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Artigo

Anti-mitotic activity towards sea urchin eggs of dichloromethane fraction obtained from *Allamanda schottii* Pohl (Apocynaceae)

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RESUMO: "Atividade anti-mitótica da fração diclorometano obtida de *Allamanda schottii* Pohl (Apocynaceae) sobre ovos do ouriço do mar." *Allamanda* (Apocynacea) é um gênero de arbustos escandentes conhecido por produzir compostos com várias atividades biológicas. Trabalhos anteriores têm mostrado um efeito anti-proliferativo do extrato etanólico de *Allamanda schottii* sobre células leucêmicas. O presente trabalho foi realizado para avaliar o efeito da fração diclorometano, obtida de *Allamanda schotti,* sobre os ovos de ouriço-do-mar de *Echinometra lucunter*, como um modelo multicelular para estudar atividade anti-tumoral. Nossos resultados mostram uma inibição do desenvolvimento dos ovos de uma maneira dose-dependente na presença da fração diclorometano. Os valores de IC_{50} para a primeira e terceira clivagem e para o estágio de blástula foram de 103,7 µg/mL, 33.1 µg/mL e 10,2 µg/mL, respectivamente. Estes resultados também demonstram um efeito acumulativo da fração sobre os embriões do ouriço-do-mar. No presente trabalho, esta expressiva atividade anti-mitótica da fração diclorometano sobre o desenvolvimento embrionário do ouriço-do-mar, um modelo multicelular, reforça o potencial anti-tumoral de *Allamanda schotti.*

Unitermos: *Allamanda schottii*, ouriço do mar, atividade anti-mitótica, anti-tumoral.

ABSTRACT: *Allamanda* (Apocynaceae) is a genus of climbing shrubs known for producing compounds with a range of biological activities. Previous works have shown the anti-proliferative effect of the ethanolic extract of *Allamanda schottii* on leukemic cells. The present work was conducted to evaluate the effects of dichloromethane fraction, obtained from *Allamanda schottii,* on sea urchin *Echinometra lucunter* eggs, as a multicellular model for evaluating anti-tumor activity. Our results show an inhibition of sea urchin development in a dose-dependent manner in the presence of dichloromethane fraction. The IC_{50} values for first and third cleavage and blastulae stage were 103.7 µg/mL, 33.1 µg/mL and 10.2 µg/mL, respectively. These results also demonstrate the cumulative effect of this fraction on sea urchin embryos. In the present work, the expressive anti-mitotic activity of dichloromethane fraction towards sea urchin eggs, a multicellular model, reinforces the anti-tumor potential of the *Allamanda schotti*.

Keywords: *Allamanda schottii*, sea urchin, anti-mitotic activity, anti-tumor.

INTRODUCTION

Allamanda (Apocynaceae) is a genus of tropical climbing shrubs, which is known for producing compounds with a range of biological activities, including algicidal (Coppen, 1983; Coppen and Cobb, 1983), antifungal (Tiwari et al., 2002) and antitumoral activities on culture cells (Kupchan et al., 1974; Anderson et al., 1988; Navarro Schmidt et al., 2006). Also their popular therapeutic uses have been reported (Agra et al, 2007; 2008). Previous works have shown the inhibitory effect of *Allamanda schottii* ethanolic extract on the growth of K562 leukemic cells (Navarro Schmidt et al., 2006). It was also demonstrated that isoplumericin, plumericin and scopoletin, all isolated from *A. schottii*, have presented cytotoxicity against 9KB (human nasopharyngeal carcinoma) and 3PS (P-388 murine leukemia) cells (Anderson et al., 1988).

For several years embryos and eggs of sea urchin have been used as a model for studying cell division and embryologic development (Epel, 1963; Hinchcliffe et al., 1998; Terasaki, 2000). Also, this model has been utilized to detect the cytotoxic and teratogenic activities of new compounds (Morale et al., 1998; Costa-Lotufo et al., 2002b; Hansen et al., 2003; Jimenez et al., 2003). Recently, some works have emphasized the study of alterations in sea urchin egg development as a multicellular model for evaluating anti-tumor activity (Costa-Lotufo et al., 2002a; Costa-Lotufo et al., 2003; Knudson, 2004; Costa-Lotufo et al.,

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2005).

Considering the therapeutic potentialities of the *Allamanda schottii*, the present work was conducted to evaluate the effects of dichloromethane fraction obtained from aerial parts of this plant on sea urchin *Echinometra lucunter* eggs development as a multicellular model for investigate the anti-tumor potential of this fraction.

MATERIAL AND METHODS

Plant material

Samples of *A. schottii* were collected in Florianópolis, SC, Brazil, in 2002. A voucher specimen was identified by Prof. Benigno Iza (UNIVALI) and has been deposited at the Barbosa Rodrigues Herbarium, Itajaí, under the number HRB 52525. Dried leaves and stems of *A. schottii* were extracted exhaustively with 95% ethanol by maceration for seven days. Crude extracts of the organs were obtained at reduced pressure and temperatures below 60° C. Fractionation of *A. schottii* involved liquid-liquid partition with dichloromethane giving the respective fraction, as described in Navarro Schmidt et al. (2006). From dichloromethane (DCM) fraction the following compounds were isolated: a mixture of the phytosterols β sitosterol and stigmasterol, the iridoids plumericin and plumieride, the coumarin scopoletin, and the triterpenoid ursolic acid, which were identified on the basis of spectroscopic data, and specifically by comparison of their IV, RMN ^{1}H and ^{13}C data with those in literature (Navarro Schmidt et al., 2006). The DMC fraction was dissolved in dimethylsulfoxide (DMSO) for anti-mitotic analysis.

Evaluation of anti-mitotic activity

The evaluation of the anti-mitotic activity of the DCM fraction was made as described by Costa-Lotufo et al. (2002). Sea urchins (*Echinometra lucunter* Linnaeus, 1758) were collected in Intermares beach, Paraíba, Northeastern Brazil (7°01'55.55''S 34°49'14.96''W). Spawning was induced by injecting 3.0 mL of a 0.5 M KCl solution into the perivisceral cavity. The eggs were washed three times in filtered sea water (FSW) in order to remove the jelly coat. The concentrated sperm was collected with a Pasteur pipette, and then was diluted 1:50 with FSW. Fertilization was performed by adding 1 mL of sperm suspension to 100 mL of FSW containing the eggs. After the confirmation of the fertilization (by the elevation of the fertilization membrane), the number of eggs was adjusted to 10^4 eggs/mL and 1 mL of the suspension of fertilized eggs were transferred to each well of 24-well plates. DCM fraction dissolved in DMSO was diluted in concentrations ranging from 5 to 200 µg/mL with FSW. DMSO was used as negative control. The eggs were incubated in a final volume of 2 mL at 25ºC. With the purpose of obtaining first and third cleavages, and blastulae, 200 µL of the egg suspension was collected and fixed in the same volume of ISOTON solution (10.5 g citric acid, 7.0 g NaCl, 5.0 mL formalin and 1000 mL distilled water) 1.5 h, 3 h and 7 h after fertilization, respectively. To obtain the percentage of normal development, one hundred eggs or embryos were counted for each concentration of the fraction tested.

Statistical analysis

The concentration that inhibits the normal development of 50% of the eggs (IC_{50}) and its 95% confidence intervals were determined by probit regression using the SPSS 8.0 for Windows program. The differences between experimental groups were compared by Student's test and values of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Dichloromethane (DCM) fraction of *A. schottii* presented strong anti-mitotic activity against sea urchin embryos. Figure 1 shows the concentration-dependent effect of the fraction on the division cycles of sea urchin embryos. The IC_{50} values for first and third cleavage and blastulae were 103.7 (76.5-148.3) µg/mL, 33.1 (27.1-40.1) µg/mL and 10.2 (6.8-14.3) µg/mL, respectively. The inhibition of the first cleavage was evident when DCM was tested at 100 µg/mL, when approximately 40% of the embryos were blocked at one-cell stage (Figure 2A and 2B). After seven hours of incubation, 100% of the embryos in control were at blastula stage (Figure 2C), while in the presence of 30 µg/mL of DCM fraction the development was blocked at 2-, 4-, 8-, 16-cell stage (Figure 2D). At this time, concentrations as low as 15 µg/mL inhibited significantly the development of sea urchin eggs and approximately 60% of these did not reach the blastula stage (results not showed). Larva stage was apparent 28 h after fertilization in control group (Figure 2E). Figure 2F demonstrates the potent effect of DCM fraction at 15 µg/mL 28 h after fertilization, showing abnormal and dead embryos, with development blocked at blastula stage. At the lowest concentration tested (5 µg/mL), the fraction impairs the normal development of sea urchin embryos into larva stage (results not showed). These results demonstrate the cumulative effect of the DCM fraction obtained from *A. schottii* on sea urchin embryos.

In the present work, we demonstrated the strong anti-mitotic effect of DCM fraction towards sea urchin egg development. This model has been used for evaluating anti-tumor activity (Costa-Lotufo et al., 2003; Knudson, 2004; Costa-Lotufo et al., 2005). Our results on this multicellular model corroborate previous work in which the anti-proliferative effect of extract of *A. schottii* on leukemic cells was described (Navarro Schmidt et al., 2006).

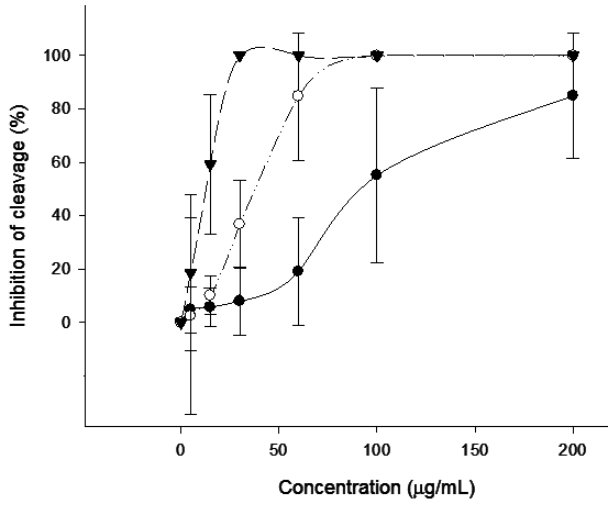

Figure 1. Effect of DCM fraction obtained from *A. schottii* on sea urchin *Echinometra lucunter* egg cleavage. Values are mean (± standard errors) of three different experiments for the first (solid circles) and third (open circles) cleavages and blastula stage (filled triangles). DMSO was used as negative control.

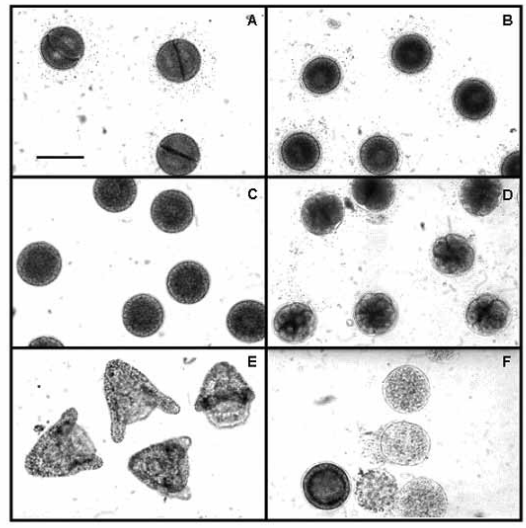

Figure 2. Photomicrographs showing the effect of DCM fraction of *A. schottii* on first cleavage (A and B), blastula (C and D) and larva (E and F) stage of sea urchin development. Control (A) and 100 µg/mL DCM fraction (B), 1.5 hours after fertilization. Control (C) and 30 µg/mL DCM fraction (D), 6 hours after fertilization. Control (E) and 15 µg/mL DCM fraction (F), 28 hours after fertilization. Bar = 100 µm.

Some compounds also present in the DCM fraction of *A. schottii* (Navarro Schmidt et al., 2006), have previously been show to have antitumoral properties on culture cells. Scopoletin is known to have anti-proliferative activity against several cancer cell lines (Anderson et al., 1988; Manuele et al, 2006). It was also demonstrated that scopoletin induces apoptosis in HL-60 cell line (Kim et al., 2005). Plumericin presents antitumoral properties and is also known to cause DNA damage (Kupchan et al., 1974; Anderson et al., 1988; Wood et al., 2001). Sitosterol was reported to inhibit the growth of tumoral cell lines, and the mechanisms by which this compound act include induction of apoptosis, inhibition of cell cycle progression, prostaglandin synthesis and the production of reactive oxygen species (ROS) (Awad et al., 2003; Choi et al., 2003; Awad et al., 2005). The ursolic acid, also present in the DCM fraction of *A. schottii* as the major compound (Navarro Schmidt et al., 2006), is recognized as a potent antiproliferative agent, which blocks cell cycle progression (Es-saady et al., 1996; Li et al., 2002; Hsu et al., 2004). It is important to stress that ursolic acid is also an inhibitor of DNA polymerase and DNA topoisomerase, two important cellular targets for chemical intervention in the development of anti-cancer agents (Mizushina et al., 2000).

In the present work, the expressive anti-mitotic activity of the DMC fraction towards sea urchin eggs, a muticellular model, reinforces the anti-tumor potential of the *Allamanda schotti*. The synergic effect of the different compounds present in this fraction is likely to be responsible for the anti-proliferative effect of this plant derivative.

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