].

## 2. Materials and methods

### 2.1. Plant material

Aerial parts of *M. balsamina* were collected in Gaza, Mozambique, in August 2006. Plant material was identified by Dr Silva Mulhovo, and a voucher specimen (30 SM) was deposited at the herbarium of the Instituto de Investigação Agronómica, Mozambique.

### 2.2. Compounds tested

Six compounds with the cucurbitane skeleton were isolated and evaluated for their efflux-modulating effects in several Gram-positive and Gram-negative bacterial strains. Their identities and structures are shown in Fig. 1: balsaminol A [**1**; cucurbita-5,24-diene-3 $\beta$ ,7 $\beta$ ,23(*R*),29-tetraol]; balsaminol F [**2**; cucurbita-5,24-diene-3 $\beta$ ,7 $\beta$ ,23(*R*)-triol]; balsaminagenin A [**3**; cucurbita-5,23(*E*)-diene-3 $\beta$ ,7 $\beta$ ,25,29-tetraol]; balsaminagenin B [**4**; 25-methoxycucurbita-5,23(*E*)-diene-3 $\beta$ ,7 $\beta$ ,29-triol]; balsaminoside A [**5**; 25-methoxycucurbita-5,23(*E*)-dien-3 $\beta$ -ol-7-*O*- $\beta$ -D-allopyranoside]; and karavilagenin C [**6**; 7 $\beta$ -methoxycucurbita-5,24-diene-3 $\beta$ ,23(*R*)-diol]. Compounds **1** and **3–6** have been isolated previously [4,5]. Isolation and characterisation of compound **2** will be reported elsewhere. Compounds **1–6** share the same triterpenic nucleus and may be considered as a homogeneous set of compounds. They differ only in the substitution pattern at C-29 (ring A), which bears an extra hydroxyl group in compounds **1** and **3–5**, and at C-7 (ring B), where a free hydroxyl is replaced in compounds **6** and **5** by a methoxyl group or a sugar moiety, respectively. Compounds **3–5** also differ from compounds **1**, **2** and **6**, having a

double bond at C-23 instead of a hydroxyl function, and a hydroxyl/methoxyl group at C-25. All the compounds were dissolved in dimethyl sulphoxide (DMSO).

### 2.3. Bacterial strains

The Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA) COL<sub>OXA</sub> and *Enterococcus faecalis* ATCC 29212 as well as the Gram-negative bacteria *Escherichia coli* AG100, *E. coli* AG100TET8, *Salmonella enterica* subsp. I serovar Typhimurium 5408 and *S. Typhimurium* 5408CIP were evaluated for efflux activity in the presence and absence of the compounds. MRSA COL (kindly provided by Prof. Hermínia de Lencastre, Department of Microbiology, Institute of Technology, Chemistry and Biology, Universidade Nova de Lisboa, Lisbon, Portugal) was adapted to 1600 mg/L oxacillin and was renamed MRSA COL<sub>OXA</sub> [8]. MRSA COL<sub>OXA</sub> overproduces its main efflux pump NorA [8]. *Enterococcus faecalis* ATCC 29212 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and was characterised for its efflux pump activity [9]. *Escherichia coli* AG100TET8 was derived from *E. coli* AG100 following prolonged exposure to increasing concentrations of tetracycline. *Escherichia coli* AG100TET8 overexpresses its main AcrAB-TolC efflux pump as well as its lesser active AcrEF. *Salmonella* Typhimurium 5408 as well as its progeny adapted to 16 mg/L ciprofloxacin (renamed *S. Typhimurium* 5408CIP) were kindly provided by Prof. Seamus Fanning (Centre for Food Microbiology, UCD Veterinary Services Centre, University College of Dublin, Dublin, Ireland). The AcrB transporter of *S. Typhimurium* 5408CIP is six-fold overexpressed compared with that of its parent *S. Typhimurium* 5408.



#### 2.4. Growth conditions

*Escherichia coli* strains were grown at 37 °C in Luria–Bertani broth purchased in powder form from Oxoid Ltd. (Basingstoke, UK). The remaining bacterial strains were grown at 37 °C in tryptic soya broth or tryptic soya agar (Oxoid Ltd.).

#### 2.5. Determination of minimum inhibitory concentrations (MICs)

MICs of the compounds were determined by the microplate broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) recommendations [10]. The MIC, defined as the lowest concentration of compound at which no visible growth (turbidity) was present, was determined after 18 h of incubation at 37 °C. The MIC for each compound was determined at least three separate times.

#### 2.6. Real-time ethidium bromide accumulation assay

The activity of the compounds against bacterial efflux pump systems was assessed by the semi-automated EtBr method as previously described [7] using a Rotor-Gene 3000™ Thermal Cycler with real-time analysis software (Corbett Research, Sydney, Australia). Briefly, strains were cultured in appropriate medium until they reached an optical density at 600 nm ( $OD_{600}$ ) of 0.6 and were then centrifuged at 13 000 rpm for 3 min. Pellets were re-suspended and were washed twice in phosphate-buffered saline (PBS) (pH 7.4) containing glucose to yield a final concentration of 0.4%. The  $OD_{600}$  was adjusted with PBS–glucose to 0.6, the suspension was mixed by vortex and 45  $\mu$ L was transferred to 0.2 mL tubes. Then, 5  $\mu$ L of DMSO blank control (to yield a final DMSO concentration of 5%) or 5  $\mu$ L of the compounds dissolved in

DMSO at different concentrations (3  $\mu$ M and 30  $\mu$ M) was individually added to the tubes, followed by addition of 45  $\mu$ L of PBS containing 1  $\mu$ g/mL or 0.5  $\mu$ g/mL EtBr (Sigma-Aldrich Química SA, Madrid, Spain).

The tubes were placed in a Rotor-Gene 3000 Thermal Cycler and the amount of fluorescence representing accumulated EtBr by the bacterium was monitored on a real-time basis [7].

From the real-time data using relative fluorescence (RF) over a 30-min period of EtBr accumulation, the slope of the accumulation–time curve was calculated by linear regression analysis (Microsoft Excel; Microsoft Corp., Redmond, WA). The greater the difference in the slope between the compound-containing assay and its relative control (no compound) was indicative of the activity of the compound to inhibit the efflux of EtBr. The relative final fluorescence (RFF) of the last time point (60 min) of the EtBr accumulation assay was also calculated according to the formula:

$$\text{RFF} = \text{RF}_{\text{treated}} - \text{RF}_{\text{untreated}}$$

where  $\text{RF}_{\text{treated}}$  is the RF at the last time point of the EtBr retention curve in the presence of an inhibitor, and  $\text{RF}_{\text{untreated}}$  is the RF at the last time point of the EtBr retention curve of the untreated control having the solvent control (DMSO).

The amount of DMSO in the assay was 5% and shown not to have any effect on the viability of the bacteria employed in this study.

### 2.7. Determination of cytotoxicity

Cytotoxicity of the isolated compounds was determined by the Trypan blue exclusion assay as described previously in detail [11].

## 3. Results

To determine efflux-modulating activity, it is necessary to use a concentration of any potential EPI that is below its antibacterial concentration [12]. Selection of this concentration was afforded by determining the MIC against the bacterial strains used in the accumulation assay. As shown in Table 1, except for compounds **2** and **6** that showed weak activity against MRSA COL<sub>OXA</sub> (MIC values of 25  $\mu$ M for both compounds), no significant antibacterial activity (MIC range 50 to >200  $\mu$ M) was observed at concentrations similar to or higher than the highest concentration used in the EtBr accumulation assay.

The cucurbitane-type triterpenoids **1–6** isolated from the aerial parts of the African plant *M. balsamina* were evaluated at two concentrations (3  $\mu$ M and 30  $\mu$ M) for their ability to modulate the activity of bacterial efflux pumps of some Gram-positive (MRSA COL<sub>OXA</sub> and *E. faecalis* ATCC 29212) and Gram-negative (*S. Typhimurium* 5408 and *S. Typhimurium* 5408CIP) strains. Compounds **4–6** were also tested against two strains of *E. coli* (AG100 and AG100TET8).

An example of the results obtained from the semi-automated method and the calculation by linear regression analysis of slopes representing accumulation by

MRSA COL<sub>OXA</sub> in the presence and absence of the two concentrations of balsaminoside A (**5**) is shown in Fig. 2.

Briefly, whereas at 3  $\mu$ M the activity of the compound was small, at 30  $\mu$ M the activity was significantly greater as noted by the graph and the calculated slopes for both concentrations as well as that for the control. Similar results were obtained when RFF was calculated. Evaluation of the other compounds for activity against the efflux pumps of MRSA COL<sub>OXA</sub> is summarised in Table 1. The most active compounds at the lower concentration of 3  $\mu$ M were karavilagenin C (**6**) and balsaminol F (**2**), yielding slopes of 0.90 and 0.88, respectively. However, at the highest concentration of 30  $\mu$ M the slope of these compounds was almost 50% lower. Although the reason for this is not clear, it may well be that the highest concentration applied exceeds the saturation of binding to the efflux pump, or that at high concentrations dimers or higher degrees of polymerisation may occur and these may not have as greater effect on the efflux pump as the monomer itself. All the other compounds expressed activities against the efflux pump system of MRSA COL<sub>OXA</sub> in a concentration-dependent manner. Table 1 also summarises the effects of the isolated compounds on the efflux system of *E. faecalis*. As noted in Table 1, balsaminagenin B (**4**) had the greatest activity on the accumulation of EtBr. Comparison of the activities of each compound against the efflux systems of MRSA COL<sub>OXA</sub> and *E. faecalis* suggests that the majority of compounds have similar activities.

Regarding Gram-negative bacteria, no significant activity against the efflux systems of *S. Typhimurium* and *E. coli* strains was observed for any of compounds listed in Table 1 (data not shown).

Cytotoxicity assays of the compounds studied for activity against the efflux pump systems of bacteria indicated that at multiples of the highest concentration of compound used, no toxicity against human lymphocytes was evident (data not shown).

#### 4. Discussion

The search for antibacterial agents from plant sources is a promising approach as shown by the many papers published in recent years [3]. The rationale for this search lies in the use of plants in traditional medicine for ailments associated with bacterial infections [13]. However, most of the compounds exhibit weak activity against bacteria, suggesting that perhaps the in vivo mechanism of action is not one of a direct antibacterial type [14]. The pioneering studies by Lewis [15] indicated that compounds isolated from plants had the ability to reduce and even reverse the resistance of MDR bacterial strains to antibiotics. Unfortunately, many of these compounds, such as berberine, are toxic [15].

In contrast, most of the compounds isolated from *M. balsamina* have been shown to have no cytotoxicity against the breast cancer cell line MCF-7 [6], and all of the compounds isolated and studied for activity against bacterial efflux pumps in the current study have also been shown to have no toxicity against human lymphocytes (data not shown).

Plants are good sources for inhibitors of transporters of cancer cells [4]. Generally speaking, inhibitors of cancer transporters that render the cancer cell resistant to

cytotoxic agents also have similar activity against ATP-binding cassette (ABC) transporters of bacteria [16]. Some of the compounds isolated from *M. balsamina* have been shown to inhibit the efflux pump system of cancer cells [4] and in the current study were shown to inhibit the efflux pump system of Gram-positive bacteria. However, whether the inhibition of efflux in the current study is due an inhibition of to ABC transporter(s) or a resistance–nodulation–cell division (RND) type(s) remains to be determined.

The compounds isolated from *M. balsamina* clearly have activity against the efflux pump system of Gram-positive bacteria. The structural relationship of these compounds may provide clues as to the site of the molecule involved in the inhibition of efflux. To determine some structure–activity relationships, despite the limited number of compounds, theoretical octanol/water partition coefficients ( $\log P$ ) and other general physicochemical properties were calculated (data not shown). All compounds are lipophilic, having  $\log P$  values in the range 5.2–7.3. They have a molecular weight between 634 Da and 472 Da and a topological polar surface area (TPSA), which is defined as the sum of surfaces of polar atoms in a molecule, in the range 60.7–128.8. Moreover, all compounds are H-bond acceptors (between 3 and 8) and donors (between 2 and 5). When comparing the RFF for compounds **1–6** against MRSA COL<sub>OXA</sub>, there is a correlation ( $r^2 = 0.95$ ) between these values and TPSA. TPSA is considered an important descriptor in drug discovery, affording the prediction of molecular ability to cross biological membranes. Conversely, no correlation was observed between the reversal of antibiotic resistance and  $\log P$  values. From this, we can conclude that partitioning does not play a significant role in the mechanism by which the compounds inhibit the efflux of EtBr by MRSA COL<sub>OXA</sub>.

Previously, compounds **1** and **3–6** were also evaluated for their activity as multidrug resistance modulators in mouse T-lymphoma cells transfected with the human *mdr1* gene [4]. It is interesting to note that compound **6** also has the greatest ability to reverse resistance of cancer cells to doxorubicin [4], suggesting that this compound might interact with bacterial ABC transporters, which are functionally related to the eukaryotic multidrug resistance P-glycoprotein previously mentioned.

For *E. faecalis* ATCC 29212, no apparent correlation was found between reversing of antibiotic resistance and the calculated physicochemical properties, suggesting that several factors may be involved. It is of note that balsaminagenin B (**4**), the most active compound, differs from compound **3** (that was inactive) only by the presence of a methoxyl at C-25 that is replaced by a free hydroxyl group in compound **3**, suggesting that the methoxyl group at that position may play an important role in the activity. It should also be noted that karavilagenin C (**6**), the most effective compound at the lowest concentration in the accumulation assay with MRSA COL<sub>OXA</sub>, weakly inhibited the efflux of EtBr by *E. faecalis*, emphasising the complexity of efflux systems in these bacteria.

Ineffectiveness of the isolated compounds against the efflux pump systems of Gram-negative bacterial strains may be due to the outer membrane and its lipopolysaccharide layer that can act as an effective barrier to compounds [3], or to RND-type efflux pumps that are not affected by the compounds studied. As result of this study, we suggest that cucurbitane-type triterpenoids may be used as lead compounds in the development of EPIs of Gram-positive bacteria.

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**Competing interests**

None declared.

**Ethical approval**

Not required.

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**Fig. 1.** Chemical structures of compounds 1–6.

**Fig. 2.** (a) Effect of compound 5 on the accumulation of ethidium bromide (EtBr) (1  $\mu\text{g}/\text{mL}$ ) by methicillin-resistant *Staphylococcus aureus* (MRSA) COL<sub>OXa</sub>. (b) Effect of compound 4 on the accumulation of EtBr (0.5  $\mu\text{g}/\text{mL}$ ) by *Enterococcus faecalis*.  $\blacklozenge$ , dimethyl sulphoxide control,  $\blacktriangle$ , 3  $\mu\text{M}$  compound,  $\bullet$ , 30  $\mu\text{M}$  compound. Solid lines represent the slope of accumulation as calculated by regression analysis.

**Table 1**

Minimum inhibitory concentrations (MICs) and effects of compounds **1–6** on accumulation of ethidium bromide (EtBr) in methicillin-resistant *Staphylococcus aureus* (MRSA) COL<sub>OXA</sub> and *Enterococcus faecalis* ATCC 29212

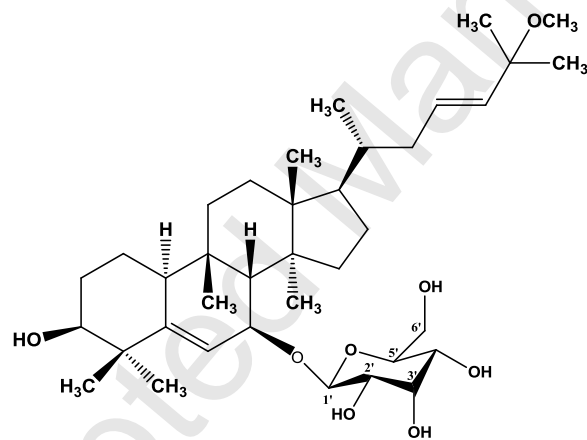
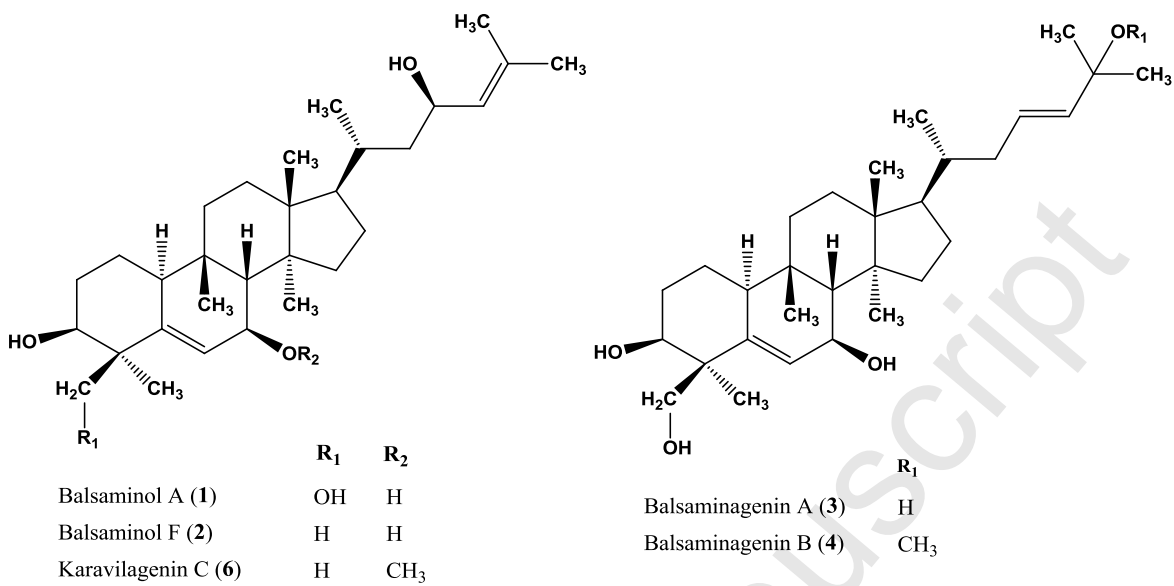
Compound	MRSA COL <sub>OXA</sub>					<i>E. faecalis</i>				
	MIC ( $\mu$ M)	Slope <sup>a</sup>		RFF <sup>b</sup>		MIC ( $\mu$ M)	Slope <sup>a</sup>		RFF <sup>b</sup>	
		3 $\mu$ M	30 $\mu$ M	3 $\mu$ M	30 $\mu$ M		3 $\mu$ M	30 $\mu$ M	3 $\mu$ M	30 $\mu$ M
DMSO <sup>c</sup>	–	0.36		–		–	0.18		–	
<b>1</b>	50	0.58	0.95	13.5	15.7	100	0.17	0.67	1.0	18.3
<b>2</b>	25	0.88	0.61	21.1	14.2	200	0.21	0.69	2.6	23.4
<b>3</b>	100	0.61	0.77	15.7	21.7	>200	0.18	0.23	0.1	3.1
<b>4</b>	50	0.55	0.98	17.3	25.1	200	0.24	0.97	5.5	32.8
<b>5</b>	200	0.58	1.09	8.1	34.1	>200	0.14	0.25	5.3	5.2
<b>6</b>	25	0.90	0.51	21.8	9.7	>200	0.20	0.14	1.7	10.4

DMSO, dimethyl sulphoxide.

<sup>a</sup> Slope of accumulation of EtBr by control (no compound plus DMSO to yield a final concentration of 5%) and by cells in presence of two concentrations of compounds in DMSO to yield a final concentration of 5%.

<sup>b</sup> Relative final fluorescence (RFF) =  $RF_{\text{treated}} - RF_{\text{untreated}}$ , where  $RF_{\text{treated}}$  is the relative fluorescence (RF) at the last time point of the EtBr retention curve in the presence of an inhibitor, and  $RF_{\text{untreated}}$  is the RF at the last time point of the EtBr retention curve of the untreated control having the solvent control (DMSO).

<sup>c</sup> DMSO at concentrations as high as 10% had no effect on the viability of the bacteria.



Balsaminoside A (5)

