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Article

Isolation of flavonoids from *Anemopaegma arvense* (Vell) Stellf. ex de Souza and their antifungal activity against *Trichophyton rubrum*

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Anemopaegma arvense (Vell) Stellf. ex de Souza belongs to the family Bignoniaceae, and is popularly known as catuaba. To evaluate the cytotoxic and antimicrobial activity of *A. arvense*, fraction F3 and flavonoids 1 (quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside) (rutin) and flavonoid 2 (quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside) were isolated from the leaves of this plant. Fraction F3 and flavonoids 1 and 2 exhibited no antibacterial activity. Furthermore, no cytotoxic activity of fraction 3 or flavonoids 1 and 2 was observed against the tumor cells tested. However, analysis of the antifungal activity of flavonoids 1 and 2 revealed minimum inhibitory concentrations of 0.5 and 0.25 mg/mL, respectively, against the *Trichophyton rubrum* strains tested (wild type and mutant). This study demonstrates for the first time the antifungal activity of isolated flavonoids, validating the same activity for *A. arvense*.

Uniterms: Bignoniaceae. *Anemopaegma arvense/phytochemistry*. Catuaba/*phytochemistry*. Flavonoids/antifungal activity. Plant extract/evaluation.

Anemopaegma arvense pertence à família Bignoniaceae, sendo conhecida popularmente como Catuaba. Para avaliação de sua atividade citotóxica e antimicrobiana, a fração cromatográfica F3 e os flavonoides 1 (quercetina 3-*O*-α-L-ramnopiranosil-(1→6)-β-D-glucopiranosídeo) (rutina) e flavonoide 2 (quercetina 3-*O*-α-L-ramnopiranosil-(1→6)-β-D-galactopiranosídeo) foram isolados das folhas de *A. arvense*. A fração 3 e os flavonoides não apresentaram atividade antibacteriana. Nenhuma atividade citotóxica foi observada para a fração F3 e para os flavonoides, quando avaliados contra as células tumorais em teste. Entretanto, e considerando a atividade antifúngica, o flavonóide 1 apresentou valor de concentração inibitória mínima (CIM) de 0,5 mg/mL, enquanto o flavonóide 2, CIM de 0,25 mg/mL contra as cepas selvagem e mutante de *Trichophyton rubrum*, demonstrando, pela primeira vez, que os flavonoides isolados possuem atividade antifúngica, o que valida a mesma atividade para *A. arvense*.

Unitermos: Bignoniaceae/fitoquímica. *Anemopaegma arvense*/fitoquímica. Catuaba/fitoquímica. Flavonóides/atividade antifúngica. Extrato vegetal/avaliação.

INTRODUCTION

The family *Bignoniaceae* comprises about 800 plant species that are found mainly in the Neotropical region (Gentry, 1980). In Brazil, several plants of this family are used in folk medicine as astringent and against fever, rheumatism, diarrhea, cancer and microbial infections (Pio Côrrea, Penna, 1969; Fenner *et al*., 2006). *Anemopaegma arvense* (Vell) Stellf. ex de Souza is a species of the family *Bignoniaceae*, which is popularly known as "catuaba". Commercially available formulations of this plant are used as aphrodisiac (Manabe *et al*., 1992). The major components identified in *A. arvense* are flavonoids, catuabins, alkaloids, tannins, and resins (Charam, 1987; Zanolari *et al*., 2005; Tabanca *et al*., 2007). Flavonoids are becoming the subject of anti-infective research and many groups have isolated and identified the structures

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of flavonoids with antifungal, antiviral and antibacterial activity (Cushnie, Lamb, 2005).

There is an urgent need to develop new and more effective antifungal drugs because of the increased resistance of fungi to the drugs currently used in clinical practices (Rahalison *et al*., 1994). Plant secondary metabolites represent a good source of novel antimicrobial molecules. Furthermore, there has been an almost exponential rise in cancer-related mortality over recent years, which has led to an increase in the search for new medicines, including those derived from natural products, able to treat the various types of the diseases (Patocka, 2003; Aziz, 2004; Diwanay *et al*., 2005).

We investigated the cytotoxic and antimicrobial activity of chromatographic fraction F3 and flavonoids isolated from *A. arvense* in order to provide better understanding of the biological activities of this plant.

MATERIAL AND METHODS

Plant material

Leaves of *A. arvense* (Vell) Stellf. ex de Souza (Bignoniaceae) were collected in Sacramento, MG, Brazil, in August 2007 (IBAMA License No. 02001.005076/2011-16) and identified by Professor Lúcia G. Lohmann, Department of Botany, São Paulo University. Voucher specimens (N HPMU-1333) were deposited at the herbarium of the Ribeirão Preto University.

Extract preparation and purification

Dried and pulverized *A. arvense* leaves (100 g) were extracted by maceration with MeOH (0.5 L x 3) at room temperature. After filtration and evaporation of the solvent under reduced pressure, the methanolic extract (5 g) was chromatographed over a Sephadex LH-20 column (3 x 64 cm) using MeOH as the mobile phase, yielding three fractions: F1 (177 mL), F2 (122 mL), and F3 (150 mL). Fraction F3 (0.7 g), rich in flavonoids, was submitted to preparative HPLC separation on a RP-18 column (Supelcosil™ RP-18, 250 x 10 mm i.d., 5 µm) using a Shimadzu LC10A system coupled to a diode array detector (280 nm). The following gradient program was used: MeOH:H_2O (0-100 min: 0-60% MeOH; 100-110 min: 60-80% MeOH; 110-112 min: 80-0% MeOH; 112-120 min: 0% MeOH). The flow rate was 2.0 mL/min and the sample injection volume was 400 µL at a concentration of 100 mg/mL. Six subfractions were obtained after purification. The F3.3 (59 mg) and F3.4 (36 mg) subfractions were pooled and purified on a Sephadex LH-20 column (2.2 x 40 cm) using acetone:water (7:3, v/v) as the mobile phase, yielding two flavonoids: 1 (6 mg) and 2 (12 mg).

Identification of flavonoids

The identity of the flavonoids was confirmed based on ^{1}H and ^{13}C NMR spectral data and by comparison with the literature (Jaramillo *et al.*, 2011). The position of the interglycosidic linkage was provided by ^{13}C NMR and was confirmed by HMBC and HMQC experiments. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded with a Bruker Avance DPX 300 spectrometer in DMSO-d_6 using TMS as internal standard. HPLC analysis was performed using a Shimadzu LC10AD*vp* system equipped with a Supelco LC18 column (Supelcosil™ RP-18, 250 x 4.6 mm i.d., 5 µm) and coupled to a diode array detector, monitored at 340 nm. The following gradient program was used: MeOH:H_2O (0-32 min: 10-66% MeOH; 32-35 min: 66-10% MeOH; 35-40 min: 10% MeOH). The flow rate was 1.0 mL/min and the sample injection volume was 20 µL at a concentration of 1 mg/mL.

Antimicrobial activity

Trichophyton rubrum

The clinical strain of *T. rubrum* (ATCC MYA3108) was kindly provided by Dr. Nilce M. Martinez-Rossi. The *TruMDR2* mutant strain was obtained by disruption of the *TruMDR2* gene of strain MYA3108 (Fachin *et al*., 2006). Standard techniques of manipulation and growth as described previously (Fachin *et al*., 2001) were used. Susceptibility of the MYA3108 (wild type) and *TruMDR2* (mutant) strains was tested by determining the minimum inhibitory concentration (MIC) of fraction F3 and of flavonoids 1 and 2 using the M38-A microdilution technique proposed by the Clinical and Laboratory Standards Institute (CLSI, 2002). Fraction F3 and flavonoids were diluted in 10% DMSO and the final concentration of DMSO in the antifungal assay was less than 1%. Colonies obtained by growth of the strains on Sabouraud agar plates at 28 °C for 15 days were harvested by sterile scraping and mixed with sterile saline and the solution was filtered through glass wool. The resulting mixture was transferred to a sterile tube and adjusted spectrophotometrically at a wavelength of 530 nm, ranging from 70 to 75% transmittance. These conidial suspensions were diluted 1:50 in RPMI 1640 (Sigma, St. Louis, MO, USA) buffered with MOPS, corresponding to twice the density needed for the test of approximately 3-5 × 10^5 CFU/mL. Growth, solvent and

sterility controls were included. Microtiter plates were incubated at 28 °C for 7 days. The MIC100 was defined as the lowest concentration of the fraction or flavonoid that resulted in the complete inhibition of fungal growth. The range of concentrations tested was 2.5-0.019 mg/mL and 0.500-0.019 mg/mL for fraction F3 and the flavonoids (1 and 2), respectively. The assays were carried out in triplicate in three independent experiments. Fluconazole and griseofulvin were used as positive controls.

Bacteria

The following strains were used as test organisms: *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermides* (ATCC 2228), *Escherichia coli (*ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). The antimicrobial activity of fraction F3 and flavonoids 1 and 2 was evaluated using the microdilution method according to CLSI M7-A7 (2006). The test strains were incubated in BHI medium for 24 h at 37 °C. The crude extract, fraction F3 and flavonoids were diluted in 10% DMSO and the final concentration of DMSO in the antibacterial assay was less than 1%. The crude extract and fraction were assayed at concentrations of 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, and 0.019 mg/mL. The flavonoids were assayed at a concentration range of 0.500 to 0.039 mg/mL. The assays were carried out in triplicate in three independent experiments. Ampicillin and chloramphenicol were used as positive controls.

Cytotoxic activity

The following cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 15% fetal calf serum (Life Technologies Inc.) at 37 °C in a humidified atmosphere of 5% CO_2: HeLa (human cervix tumor), 3T3 (mouse embryonic fibroblasts), and U343MG-a (human glioblastoma cell line). Penicillin (100 U/mL) and streptomycin (0.1 mg/mL) were added to the medium to prevent bacterial growth. A stock solution (20 mg/mL) was prepared by dissolving fraction F3 and flavonoids 1 and 2 in 10% DMSO (v/v). The final concentration of these compounds (0.2, 0.02 and 0.002 mg/mL, respectively) was obtained by direct dilution in the culture medium. The final concentration of DMSO in the control and experimental groups was 1%. The cells (10^5 cells/well) were seeded into a 96-well plate 24 h prior to the beginning of the experiment. Actinomycin D (Sigma) was used as positive control. The cells were incubated for 48 h with the fraction or flavonoids and analyzed by the MTT assay (Mosmann, 1983; Rubinstein *et al*., 1990).

RESULTS AND DISCUSSION

The aglycons were identified as quercetin by ^{1}H and ^{13}C NMR. The ^{1}H NMR spectra indicated the presence of a rhamnosyl and a glucosyl group at δ 5.34 and δ 4.41 in flavonoid 1 and of a rhamnosyl and a galactosyl group at δ 5.30 and δ 4.36 in flavonoid 2. The relatively deshielded rhamnosyl protons suggested that they are not directly attached to the aglycone, but that there is a sugar-sugar linkage. The position of the sugar in the aglycons provided by HMBC was demonstrated by cross-peaks between H-1'' and C-3. The position of the interglycosidic linkage was provided by the ^{13}C-NMR downfield shift of C-6'' at δ 67.5 and δ 65.7 in flavonoids 1 and 2, respectively. This fact was confirmed by HMBC and HMQC experiments. On the basis of the spectroscopic data, flavonoids 1 and 2 were characterized as quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (rutin) and quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside, respectively (Figure 1). HPLC analysis of fraction F3 revealed the presence of six flavonoids (Figure 2A). Flavonoids 1 and 2 isolated from *A. arvense* were detected at 24.1 and 23.2 min, respectively (Figure 2B and 2C).

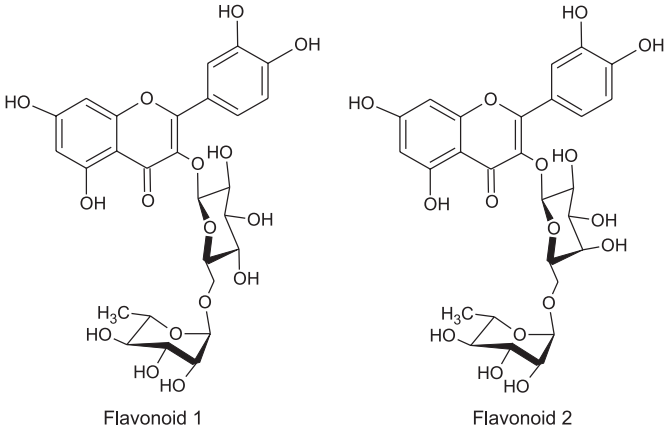

FIGURE 1 - Chemical structure of flavonoids 1 and 2 isolated from *Anemopaegma arvense*. Flavonoid 1: quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (rutin); flavonoid 2: quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside.

Analysis of the bioactivity of *A. arvense* showed no cytotoxic activity of fraction F3 or of the isolated flavonoids at a dose of 0.2 mg/mL against the cell lines tested. In addition, at a dose of 2.5 mg/mL, the crude extract, fraction F3 and the flavonoids exhibited no antibacterial activity against the strains tested (data not shown). These results partially agree with those reported by Tabanca *et al*. (2007)

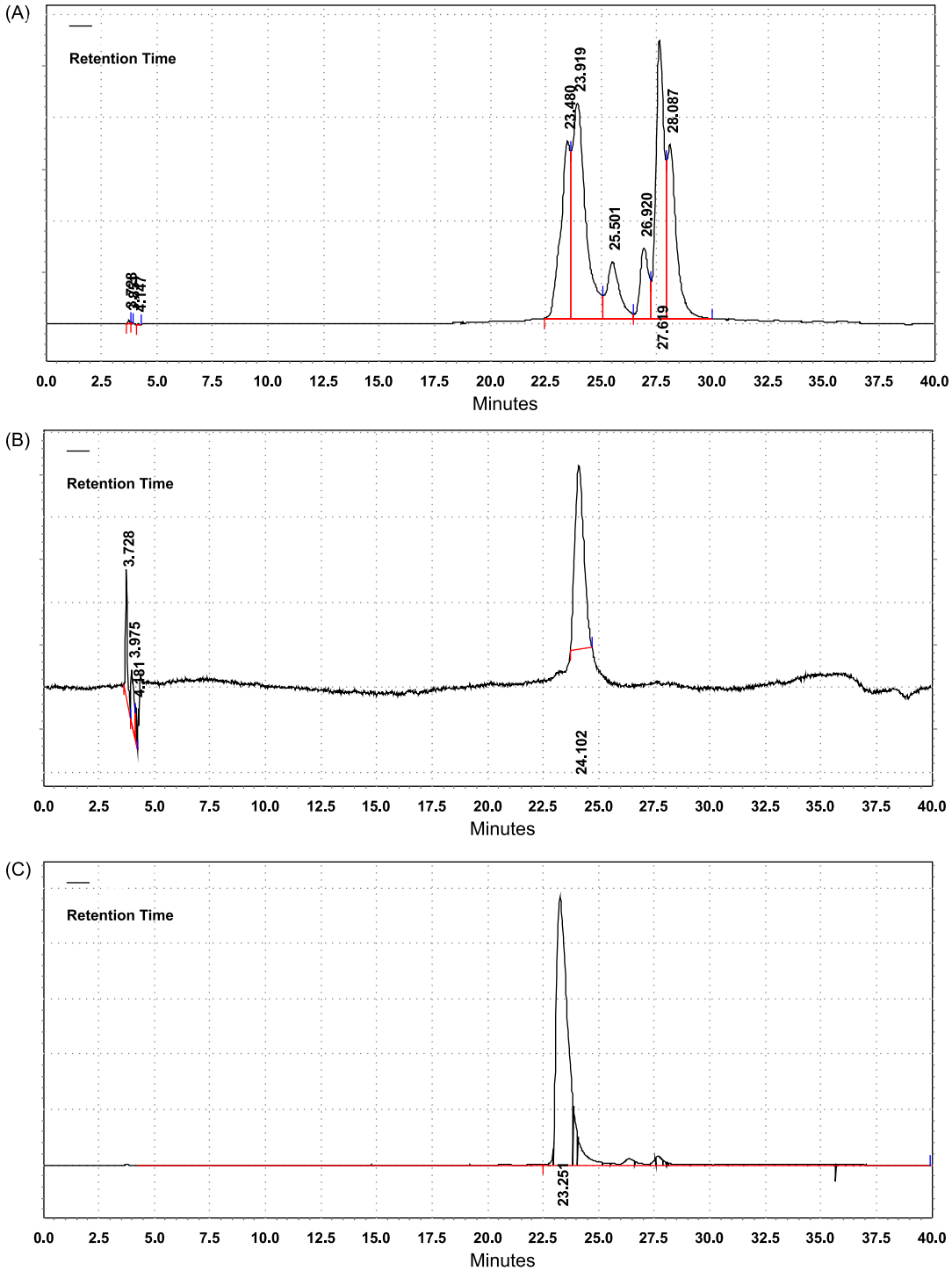

FIGURE 2 - HPLC profile of fraction F3 (A), flavonoid 1 (B), and flavonoid 2 (C) isolated from *Anemopaegma arvense*.

who isolated a new catuabin (catuabin A) and three known flavan-type phenylpropanoids (cinchonain Ia, cinchonain IIa, and candelin A1) from *A. arvense*. These compounds possessed no anti-inflammatory, cytotoxic, antimicrobial or antimalarial property, but exhibited antioxidant activity.

In the present study, fraction F3 exhibited antifungal activity against the mutant and wild-type strains of *T. rubrum* at concentrations of 1.25 and 0.625 mg/mL, respectively. Isolated flavonoid 1 showed weak antifungal activity, with an MIC of 0.5 mg/mL against the two fungal strains tested, whereas flavonoid 2 exhibited moderate antifungal activity, with an MIC of 0.25 mg/mL (Table I). Antifungal activity of ethanol extracts of *Hyptis ovalifolia* and *Eugenia uniflora* has been demonstrated by Souza *et*

al. (2002). These extract completely inhibited the growth of the 30 dermatophytes tested. The MIC of the *H. ovalifolia* extract against *T. rubrum* strains was 0.25 mg/mL and the *E. uniflora* extract exhibited antifungal activity against 19 of the 30 isolates at a concentration of 0.5 mg/mL. Rocha *et al*. (2004) demonstrated antifungal activity of *Clytostoma ramentaceum* and *Mansoa hirsuta* (Bignoniaceae) when testing the low and medium polar fractions at concentrations of 0.1 to 0.3 mg/mL. Pacciaroni *et al*. (2008) isolated several flavonoids from the aerial parts of *Heterothalamus alienus* and tested these compounds against clinical isolates of dermatophytes. The flavanones showed very good fungicidal activity against standard (MIC: 31.2 µg/mL) and clinical isolates of *T. rubrum* and *T. mentagrophytes* (MIC: 31.2-62.5 and 31.2-125 µg/mL, respectively). However, rutin, spathulenol (1) and two of the 3-acetylated flavanones were inactive or marginally active against the fungal strains (MIC > 250 µg/mL).

Methods for antimicrobial assessment of natural products and effective MIC values are not well established in the literature. Holetz *et al*. (2002), who screened hydroalcoholic extracts from 13 Brazilian plants using the microdilution technique, defined an MIC < 0.1 mg/mL as good antimicrobial activity, MIC of 0.1 to 0.5 mg/mL as moderate antimicrobial activity, and MIC of 0.5 to 1 mg/mL as weak antimicrobial activity. Extracts exhibiting MIC higher than 1 mg/mL are considered to be ineffective. Reports of activity in the field of antibacterial flavonoid research are widely conflicting, probably because of inter- and intra-assay variation in susceptibility testing (Cushnie, Lamb, 2005).

TABLE I - Minimum inhibitory concentration (mg/mL) of fraction F3 and flavonoids isolated from *Anemopaegma arvense* against *Trichophyton rubrum* strains (MYA-3108 and *ΔTru*MDR2)

Compound	MYA-3108	Δ*Tru*MDR2
Fraction F3	0.625	1.250
Flavonoid 1	0.500	0.500
Flavonoid 2	0.250	0.250
Griseofulvin	0.0005	0.0005
Fluconazole	0.075	0.075

The minimum inhibitory concentration corresponds to the lowest concentration of the fraction or flavonoid that resulted in 100% inhibition of visible fungal compared to control. The results are representative of three independent experiments performed in triplicate.
Flavonoid 1: quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (rutin); flavonoid 2: quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside.
Fluconazole and griseofulvin were used as positive controls.
Fraction F3: concentration range of 2.5-0.019 mg/mL.
Flavonoids: concentration range of 0.500-0.019 mg/mL.

Membrane transporters, especially efflux transporters, affect the adsorption and bioavailability of drugs. MIC of flavonoids 1 and 2 was the same for the wild-type and mutant strain of *T. rubrum* (in which the ABC gene is disrupted). This finding may be explained by the fact that ABC transporters are not involved in the transport of flavonoids (Walgren *et al*., 2000). The *TruMDR2* gene was disrupted in the mutant strain of *T. rubrum* and this strain has been shown to be susceptible to several compounds (Fachin *et al*., 2006). Therefore, despite relatively high MIC, flavonoid-based inhibitors of fungi may be an alternative for the treatment of multidrug-resistant strains since efflux pumps do not transport these compounds.

In the present study, we were able to isolate and identify two quercetin-derived glycosylated flavonoids from *A. arvense*, which showed antifungal activity. Quercetin is a substance widely distributed in the plant kingdom. However, this study describes for the first time the antidermatophyte activity of *A. arvense*, which could be attributed to the presence of quercetin. In fact, the antifungal activity of quercetin and its derivatives has been described in other medicinal plants. Semwal *et al*. (2009) demonstrated the antifungal activity of an ethanol extract of *Boehmeria rugulosa* leaves and of three new flavonoid glycosides against *T. rubrum*, *Microsporum canis* and *Microsporum gypseum*, with MIC of 100 µg/mL. Pereira *et al*. (2008) isolated the flavonoid rutin from the aerial parts of *Solanum palinacanthum* and evaluation of its antimicrobial activity showed an MIC of 35 µg/mL against the fungus *Aspergillus ochraceus*.

Human mycoses are not always treated effectively. The most important causes of treatment failure are the recurrence of infections, drug resistance of pathogens, and toxicity of currently available antifungal agents (Turel, 2011; Butts, Krysan 2012). Therefore, the continual search for new and more effective antifungal drugs, which should also be safer than currently used agents, is important (Zacchino, 2001). The increasing prevalence of multidrug-resistant pathogens requires the identification of new antimicrobial agents as alternative therapies in difficult-to-treat infections (Pereira *et al*., 2006). In conclusion, the flavonoids isolated from *A. arvense* were bioactive against *T. rubrum* and may be a promising target in studies on new antifungal agents.

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