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Original Paper

Casearia sylvestris essential oil and its fractions inhibit *Candida albicans* ABC transporters related to multidrug resistance (MDR)

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Abstract

ABC transporters constitute a superfamily of transmembrane proteins that act mediating the translocation of several substrates across the membrane, using the energy of ATP hydrolysis. This mechanism of unrelated substrates efflux (multidrug resistance) has been associated with several diseases and it is a problem in chemotherapy efficacy. Nowadays, approximately 25% of the prescription drugs in the world are derived from plants. *Casearia sylvestris* is commonly found in the Americas and different parts of this plant are popularly used to treat several diseases. Previous studies have also confirmed the biological activities of *C. sylvestris*, such as anti-tumor, anti-leishmania, and antifungal properties. Then, the propose of this study was demonstrate that fraction 1-6 of *C. sylvestris*, essential oil, was able to reverse the fluconazole resistance phenotype in the *Saccharomyces cerevisiae* model mediated by the heterologous protein CaCdr2p from *Candida albicans*. The MIC value of fraction 1-6 combined with fluconazole in the checkerboard assay decreased approximately 4-fold, suggesting a synergistic effect. In addition, fraction 1-6 increased intracellular rhodamine 6G accumulation from 17% to 49% in the presence of glucose. Data indicate that *C. sylvestris* fraction 1-6 is a potential reverser of the fluconazole resistance phenotype.

Key words: ABC transporters, Atlantic Forest, fluconazole, sesquiterpenes, yeast.

Resumo

Os transportadores ABC constituem uma superfamília de proteínas transmembranares que atuam mediando a translocação de vários substratos através da membrana, utilizando a energia da hidrólise de ATP. Esse mecanismo de efluxo de substratos não relacionados (resistência a múltiplas drogas) tem sido associado a várias doenças e é um problema na eficácia da quimioterapia. Atualmente, aproximadamente 25% dos medicamentos prescritos no mundo são derivados de plantas. *Casearia sylvestris* é comumente encontrada nas Américas e diferentes partes desta planta são popularmente usadas para tratar várias doenças. Estudos anteriores também confirmaram as atividades biológicas de *C. sylvestris*, como propriedades antitumorais, anti-leishmania e antifúngica. Desta forma, a proposta deste trabalho foi demonstrar a fração 1-6 de *C. sylvestris*, o óleo essencial, foi capaz de reverter o fenótipo de resistência ao fluconazol no modelo de *Saccharomyces cerevisiae* mediado pela proteína heteróloga CaCdr2p de *Candida albicans*. O valor da CIM da fração 1-6 combinada com fluconazol no ensaio tipo tabuleiro de xadrez diminuiu aproximadamente 4 vezes, sugerindo um efeito sinérgico. Além disso, a fração 1-6 aumentou o acúmulo intracelular de rodamina 6G, de 17% para 49% na presença de glicose. Os dados indicam que a fração 1-6 de *C. sylvestris* é um potencial reversor do fenótipo de resistência ao fluconazol.

Palavras-chave: transportadores ABC, Mata Atlântica, fluconazol, sesquiterpenos, levedura.

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Introduction

Nowadays, mycoses are a serious problem in the world public health scenario, causing a high ratio of morbidity and mortality. The increase of the prevalence of fungal infections is mainly due to the large number of immunocompromised individuals, which are part of the risk group for opportunistic infections, especially those caused by fungi, including invasive fungal infections (IFI), such as candidiasis, cryptococcosis and aspergillosis, which are responsible for the death of around 1.5 million people per year, a number close to those caused by tuberculosis and malaria (Brown *et al*. 2012; Vallabhaneni *et al*. 2016).

The similarity between mammalian cells and fungal cells embarrass the development of new antifungal drugs thus limiting the therapeutic arsenal for the treatment of fungal infections (Paul & Moye-Rowley 2014). Azole-type compounds are the most common antifungal agents, especially fluconazole, which has an important spectrum of action and effective pharmacokinetic properties (Vandeputte *et al.* 2012). Since the 90s, fluconazole has been the "gold standard" in the treatment of fungal infections, and many countries have fluconazole as the only therapeutic choice (Kneale *et al*. 2016). However, the indiscriminate and prolonged use of this chemotherapeutic over the years has increased the incidence of resistance to azole drugs (Sanglard 2002).

The major mechanism of resistance to fluconazole in *Candida* spp. is the overexpression of transmembrane transporters, especially those belonging to the ATP-binding cassette (ABC) superfamily (Holmes *et al.* 2008). These pumps are able to translocate structurally unrelated drugs using the hydrolysis of ATP as energy source. The increasing reduction of intracellular levels of antifungal drugs by efflux pumps is a critical mechanism which causes therapeutic failure in the treatment of fungal infections (Prasad & Goffeau 2012).

Inhibition of ABC transporters is a well-studied strategy to reverse the resistance mediated by these proteins (Cannon *et al*. 2009). Co-administration of an efflux pump inhibitor together with an antifungal, such as fluconazole, has a chemosensitizer function, enabling the antifungal drug to reach appropriate intracellular concentrations to perform its pharmacological activity (Lacka *et al.* 2015). ABC transporters inhibitors have already been described in the literature, and may be obtained both from synthetic sources (Reis de Sa *et al*. 2014) and natural products (Belofsky *et al.* 2013).

Casearia sylvestris Sw. (Salicaceae) is popularly known as "erva-de-lagarto", "guaçatonga" or "tiú", and can be found throughout the Brazilian territory. According to ethnobotanical surveys, this species has medicinal properties, including antiviral, antitumor and antifungal activities (Pereira *et al.* 2016,b). According to the chemical profile of the essential oils from *Casearia* genus, sesquiterpenes represent the main fraction of the mixture, but monoterpenes are also identified. However, despite many phytochemical studies with this species, there are few reports about the chemical profile of essential oils. In previous report we demonstrated that pure essential oil from fresh leaves of *C. sylvestris*, rich in α-humulene, as well as its fraction 1-6 rich in 14-hydroxy-9-epi-β-caryophyllene have antifungal activity (Pereira *et al.* 2017a).

The non-pathogenic yeast *Saccharomyces cerevisiae* was the first eukaryote to have its genome completely sequenced, thus facilitating genetic modifications for several scientific studies (Goffeau *et al.* 1996), including the construction of mutant organisms that overexpress CaCdr1p and CaCdr2p proteins of *C. albicans* involved in the fluconazole resistance phenotype. Due to these characteristics, the experimental model of *S. cerevisiae* expressing heterologous proteins is outstanding for the evaluation of new fungal ABC transporters inhibitors (Lamping *et al.* 2007).

Finding new compounds that increase the antifungal potential of drugs, such as fluconazole, is important to provide alternatives to treat fungal infections, and overcome drug resistance. Thus, in the present work the effect of *C. sylvestris* essential oil fractions was evaluated using *S. cerevisiae* strains that overexpress ABC transporters originally found in *C. albicans*.

Material and Methods

Strains and culture conditions

Three strains of *S. cerevisiae* mutants were used: a fluconazole-sensitive strain, which does not express ABC transporters related to MDR phenotype (AD 1-8u$^-$), and two strains that overexpress CaCdr1p (CaCDR1) and CaCdr2p (CaCDR2), which are proteins of *C. albicans* that confer resistance to xenobiotics (Lamping *et al.* 2007). All strains were kindly provided by Drs. Richard Cannon and Brian Monk (University of Otago - New Zealand). Strains were grown

overnight at 30 °C in YPD medium (1% Yeast extract, 2% Peptone and 2% Glucose) at 100 rpm and were collected at the exponential growth phase.

Essential oil and fraction 1-6 chemical analysis

The plant *C. sylvestris* Sw. (Salicaceae) was collected in Tijuca National Park (22°57'05.04"S, 43°17'10.09"W), Rio de Janeiro, Brazil (SISBIO license n. 38765-1/CGEN license n. 010105/2014-0). Plant identification was conducted by Dr. Ronaldo Marquete, and the herbarium voucher was deposited in the Botanical Garden Herbarium of Rio de Janeiro with registration number RB 570651.

Fresh leaves of plant were used to obtain the essential oil by hydrodistillation as described in a previous work (Pereira *et al.* 2017a). Essential oil was fractionated over silica gel column, resulting in fractions 1-6; 7-10; 11-13; 14-28; 29-56; 57-62; 87-88; 91-93, which that were analyzed by GC-MS (Pereira *et al.* 2017a). Pure essential oil and its fractions were used for the tests.

Agar diffusion chemosensitization assay

CaCDR1 and CaCDR2 cells (2.5×10^6 cells/ml) were inoculated onto the surface of YPD medium, in the presence or absence of sub-inhibitory concentrations of fluconazole. Then, 6 mm-diameter sterile disks of Whatman 3MM paper were placed on the surface of the medium and 5 µl of *C. sylvestris* essential oil fractions (10 mg/ml) were added, as well as a control with dimethyl sulfoxide (DMSO) (Sigma Aldrich®, St. Louis, USA). Plates were incubated at 30 °C for 48 h for observation of the growth inhibition zones around the disks (Ricardo *et al.* 2009).

Fraction 1-6 susceptibility testing

The minimal inhibitory concentration (MIC) was determined according to Niimi *et al.* (2004) with slight modifications, using a microdilution assay on 96-well microplates. CaCDR2 cells (5×10^4 cells/ml) were inoculated in YPD medium in the presence of several concentrations of the fraction 1-6 (ranging from 100 to 0.78 µg/ml). The plates were incubated at 30 °C for 48 h in a rotary shaker (100 rpm). Cellular growth was evaluated at 600 nm using a FLUOstar Optima microplate reader (BMG Labtech, Germany).

Checkerboard liquid susceptibility assay

CaCDR2 cells (5×10^4 cells/ml) were incubated in a 96-well plate in the presence of different combinations of fluconazole (UFJF, Juiz de Fora-MG, Brazil) and fraction 1-6. The plates were incubated at 30 °C for 48 h in a rotary shaker (100 rpm). Cellular growth was measured at 600 nm (Fluostar Optima, BMG Labtech, Offenburg, Germany). The type of interaction between the azole drug and fraction 1-6 was measured by fractional inhibition concentration index (FICI). The FICI values are interpreted as follows: synergism, < 0.5; indifferent or additive, 0.5–4.0; antagonism, > 4.0 (Mukherjee *et al.* 2005).

Intracellular accumulation of rhodamine 6G (R6G)

CaCDR2 cells at the exponential growth phase were incubated on ice for 2 h to decrease cellular energy reserves. Then, 5×10^6 cells were resuspended in ice-cold PBS, and incubated with 15 µM of R6G (Sigma Aldrich®, St. Louis, USA) at 30 °C for 40 min with agitation (100 rpm). Further, cells were incubated in the presence or absence of fraction 1-6 (12.5 µg/ml) at 30 °C for 1 h with agitation (100 rpm). Then, the efflux of rhodamine was started by adding glucose (2%) with subsequent incubation at 30 ºC for 30 min. Cells were centrifuged at 9,000 rpm for 2 min and resuspended in PBS for flow cytometry analysis. A total of 20,000 events were read using the FACSCalibur FL-2 filter (Becton Dickinson, California, USA) at the Multi-User Cytometry Unit -CCS- UFRJ and data were analyzed by Flowjo software (Reis de Sa *et al.* 2017)

Statistical analysis

All experiments were performed at least three times, and the results were expressed as mean ± standard deviation, when applicable. Data were analyzed by Student's t-test, and P-values lower than 0.05 were considered significant.

Results

Agar diffusion chemosensitization assay

Eight *C. sylvestris* essential oil fractions and the pure essential oil were tested for their abilities to chemosensitize the CaCDR2 strain to the antifungal drug fluconazole. None of the

fractions presented antifungal activity alone or combined to fluconazole against CaCDR1 strain. On the contrary, when CaCDR2 strain was tested, it appeared a growth inhibition zone around the disk containing the fraction 1-6, suggesting an antifungal activity (Fig. 1a). Moreover, there was a significant increase in the growth inhibition zone when fraction 1-6 was combined to a sub-inhibitory concentration of fluconazole (Fig. 1b).

Antifungal susceptibility testing

CaCDR2 strain showed a dose-response inhibition curve in the presence of different concentrations of the fraction 1-6 of *C. sylvestris* essential oil (Fig. 2). A complete inhibition of cell growth was observed at the concentration of 50 μg/ml (MIC), with an IC_{50} of 20 μg/ml.

Checkerboard: the synergistic effect between fraction 1-6 and fluconazole

The MIC value of fraction 1-6 decreased from 50 μg/ml to 12.5 μg/ml when combined with fluconazole, while the MIC of fluconazole decreased from 75 μg/ml to 17.5 μg/ml when combined with fraction 1-6. The FICI value was 0.48, suggesting a synergistic interaction between the azole drug and the fraction 1-6 (Tab. 1).

Flow cytometry analysis

In the absence of glucose, 98% of CaCDR2 cells were stained with rhodamine 6G (Fig. 3a), whereas only 17% of cells were labeled after glucose addition. However, in the presence of fraction 1-6 there was a decrease in the efflux of rhodamine 6G, and the number of stained cells increased by 32% as compared to untreated cells (Fig. 3b).

Discussion

Different parts of *C. sylvestris* are popularly used to treat several diseases, and previous studies have confirmed some of its biological activities, such as anti-tumor, anti-leishmania and antifungal (Ferreira *et al.* 2011; Moreira *et al.* 2019; Pereira *et al.* 2017a).

The present study was aimed at evaluating the potential of *C. sylvestris* essential oil, mainly composed of α-humulene, spathulenol and α-copaene (Pereira *et al.* 2017a), and its fractions to revert the multidrug resistance phenotype of *Saccharomyces cerevisiae* strains that overexpress

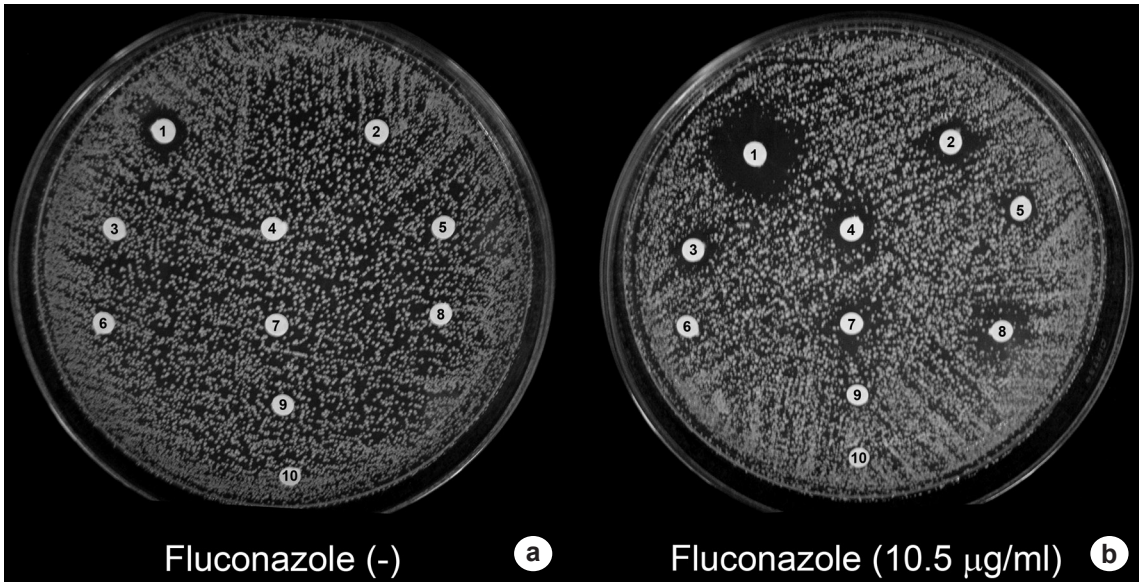

Figure 1 – Agar diffusion chemosensitization assay with CaCDR2 strain. The resistant strain grown in the absence (a) or presence (b) of fluconazole (10.5 μg/ ml) and disks (1-9) impregnated with 5 μl of *Casearia sylvestris* essential oil and fractions (10 mg/ml), in addition to DMSO control. In the absence of fluconazole, 1 showed growth inhibition around the disk, while in the presence of antifungal the inhibition zone was even greater. Disk 1: fraction 1-6; disk 2: fraction 7-10; disk 3: fraction 11-13; disk 4: fraction 14-28; disk 5: fraction 29-56; disk 6: fraction 57-62; disk 7: fraction 87-88; disk 8: fraction 91-93; disk 9: pure essential oil and disk 10: DMSO.

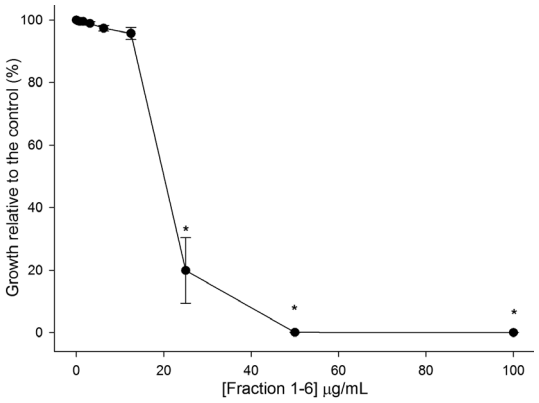

Figure 2 – Minimum inhibitory concentration (MIC) of fraction 1-6 for *Saccharomyces cerevisiae* CaCDR2 strain. Cells were incubated at 30 °C for 48 h in the presence of serial dilutions of fraction 1-6. The inhibition of cell growth by fraction 1-6 was dose-dependent showing a MIC of 50 µg/ml and IC_{50} of 20 µg/ml. The data represent means of three independent experiments.

efflux pumps from *Candida albicans*. An advantage of using these organisms as model, instead of *C. albicans* clinical isolates, is the possibility of evaluating a specific efflux pump since we can use modified cells that overexpress only one type of ABC transporter, or even just the MFS transporter that is also related to multidrug resistance phenotype in yeast.

Initially, an agar-based disk diffusion screening assay was performed in order to evaluate the ability of pure essential oil and its fractions from *C. sylvestris* to enhance the antifungal activity of fluconazole. The results obtained with CaCDR2 strain have demonstrated the antifungal activity of the essential oil when used alone or in combination with fluconazole. In addition, fraction 1-6 displayed a significant activity among the other fractions, and also increased the diameter of zone of inhibition when combined with fluconazole. The microbroth dilution analysis revealed a dose-dependent relationship between *S. cerevisiae* growth and fraction 1-6 concentration, with a MIC value of 50 µg/ml. In a previous work, the antifungal activity of fraction 1-6 against a fluconazole-sensitive *Saccharomyces cerevisiae* strain, showed a MIC value of 62.5 µg/ ml (Pereira *et al.* 2017a). Comparing both results, it may be suggested that the major compound 14-hydroxy-9-epi-β-caryophyllene or set of compounds responsible for the antifungal effect of fraction 1-6 are not a substrate of CaCdr2p. A higher MIC value against CaCDR2 strain would be expected if the active compound of fraction 1-6 (14-hydroxy-9-epi-β-caryophyllene) was pumped out by this transporter, as observed with the terpenoid eucalyptal D (Xu *et al.* 2019), and drugs like azoles, rhodamines, and tacrolimus (Niimi *et al.* 2012).

The results obtained from the checkerboard test to evaluate the type of interaction between the fraction 1-6 and fluconazole against the growth of CaCDR2 strain showed a synergist interaction between the two, as the concentration of fluconazole needed to completely inhibit the growth of yeast cells decreased by approximately four times. The potential of sesquiterpenes (the major components of *C. sylvestris* essential oil and fraction 1-6) to enhance antifungal activity of fluconazole has already been reported. Khoury *et al.* (2019) observed that essential oil from leaves of *Hirtellina lobelii* (DC.) Dittrich (Asteraceae) showed a synergistic interaction with fluconazole and griseofulvin against *Trichophyton rubrum* (Castell.) Sabour., *T. mentagrophytes* (C.P. Robin) Sabour., *T. violaceum* Sabour. *ex* E. Bodin, *T. soudanense* Joyeux, and *T. tonsurans Malmsten* (Khoury *et al.* 2019). Sesquiterpenes are the main components of *H. lobelii* essential oil, and between them, α-bisabolol is the major one, corresponding to 34.5% of the composition of the oil. This

Table 1 – Checkerboard assay of CaCDR2 strain. Chemosensitization test was done from the growth of the fluconazole resistant strain in the presence of different combined concentrations of antifungal and fraction 1-6.

Fraction 1-6			Fluconazole				
MIC (µg/ml)	MIC Combined (µg/ml)	FIC	MIC (µg/ml)	MIC Combined (µg/ml)	FIC	FICI	Outcome
50	12.5	0.25	75	17.5	0.23	0.48	Synergy

compound was also able to enhance antifungal activity of fluconazole against *C. albicans* and *C. tropicalis* strains (Rodrigues *et al.* 2018). Futhermore, the antifungal activity of essential oil obtained from leaves of *Piper claussenianum* (miq.) C.DC. (Piperaceae), in combination with fluconazole, has also been described. It has shown synergism activity with the azole drug against *C. albicans* strains, and it was suggested that this result was obtained due to the high concentration of the sesquiterpene nerolidol in the essential oil (Curvelo *et al.* 2014). The synergistic effect between natural products from *C. sylvestris* and antimicrobial drugs has not been reported so far.

Synergism between two or more drugs may occur by mechanisms unrelated to efflux pumps. For instance, fluconazole enhances berberine antifungal activity by increasing cell membrane permeability (Yang *et al.* 2018). To confirm that fraction 1-6 impairs CaCdr2p efflux activity, a flow cytometry assay was performed. Rhodamine 6G, a fluorescent substrate of candida ABC transporters (Maesaki *et al.* 1999), was used to evaluate the effect of fraction 1-6 on CaCdr2p functioning. When the efflux pump is active, R6G is extruded from the intracellular environment, decreasing cellular fluorescence. The results obtained in the present work have demonstrated that fraction 1-6 significantly decreased R6G efflux, confirming that synergism between this fraction and fluconazole occurs due to the inhibition of CaCdr2p efflux.

In summary, this study highlighted the potential of *C. sylvestris* essential oil, specifically fraction 1-6, to be used in the future as an adjuvant in candidiasis therapy. Due to its ability of inhibiting CaCdr2p, the fraction could be used to revert the resistance phenotype of cells that overexpress this efflux pump, then improving the outcome of the treatment. Further studies will be conducted to evaluate the effect of fraction 1-6 combined with fluconazole against the growth of *C. albicans* clinical isolates.

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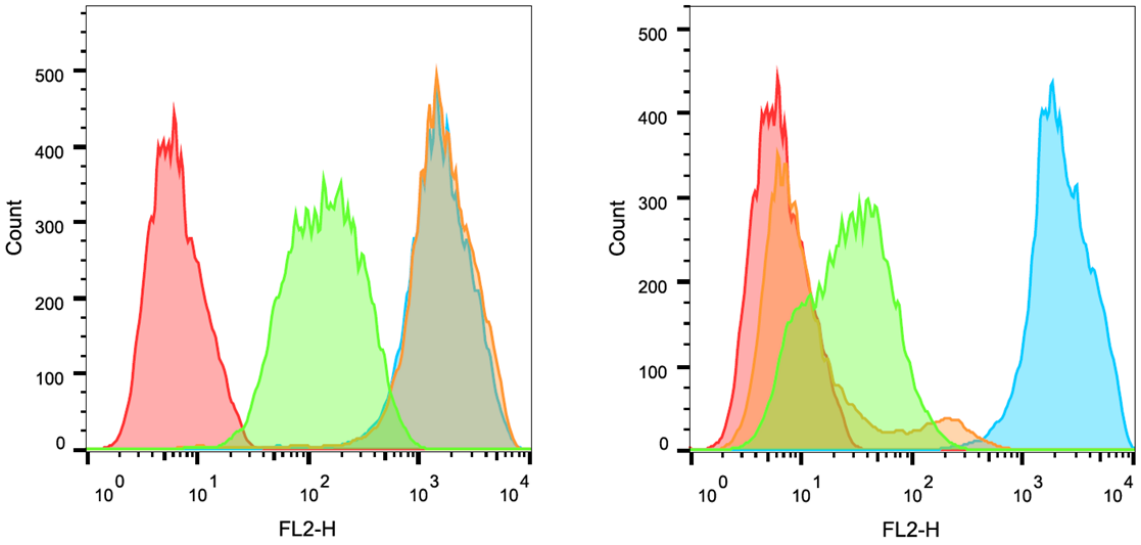

Figure 3 – Intracellular rhodamine 6G accumulation in the presence of fraction 1-6. Intracellular rhodamine 6G accumulation was measured after incubation of exponentially growing phase CaCDR2 cells for 1 h at 30 °C, without (a) or with (b) glucose. Even the presence of glucose, the fraction 1-6 was able to block the extrusion of rhodamine 6G decreasing the number of cells stained with the fluorescent substrate. Autofluorescence (red line); Untreated CaCDR2 cells (orange line); CaCDR2 cells treated with fraction 1-6 (green line); Negative efflux control with the AD 1-8u$^-$ cells (blue line).

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