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The husk fiber of *Cocos nucifera* L. (Palmae) is a source of anti-neoplastic activity

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Abstract

In the present study, we investigated the *in vitro* anti-tumoral activities of fractions from aqueous extracts of the husk fiber of the typical A and common varieties of *Cocos nucifera* (Palmae). Cytotoxicity against leukemia cells was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2 x 10^4/well) were incubated with 0, 5, 50 or 500 µg/mL high- or low-molecular weight fractions for 48 h, treated with MTT and absorbance was measured with an ELISA reader. The results showed that both varieties have almost similar antitumoral activity against the leukemia cell line K562 (60.1 ± 8.5 and 47.5 ± 11.9% for the typical A and common varieties, respectively). Separation of the crude extracts with Amicon membranes yielded fractions with molecular weights ranging in size from 1-3 kDa (fraction A) to 3-10 kDa (fraction B) and to more than 10 kDa (fraction C). Cells were treated with 500 µg/mL of these fractions and cytotoxicity was evaluated by MTT. Fractions ranging in molecular weight from 1-10 kDa had higher cytotoxicity. Interestingly, *C. nucifera* extracts were also active against Lucena 1, a multidrug-resistant leukemia cell line. Their cytotoxicity against this cell line was about 50% (51.9 ± 3.2 and 56.3 ± 2.9 for varieties typical A and common, respectively). Since the common *C. nucifera* variety is extensively cultured in Brazil and the husk fiber is its industrial by-product, the results obtained in the present study suggest that it might be a very inexpensive source of new antineoplastic and anti-multidrug resistant drugs that warrants further investigation.

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- Leukemia cells

Elimination of malignant cells is one of the major challenges in cancer. The ability of tumor cells to evade cell death and their resistance to anticancer agents remains a major cause of treatment failure in cancer patients (1-3). In fact, in spite of the large number of antineoplastic drugs available, about 50% of cancer patients face remission of cancer after treatment but eventually die of generalized metastasis. Thus, the identification of new drugs for the treatment of cancer, especially of multidrug-resistant tumors, is of great clinical interest.

Natural products currently are the leading source in the search for new biologically active compounds. As part of a program to

study the therapeutic properties of Brazilian plants, our group started a biomonitor assay of *Cocos nucifera* (Palmae) extracts. Although aqueous extracts of *C. nucifera* husk fiber are popularly used for the treatment of diarrhea and arthritis, pharmacological investigation of their beneficial or adverse biological effects is still very preliminary. Recent data from our group have shown that the aqueous extract of the husk fiber of *C. nucifera*, typical A variety, popularly known as "olho de cravo", has antibacterial and antiviral (4), antitumoral (5) and antileishmanial properties (6). This extract also exhibited *in vivo* and *in vitro* analgesic and free radical-scavenging properties (7). Preliminary study by Kirszberg et al. (5) has suggested that the efficacy of the antitumoral activity of *C. nucifera*, typical A variety, could be extended to leukemia cells having a multidrug-resistant phenotype.

Despite the biological properties observed for *C. nucifera*, typical A variety, its culture is relatively rare in comparison with the common variety of *C. nucifera*. The widespread industrial use of the latter variety generates large amounts of husk fiber as an industrial reject. In the present study, we used bio-assay-guided fractionation to investigate the antitumoral activity of different molecular weight fractions obtained from the husk fiber aqueous extracts of *C. nucifera*, varieties typical A and common, to evaluate whether the common variety has the same biological properties described for the typical A variety.

C. nucifera L. (Palmae), typical A variety, commonly known as "olho-de-cravo" and the common variety were both collected in Aracaju, Brazil, and authenticated by Dr. Benedito Calheiros Dias, Centro de Pesquisas do Cacau, Bahia, Brazil, where voucher specimens were deposited. A water extract from the husk fiber of both varieties was prepared as described previously (4). Both extracts were lyophilized and stored at 5ºC, yielding about 10% of the dry weight of the starting material (215 g). The crude aqueous extracts (2 g) were re-suspended in distilled water (100 mL) and filtered through a 0.22-µm membrane (Millipore, São Paulo, SP, Brazil). These extracts were separated into two major fractions of molecular weight greater than 1 kDa (high-molecular weight fraction, HMWF) and less than 1 kDa (low-molecular weight fraction, LMWF) by filtration through an Amicon Diaflo (Millipore) membrane of 1-kDa cut-off. Crude extracts were also subjected to serial membrane filtrations (cut-off of 1, 3, and 10 kDa), yielding fractions with molecular weights ranging in size from 1-3 kDa (FA) to 3-10 kDa (FB) and to more than 10 kDa (FC). For use, lyophilized extracts and fractions were diluted in water or RPMI 1640 medium and sterile-filtered.

Cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (8). The human erythroleukemia cell line K562 and Lucena 1, a multidrug-resistant (MDR) and vincristine-resistant derivative of K562 (9), were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine, at 37ºC in the presence of 5% CO_2. Cells were added to 96-well microtiter plates (2 x 10^4/well) and incubated with medium (control), different concentrations of HMWF or LMWF (5, 50, and 500 µg/mL) or 500 µg/mL FA, FB, or FC. After 48 h, each well received 20 µL MTT (5 mg/mL) and the plate was incubated for another 4 h at 37ºC in the dark. The plate was centrifuged, the pellet was solubilized in DMSO and absorbance was measured with an ELISA reader (BenchMark, Bio-Rad, Hercules, CA, USA) at 570 nm, with the reference filter at 655 nm. Data are reported as the mean ± SD of at least three experiments performed in triplicate. Statistical differences were calculated by the Tukey test, with the level of significance set at $P < 0.05$.

As shown in Figure 1A and B, the activity of LMWF (<1 kDa) and HMWF (>1 kDa) from the two *C. nucifera* varieties on the leukemia cell line K562 was very similar. No anti-tumoral activity was observed with the LMWF. However, at 500 µg/mL the HMWF from the typical A and common varieties reduced the cell viability of K562 by 60.1 ± 8.5 and 47.5 ± 11.9%, respectively, suggesting that the active principle responsible for this activity was present in similar amounts in the HMWF of both varieties.

The crude extracts were separated into three fractions, FA (1-3 kDa), FB (3-10 kDa) and FC (>10 kDa), and their cytotoxic activity was assessed on K562 cells. As can be seen in Figure 1C, the activity of FA and FB from both varieties was very similar. However, a significant difference was observed between FA and FC of variety typical A ($P < 0.001$) and between FB and FC of both varieties ($P < 0.01$ and $P < 0.05$ for the typical A and common varieties, respectively). These results indicate that in both varieties the antitumoral activity was concentrated in fractions ranging in molecular weight from 1 to 10 kDa (Figure 1C).

Natural or chemically induced MDR is one of the major problems in cancer treatment. Although MDR is a multifactorial phenomenon, resistance to numerous anticancer agents may be associated with overexpression of the ABC superfamily of transporter proteins (P-gp/ABCB1 or MRP1/ABCC1), which act as efflux pumps, decreasing the intracellular concentration of the drug (10). Therefore, we investigated whether the aqueous fractions from the husk fiber of *C. nucifera* were able to overcome resistance mediated by overexpression of P-gp, an MDR protein present in the leukemia line Lucena 1 (9). Figure 2A and B shows that, as observed for K562, while no activity was detected in the LMWF, at 500 µg/mL the HMWF from both varieties of *C. nucifera* was able to decrease by 50% the viability of Lucena 1. The activity of fractions FA (1-3 kDa), FB (3-10 kDa) and FC (>10 kDa) was also analyzed. Although no difference in cytotoxicity was observed between fractions A and B from the typical A variety, the activity of fraction C was significantly lower than the activity of the other fractions ($P < 0.001$ for both FA and FB). In the common variety a significant decrease in activity was only found for FB and FC ($P < 0.05$; Figure 2C). Thus, in both varieties, most of the anti-MDR activity was concentrated in fractions ranging in molecular weight from 1 to 10 kDa.

Since one of the major goals of cancer chemotherapy is to circumvent anti-apoptotic strategies developed by tumor cells, the

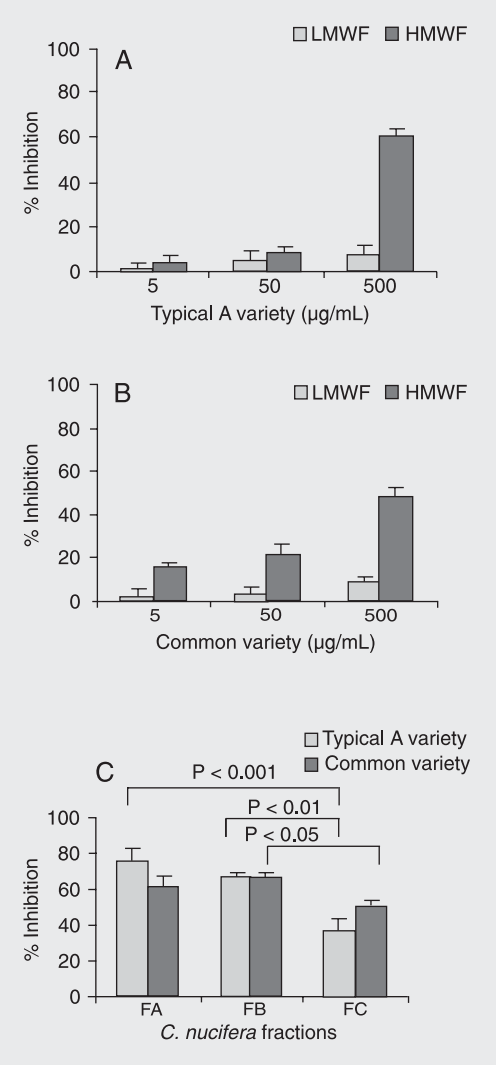

Figure 1. Cytotoxic activity of fractions from aqueous extracts of *Cocos nucifera* on K562. Cells were treated with the concentrations indicated in the figure of low- (LMWF) or high-molecular weight fractions (HMWF) of *C. nucifera*, typical A variety (A) or common variety (B) and viability was assessed by the MTT assay 48 h later. Alternatively, in *C*, cells were treated for 48 h with 500 µg/mL of different molecular weight fractions (FA (1-3 kDa), FB (3-10 kDa), FC (>10 kDa)) and viability was assessed as described above. Data are reported as mean ± SD of at least 3 experiments performed in triplicate.

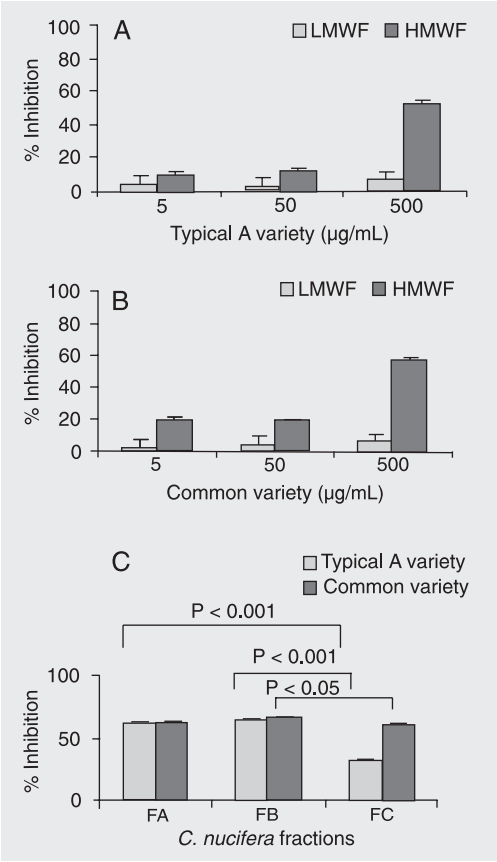

Figure 2. Cytotoxic activity of fractions from aqueous extracts of *Cocos nucifera* on Lucena 1. Cells were treated with different concentrations of low- (LMWF) or high-molecular weight fractions (HMWF) of *C. nucifera*, typical A variety (A) or common variety (B) and viability was assessed by MTT 48 h later. In *C*, cells were treated with 500 µg/mL of different molecular weight fractions (FA (1-3 kDa), FB (3-10 kDa), or FC (>10 kDa)) and viability was assessed as described for *A* and *B*. Data are reported as mean ± SD of at least 3 experiments performed in triplicate.

identification of new compounds able to overcome the resistance mechanisms and leading to tumor cell death is of great interest for cancer therapy. Thus, the present results, showing the presence of anti-MDR activity in extracts of the *C. nucifera* varieties typical A and common, may be relevant for the pharmaceutical industry. Different compounds may be associated with the antitumoral and anti-MDR activities found in the *C. nucifera* husk fiber extracts. Esquenazi et al. (4) showed that the extract of *C. nucifera* husk fiber is rich in catechins, epicatechins and condensed tannins. These substances, which are also found in green tea and in some fruits, have been reported to be potent inhibitors of cell growth (11-13), having anticancer activity (14,15). The main polyphenol of green tea, epigallocatechin gallate, has been shown to modulate the activity of transporter proteins in drug-resistant cancer cell lines (16-18). Further studies should be carried out in order to identify the bioactive compounds of the *C. nucifera* husk fiber extracts and to elucidate the mechanisms of their antitumor action.

C. nucifera is cultured extensively in the northern region of Brazil. The observation that the antitumoral activity of *C. nucifera*, typical A variety, was also present in comparable amounts in the common variety is of great interest since this variety is cultured on larger scale than the typical A variety. Since the husk fiber is a reject from the processing of *C. nucifera*, its use, in addition to solving an environmental problem, may lead to the production of a new low-cost medicine for cancer treatment, including tumors expressing the MDR phenotype.

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