Antifungal Activity of Coumarin Mammeisin Isolated from Species of the Kielmeyera Genre (Family: Clusiaceae or Guttiferae)

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Coumarin mammeisin isolated from Kielmeya elata was evaluated for its toxicity and antifungal activities. The toxicity of mammeisin was investigated by utilizing the Artemia salina methodology to determine its LD$_{50}$ value. The minimum inhibitory concentration (MIC) of fungi Candida sp. was assessed for mammeisin, presenting equivalent activity to ketoconazole but displaying better results than fluconazole.

1. Introduction

The current pharmacotherapy used against human fungal diseases is based on azole compounds. Unfortunately, the emergence of microorganisms resistant toazole antifungals is increasing in number and patients at risk of acquiring fungal infections have created new challenges. The demand for drugs of plant origin has led to a renewed interest in pharmaceutical coumarins, chromones, and xanthones, given the fact that these substances show potent pharmacological activities and low toxicity to mammals [1].

Amongst the many pharmacological functions of coumarins a few stand out, such as scopolamine (6,7-dimethoxy-coumarin), isolated from the Chinese plant Artemisia scoparia Waldst. Osthole coumarin (a C-prenylated coumarin) inhibited platelet aggregation and was able to induce relaxation of the smooth muscles of the heart, possibly due to inhibition of the enzyme cAMP- and cGMP-phosphodiesterase and calcium influx [2]. Coumarins such as calanolides A and B (pyranocoumarins) exhibited anti-HIV activity by impeding the in vitro replication of HIV-1, probably by inhibiting the enzymatic activity of DNA polymerase-dependent DNA and DNA-dependent RNA polymerase present in viruses [3].

The genus Kielmeya belongs to the family Clusiaceae (Guttiferae), part of the subfamily Kielmeyroidae, which is endemic in South America, and most species are exclusively native of the Brazilian flora. Coumarins isolated from Kielmeya elata, which are pertinent to this report, include mammeisin (Figure 1). Mammeisin can essentially be classified as a C-prenylated coumarin and was isolated from the stem of Kielmeya elata by our group [4]. Mammeisin has also been more recently identified in Mammea americana L. seeds and has been evaluated for its cytotoxic and antioxidant properties [5].

Until now, its antifungal activities had not been investigated; thus, we isolated the natural product from the stem of Kielmeya elata in order to evaluate its potential antifungal properties.
2. Experimental

2.1. Isolation and Characterization of Natural Product Mammeisin. Mammeisin was isolated from the stems of *Mammea* as described by our group [4] and its structure confirmed by spectroscopic analysis. Pale yellow prisms, mp 102–104°C; δH (300 MHz, CDCl3): 0.89 (6H, d, J 6.9), 1.76 (3H, s), 1.89 (3H, s), 2.19 (1H, sept, J 6.9), 3.57 (2H, d, J 7.2), 5.26 (1H, t, J 7.2), 5.96 (1H, s), 7.39–7.41 (2H, m), 7.52–7.54 (3H, m), 9.93 (1H, s), 11.04 (1H, s); δC (75 MHz, CDCl3): 207.4, 163.5, 159.8, 159.5, 156.9, 154.7, 137.5, 135.7, 130.0, 129.4, 127.7, 120.9, 112.9, 108.2, 107.5, 101.1, 53.9, 29.9, 26.1, 24.9, 22.9, 21.9, 18.2, 14.4.

2.2. Evaluation of Biological Activities

2.2.1. Antifungal Activity. The evaluation used the antifungal method for determination of minimum inhibitory concentration (MIC), according to the broth microdilution technique recommended by the CLSI document M27-A2. The methodology employed for testing antifungal activity was divided into four stages: preparation of the dilutions from all compounds tested, preparation of dilutions of the antifungal agents (in this case, ketoconazole and fluconazole), preparation of inocula with species of *Candida* (*C. albicans, C. tropicalis, C. parapsilosis*, *C. krusei*), and finally plating. The dilutions for all compounds tested consisted of solubilizing, 12.0 mg in 6.0 mL of DMSO, resulting in a solution with 2000 μg/mL. Then dilutions were made successively with the culture medium RPMI (Roswell Park Memorial Institute) to yield ten concentrations 512.0, 256.0, 128.0, 64.0, 32.0, 16.0, 8.0, 4.0, 2.0, and 1.0 μg/mL (extract + RPMI + DMSO). As for the antifungal compounds fluconazole and ketoconazole, 5.0 mg was diluted in 5.0 mL DMSO resulting in two solutions with a concentration of 1000 μg/mL; then the solutions were diluted with culture medium (RPMI) to give dilutions of fluconazole 64.0 μg/mL, 32.0 μg/mL, 16.0 μg/mL, 8.0 μg/mL, 4.0 μg/mL, 2.0 μg/mL, 1.0 μg/mL, 0.5 μg/mL, 0.25 μg/mL, and 0.125 μg/mL and for ketoconazole 32.0 μg/mL, 16.0 μg/mL, 8.0 μg/mL, 4.0 μg/mL, 2.0 μg/mL, 1.0 μg/mL, 0.5 μg/mL, 0.25 μg/mL, 0.125 μg/mL, and 0.0625 μg/mL.

The plates were incubated at 28°C and readings were taken after 48 hours to analyse the fungal growth at different courmarin concentrations against a positive control. MICs were obtained by visual reading and expressed in μg/mL.

2.2.2. Toxicity Evaluation: Artemia salina. In order to obtain *Artemia salina*, the microcrustacean eggs were placed in an aquarium with saline solution (sea salt of artificial Red Sea) and allowed to hatch at a concentration of 38.0 g/L under oxygenation and light radiation for 48 h. Next, 40.0 mg of dry extract was diluted in 4.0 mL of DMSO and from this solution aliquots of 5, 50, 250, and 500 μL were withdrawn, which were then transferred to test tubes to allow 4 treatments for each compound to be tested in three replicates. Subsequently, the solvent was evaporated, and 5.0 mL (per well) of saline was added resulting in concentrations of 10, 100, 500, and 1000 μg/mL (T10, T100, T500, and T1000, each treatment being represented with numbers from 1 to 8). The controls used were lapachol prepared by the same procedure to provide 4 treatments with three replicates (13, 14, 15, and 16) and one treatment with saline and 4 repeats (white 17). In each test tube ten larvae of *A. salina* of the nauplius type were placed, which remained in solution for 24 hours and were finally analyzed for mortality rate to determine the toxicity of compounds by calculating the LD$_{50}$ (probit method).

2.2.3. Statistical Analysis. The results presented in this study are the average of three replicates (n = 3) ± standard deviation. The data obtained from the *Artemia salina* experiments were treated to statistical analysis using the SAEG 9.1 software and by applying the ANOVA variance, followed by Tukey’s multiple comparisons test (P < 0.05). Graphics displaying linear regression of the stock solution of DPPH and EC$_{50}$ calculations were performed using Microsoft Excel 2007 program.

3. Results and Discussion

3.1. Toxicity Evaluation: Artemia salina. The linear regression data (probit) indicated LD$_{50}$ values for the lethal dose at 50% of the *nauplii* population under study showed that mammeisin at the concentrations tested was 100% lethal and therefore very toxic. Toxicity to *A. salina* shows good correlation with antitumor and insecticide activity [6], for substances when the LD$_{50}$ is <10$^3$ μg/mL. In the statistical analysis by Tukey’s test (P < 0.05), the high toxicity of mammeisin was confirmed and evidenced by the fact that toxicity at all concentrations was equivalent to the highest concentration tested for lapachol.

3.2. Antifungal Activity. Given that mammeisin demonstrated high toxicity, we proceeded to perform an antifungal assay. For the initial dilution of mammeisin, 12.0 mg was diluted in to the culture medium, RPMI (Roswell Park Memorial Institute), to provide final dilutions of 512.0, 256.0, 128.0, 64.0, 32.0, 16.0, 8.0, 4.0, 2.0, and 1.0 μg/mL. Pure RPMI is also used as a negative control. In the case of the antifungal standards, 5.0 mg of either fluconazole or ketoconazole is diluted in to 5.0 mL of DMSO.

![Figure 1: Chemical structure of mammeisin.](image-url)
Table 1: Summary of the minimum inhibitory concentration (MIC) for mammeisin and two antifungal agents.

(a)

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<tr>
<th>Candida</th>
<th>ATCC</th>
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<th>Ketoconazole (CIM)</th>
<th>Fluconazole (CIM)</th>
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(b)

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* Mammeisin.
** Antifungal standard (ketoconazole/fluconazole).
C+: positive control.
C−: negative control.

Figure 2: Antifungal experiment: concentrations 512-1 are given in units of μg/mL. Photo above corresponds to experiment with ketoconazole as positive control and photo below shows experiment carried out with fluconazole as positive control.

The minimum inhibitory concentration (MIC) for mammeisin was 512 μg/mL for all types of Candida employed in this experiment. In comparison to the positive control, it was noted that fluconazole was not effective against the Candida tropicalis, since there was growth at all concentrations evaluated whereas mammeisin demonstrated antifungal activity against Candida tropicalis. Moreover, mammeisin was almost equally active as positive control ketoconazole for the same types of Candida and presented similar MIC. The results are outlined in Table 1.

4. Conclusions

To conclude, we report the antifungal activities of courmarin mammeisin, against 4 different types of Candida. Mammeisin showed better MIC values for Candida tropicalis when compared to positive control fluconazole.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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