

Effect of *Paullinia cupana* on MCF-7 breast cancer cell response to chemotherapeutic drugs

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Abstract. Previous studies suggested that certain plants, such as guarana (*Paullinia cupana*), exert a protective effect against cancer-related fatigue in breast cancer patients undergoing chemotherapy. However, guarana possesses bioactive molecules, such as caffeine and catechin, which may affect the pharmacological properties of antitumor drugs. Therefore, the aim of this study was to evaluate the effects of guarana on breast cancer cell response to 7 chemotherapeutic agents currently used in the treatment of breast cancer. To perform this study, MCF-7 breast cancer cells were cultured under controlled conditions and exposed to 1, 5 and 10 $\mu\text{g/ml}$ guarana concentrations, with and without chemotherapeutics (gemcitabine, vinorelbine, methotrexate, 5-fluorouracil, paclitaxel, doxorubicin and cyclophosphamide). The effect of these treatments on MCF-7 cell viability and proliferation was spectrophotometrically analyzed with the MTT assay. The main results demonstrated an antiproliferative effect of guarana at concentrations of 5 and 10 $\mu\text{g/ml}$ and a significant effect on chemotherapeutic drug action. In general, guarana improved the antiproliferative effect of chemotherapeutic agents, causing a decrease of >40% in cell growth after 72 h of exposure. The results suggested an interaction of guarana with

the chemotherapeutic drugs, which requires confirmation by *in vivo* complementary studies.

Introduction

Cancer-related fatigue (CRF) is a common phenomenon in patients undergoing cytotoxic chemotherapy and radiotherapy, with a prevalence of 59-100%, depending on the clinical status of the disease (1). CRF is also associated with other physical and psychological symptoms, such as pain, sleep disturbance, reduced physical activity and depression (2,3). Therefore, CRF negatively affects the functional status and quality of life of the patients (4).

Patients exhibiting moderate or severe fatigue may benefit from non-pharmacological as well as pharmacological interventions, including use of psychostimulants, such as methylphenidate and dexmethylphenidate, modafinil and erythropoietin-stimulating agents (5). However, the clinical management of CRF remains unsatisfactory. For this reason, de Oliveira Campos *et al* (6) performed a phase II randomized, double-blind, placebo-controlled crossover study to evaluate the effect of guarana (*Paullinia cupana*) against CRF. Guarana is an Amazon fruit used since the pre-Columbian era that is currently commercialized in herbal and energetic beverages due to its stimulant properties (7).

A phase II randomized, double-blind, placebo-controlled crossover study was conducted by de Oliveira Campos *et al* (6) on breast cancer patients undergoing systemic chemotherapy, with 100 mg/day of guarana powder supplementation. The guarana supplementation significantly decreased CRF. The study also reported no occurrence of toxic adverse effects, sleep disturbance or anxiety and depression in the patients receiving guarana supplementation. For this reason, the authors suggested that guarana may be an effective, non-toxic, cost-effective option for the treatment of CRF (6). A complementary study was also recently published, demonstrating that a purified dry extract of guarana may be effective in treating

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CRF patients with various solid tumors who undergo chemotherapy (8).

The guarana effect on CRF is possibly associated with its chemical composition, which includes a higher content of purinic alkaloid caffeine (1,3,7-trimethylxanthine) compared to coffee (*Coffea arabica*), tea (*Camellia sinensis*) and yerba mate (*Ilex paraguariensis*). Guarana also contains a small proportion of other purinic alkaloids, including theobromine and theophylline (6), as well as other chemical bioactive molecules, such as tannins and proanthocyanidins, with a higher content of catechins and epicatechins (9).

Despite the results suggesting a beneficial effect of guarana on breast cancer patients with CRF, the bioactive molecules in guarana may also affect the chemotherapeutic efficacy. The potential antitumor effect of catechins on breast cancer cells has been extensively described in the literature (10,11). However, the effect of caffeine is more controversial (12). A previous study suggested that caffeine may attenuate the MCF-7 cell response to chemotherapy due to its ability to intercalate into DNA (13). By contrast, another study performed on MCF-7 breast cancer cells treated with paclitaxel reported that caffeine supplementation enhanced the apoptosis induction triggered by the chemotherapeutic drug (14). A recent study also demonstrated that co-treatment with anticancer agents and 6-selenocaffeine decreased MCF-7 cell viability (15).

The abovementioned evidence prompted us to investigate whether guarana affects the properties of antitumor drugs when concomitantly administered to MCF-7 breast cancer cells. Therefore, the present study aimed to evaluate the effect of guarana on MCF-7 cell viability and proliferation, with and without exposure to 7 chemotherapeutic agents currently used in the treatment of breast cancer.

Materials and methods

Chemicals. Analytical grade chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). The MCF-7 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The RPMI-1640 culture medium, fetal bovine serum (FBS), heat-inactivated equine serum, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA); Vacutainer[®] tubes were provided by BD Diagnostics (Plymouth, UK).

Guarana extract. The guarana powder used in the present study was supplied by Western Agropecuary Research Brazilian Enterprise (EMBRAPA), a non-profit Brazilian governmental sector that offers technical support to the production of guarana in the Amazonas state. The bioactive compounds present in guarana powder were previously determined and described (16). The extract contained 12.240 mg/g caffeine, 6.733 mg/g theobromine and 4.336 mg/g total catechins. The concentration of condensed tannin was 16 mg/g. To perform the *in vitro* assay, the lyophilized extract was diluted in distilled water to a concentration of 200 mg/ml. The mixture was infused for 7 min by boiling, centrifuged at 1,500 x g for 15 min and filtered. The solution was sterilized by filtration (0.20 μ M), diluted in distilled water and added to cell culture medium to obtain 1, 5 and 10 μ g/ml guarana concentrations. These concentrations were selected considering that *in vivo*

guarana supplementation of breast cancer patients was relatively lower (100 mg/day).

Cell culture. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin and 100 g/ml streptomycin (pH 7.2) in a 5% CO₂ incubator at 37°C. Cell viability was measured using the MTT cell proliferation assay (17). Cells (1x10⁵) were seeded in a 96-well plate in 200 μ l complete culture medium. Following overnight adhesion, the medium was changed with media containing the antitumor drugs and different guarana extract concentrations. All the experiments were performed in triplicate.

Antitumor and guarana co-administration. The effect of the three different guarana concentrations on the cytotoxic and antiproliferative properties of 7 antitumor agents currently used in the treatment of breast cancer were tested on MCF-7 cells. The main actions of the chemotherapeutic agents on the cell cycle are presented in Fig. 1.

The selection of the antitumor agents was based on previous studies demonstrating their cytotoxic effect on MCF-7 breast cancer cells: Cyclophosphamide, a nitrogen mustard alkylating agent that forms irreversible DNA crosslinks leading to cell death (18); doxorubicin, an anthracycline antibiotic with a DNA intercalating effect; 5-fluorouracil, a pyrimidine analog that belongs to the family of antimetabolite drugs, causing irreversible inhibition of thymidylate synthase and, consequently, cell cycle arrest and apoptosis (19); paclitaxel, a mitotic inhibitor targeting tubulin, causing defects in mitotic spindle assembly and chromosome segregation (20); vinorelbine, a semi-synthetic vinca alkaloid with an antimitotic effect (21); gemcitabine, a nucleoside analog that adds a 'faulty' nucleoside during DNA synthesis, leading to cell apoptosis (22); and methotrexate, a folic acid analogue that prevents purine and pyrimidine synthesis, leading to the inhibition of DNA, RNA and protein synthesis (23). Based on previous studies investigating the effects of these chemotherapeutics on MCF-7 cells, their concentrations were as follows: 10 μ M gemcitabine (22), vinorelbine and methotrexate (24); 2 μ M 5-fluorouracil (25); 50 μ M paclitaxel (26); 200 nM doxorubicin (19); and 5 mM cyclophosphamide (27).

Cell viability and proliferation analysis. To evaluate the effect of the co-administration of guarana and antitumor agents on cell viability and proliferation, the MTT assay was used as previously described by Fukui *et al* (26), who investigated the effect of resveratrol and paclitaxel co-administration on the viability of several cancer cell lines with slight modifications. A total of 10 μ l of MTT (at 5 mg/ml) was added to each well at a final concentration of 500 μ g/ml. Subsequently, the mixture in each well was incubated for 1 h. The MTT is reduced by mitochondrial dehydrogenase in living cells to produce insoluble purple formazan crystals that are quantitatively measured following their removal from the cells by the addition of 100 μ l dimethyl sulfoxide (DMSO) (18). However, prior to the addition of DMSO, the treatment samples in the 96-well plate were visualized by optic microscopy (magnification, x400) and photographed. The images displayed living cells with purple

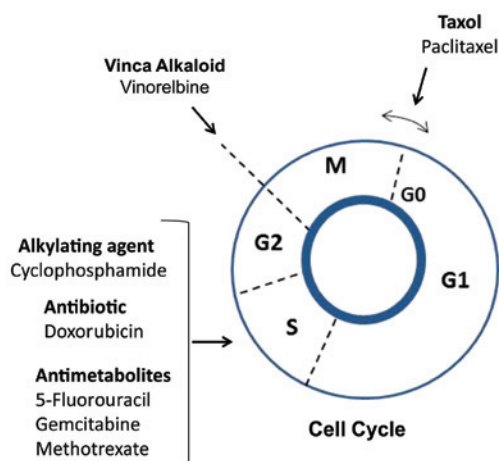


Figure 1. Chemotherapeutic agents used in the treatment of breast cancer and their action on the cell cycle.

formazan crystals and dying cells without crystals. The absorbance was read at 560 nm. The relative cell viability and/or proliferation under antitumor agent and guarana treatment were expressed as a percentage of the control well that was not treated with chemotherapeutic drugs. To evaluate the guarana co-administration effect on the properties of the chemotherapeutic agents, the results were expressed as a percentage of each tumor agent without guarana addition. All the experiments were performed in triplicate.

Statistical analysis. The different treatments were compared using one-way analysis of variance followed by Tukey's post hoc test. All the tests with $P < 0.05$ were considered to indicate statistically significant differences.

Results

Effect of chemotherapeutic agents on cell viability and proliferation. The effect of the chemotherapeutics on MCF-7 cells was initially determined to confirm that their concentrations were effective in decreasing cell viability and proliferation (Fig. 2). As expected, all the investigated drugs significantly decreased MCF-7 cell viability and proliferation ($P < 0.01$). The effect on viability was similar among all the chemotherapeutic agents used in the present study. However, the effect on cell proliferation was drug-dependent. Paclitaxel and cyclophosphamide were the chemotherapeutics that most significantly inhibited MCF-7 cell proliferation ($>70%$) compared to the control untreated cells and the cells treated by the other 5 agents.

Effect of guarana on cell viability and proliferation. The guarana extract produced from toasted seeds used in all the protocols exhibited a high caffeine content (12.3 mg/g) and was also rich in theobromine (6.8 mg/g) and total catechins (4.3 mg/g) (Fig. 3A and B). Therefore, three low concentrations of guarana (1, 5 and 10 $\mu\text{g/ml}$) were selected to investigate the effect of this extract on antitumor drugs. The isolated effect of guarana was evaluated prior to testing its action on MCF-7 cell response to chemotherapeutic drugs. As can be seen in Fig. 3, the three guarana concentrations tested here did not affect MCF-7 cell viability at 24 h of exposure. However, a signifi-

cant effect on MCF-7 cell proliferation was observed in the cells exposed to guarana at concentrations of 5 and 10 $\mu\text{g/ml}$ ($P < 0.01$).

Effect of guarana on the action of chemotherapeutic agents. Based on these data, the effect of guarana on the action of chemotherapeutics in MCF-7 cells was finally evaluated and the results are presented in Figs. 4 and 5. Guarana did not affect the viability of MCF-7 cells treated with cyclophosphamide, gemcitabine and paclitaxel after 24 h of exposure (Fig. 4A-C). However, the presence of different guarana concentrations significantly increased the cytotoxicity of 5-fluorouracil after 24 h of exposure. 5-Fluorouracil plus guarana at concentrations of 5 or 10 $\mu\text{g/ml}$ killed $\sim 50%$ of the MCF-7 cells compared to the untreated control group (Fig. 4D).

By contrast, when compared to the negative and positive control groups, guarana significantly increased MCF-7 cell viability in the methotrexate- (Fig. 5A), doxorubicin- (Fig. 5B) and vinorelbine-treated groups (Fig. 5C), mainly at concentrations of 5 and 10 $\mu\text{g/ml}$.

Different from the results at 24 h, the combination of guarana with all the investigated chemotherapeutic drugs exerted a strong antiproliferative effect on MCF-7 cells after 72 h of exposure ($P < 0.01$). This effect was more prominent when the cells were exposed to all guarana concentrations and paclitaxel. The cell proliferation was reduced by $\sim 80%$ when compared to the untreated control group. Furthermore, cyclophosphamide plus guarana at 5 $\mu\text{g/ml}$ was also effective in decreasing the MCF-7 cell population by $>80%$ compared to the control group (Fig. 4A). As regards the other antitumor drugs, the presence of guarana inhibited cell proliferation by $\sim 40-50%$ when compared to the negative control group.

Discussion

Historically, natural products have provided resources for the development of several antitumor molecules. Plants, marine organisms and microorganisms are the origin of $>60%$ of the drugs currently used in cancer therapy (28). In addition, plants may also be used to treat adverse effects caused by chemotherapeutics, such as CRF. However, this effect has been less extensively investigated compared to the anticancer action.

The present study demonstrated that guarana at low concentrations is able to differentially modulate MCF-7 cell proliferation, as well as affect the antitumor properties of 7 chemotherapeutic agents currently used in the treatment of breast cancer. In general, guarana intensified the antiproliferative effects of all the investigated drugs, although the initial effect on cell viability was heterogeneous.

Guarana possesses biological properties described in the literature as anti-inflammatory (29), antidepressant (30), panicytolytic (31) and energetic (6), which may help minimize CRF, as previously reported (7,8). Other studies have also described the antitumor activity of guarana using animal and cell experimental models (32-34). It has been suggested that guarana may be used to improve CRF caused by chemotherapy, which prompted us to conduct the present study.

Considering that guarana is rich in caffeine and also contains catechins, we performed a literature review regarding the potential effect of caffeine and catechins on MCF-7 cells,

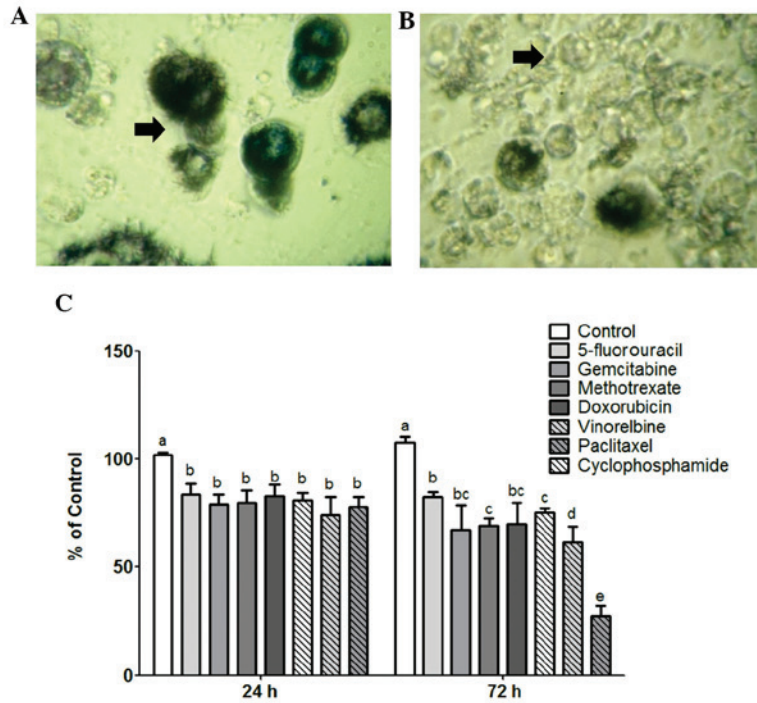


Figure 2. Chemotherapeutics effect on MCF-7 cell viability (24 h) and proliferation (72 h). (A) Microphotography of untreated viable MCF-7 cells showing the reaction with MTT that produces formazan, an insoluble crystal of purple color (arrow). (B) Microphotography of dying MCF-7 cells exposed to paclitaxel, exhibiting a lower formazan content. (C) Viability and proliferation of MCF-7 cells exposed to several chemotherapeutic drugs (data presented as % of control group). Different letters indicate statistically significant differences at $P < 0.05$ among untreated MCF-7 cells and cells treated with different chemotherapeutic drugs determined by one-way analysis of variance followed by Tukey's post hoc test.

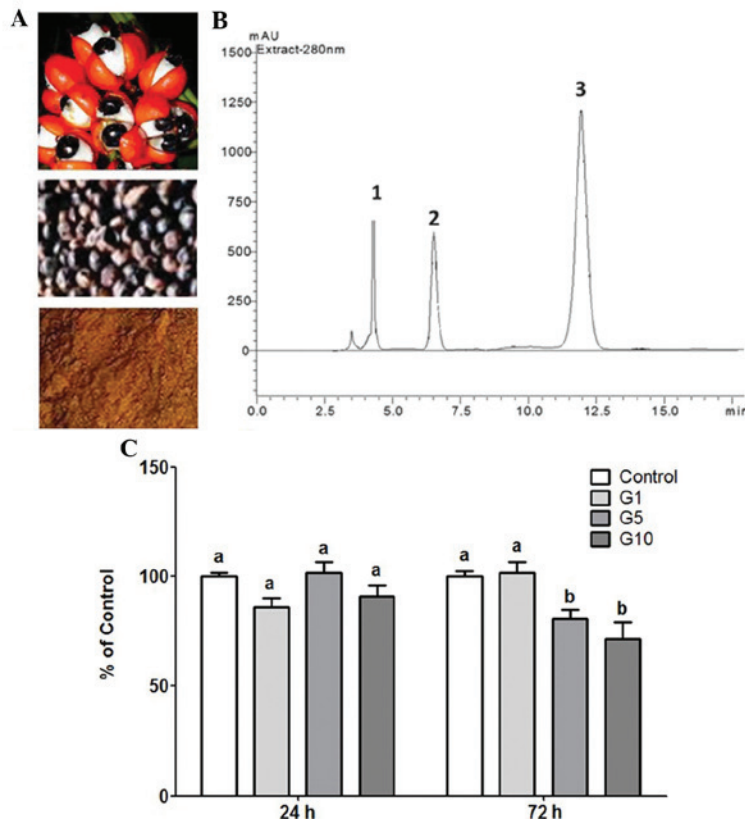


Figure 3. Effect of guarana on MCF-7 breast cancer cells. (A) Guarana powder is produced from toasted and triturated seeds. (B) Chromatography of guarana hydro-alcoholic extract exhibiting three peaks: 1, theobromine; 2, catechins; and 3, caffeine. (C) Effect of guarana at different concentrations (1, 5 and 10 $\mu\text{g/ml}$) on MCF-7 cell viability (measured after 24 h of exposure) and proliferation (measured after 72 h of exposure). Different letters indicate statistical differences at $P < 0.05$ among untreated MCF-7 cells and cells treated with different guaraná extract concentrations determined by one-way analysis of variance followed by Tukey's post hoc test.

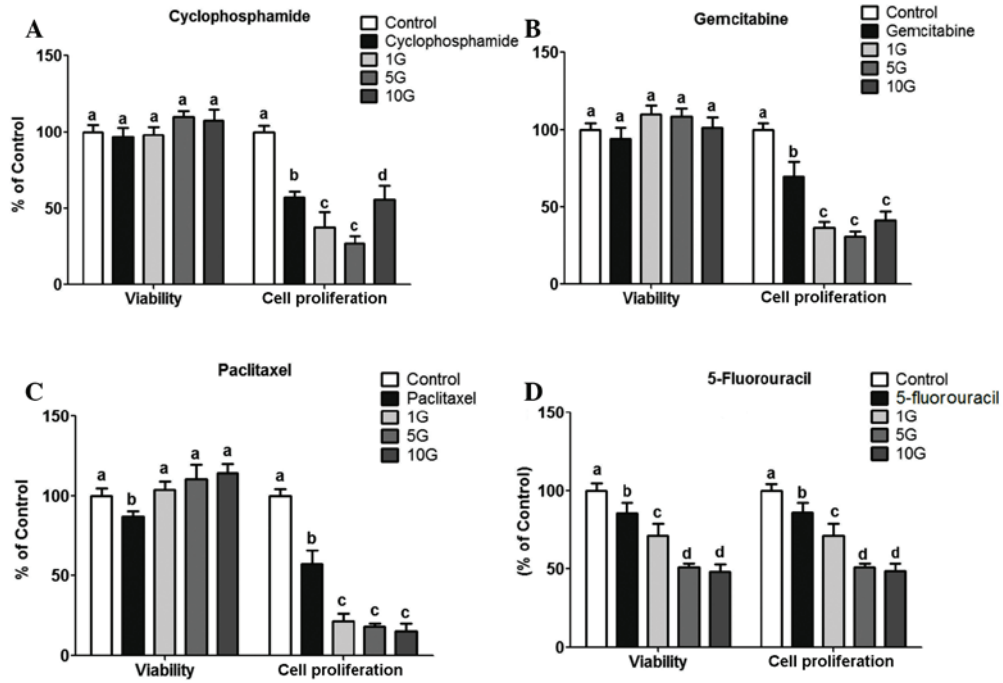


Figure 4. Effect of guarana on antitumor activity in MCF-7 cells exposed to different chemotherapeutics. Guarana concentrations: 1G, 1 µg/ml; 5G, 5 µg/ml; and 10G, 10 µg/ml. Different letters indicate statistically significant differences at P<0.05 among untreated MCF-7 cells and cells treated with different guaraná extract concentrations plus chemotherapeutic drugs determined by one-way analysis of variance followed by Tukey's post hoc test.

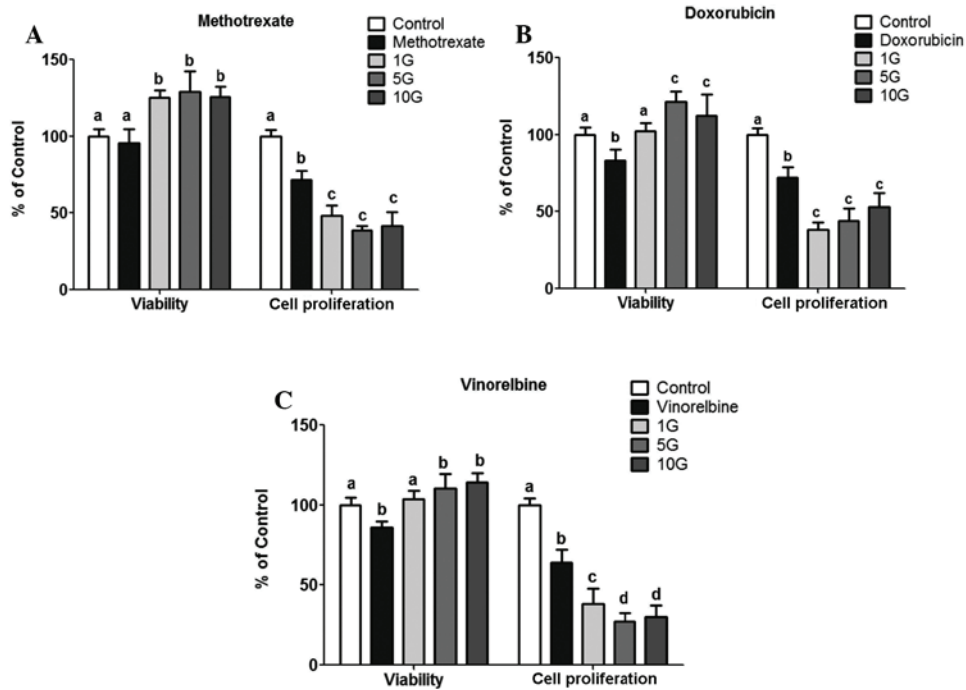


Figure 5. Effect of guarana on antitumor activity in MCF-7 cells exposed to different chemotherapeutics. Guarana concentrations: 1G, 1 µg/ml; 5G, 5 µg/ml; and 10G, 10 µg/ml. Different letters indicate statistically significant differences at P<0.05 among untreated MCF-7 cells and cells treated with different guaraná extract concentrations plus chemotherapeutic drugs determined by one-way analysis of variance followed by Tukey's post hoc test.

as well as the effect of these molecules on cell response to antitumor drugs.

MCF-7 is a cell line derived from an invasive ductal breast carcinoma, which expresses estrogen and progesterone receptors and exhibits a proliferative response in the presence of progesterone. This cell line may be used to investigate

resistance to antitumor agents, involving overexpression of the ABCG2 protein that confers multidrug resistance to tumor cells by extruding a variety of chemotherapeutic agents (35). Caffeine, the most widely used neuroactive compound in the human diet, has antiproliferative activity and the ability to induce cell cycle arrest and apoptosis (10).

However, the effect of caffeine on antitumor drugs appears to be cell line- and drug-dependent. As regards MCF-7, the line used in the present study, previous studies demonstrated the effect of caffeine on enhancing cell apoptosis caused by exposure to paclitaxel (14) and increasing the cytotoxic effect of alkylating drugs, such as cyclophosphamide (36). In addition, caffeine and other xanthines, including theophylline and dyphylline, significantly decreased the expression of the ABCG2 protein in the MCF-7/MX100 subline, which exhibits a high resistance to anti-breast cancer drugs (37).

By contrast, Hill *et al* (13) reported that caffeine may attenuate the cytotoxic effect of intercalating antitumor drugs, such as doxorubicin. That study described a possible interceptor role of caffeine, protecting cancer cell DNA from intercalation. In the present study, we observed a significant increase in MCF-7 cell viability after 24 h of exposure to doxorubicin plus guarana at concentrations of 5 and 10 $\mu\text{g/ml}$. However, this potential procarcinogenic effect was significantly attenuated after 72 h of exposure. This contradictory effect may be caused by other bioactive molecules present in guarana, such as catechins.

A previous study performed by Seeram *et al* (38) described that several catechin and anthocyanin molecules are able to inhibit the proliferation of cancer cells, including the MCF-7 cell line (39). The effect of catechins appears to be associated with the ability of these molecules to increase the expression of pro-apoptotic genes, such as caspase-3, -8, -9, as well as other genes involved in the apoptotic pathway (39).

A similar study that specifically evaluated the effect of epigallocatechin-3-gallate (EGCG), the main catechin present in green tea, on a breast carcinoma cell line resistant to tamoxifen (MCF-7Tam cells) reported cell growth inhibition and dose-dependent apoptosis. Following exposure to 100 $\mu\text{g/ml}$ EGCG for 24 h, the expression of Bax was increased and the expression of Bcl-2 was decreased (40). A recent study also reported that microRNA expression in MCF-7 cells may be affected by green tea, which is rich in catechins and caffeine, resulting in inhibition of carcinogenesis (41). A recent study also reported that microRNA overexpression in MCF-7 cells may be decreased following treatment with polyphenon-60, a catechin included in green tea (41). This mechanism of action may explain the antitumor effect of these molecules on MCF-7 breast cancer cells.

Despite the evidence on the effect of catechins on MCF-7 cells, we were unable to identify previous studies investigating the effect of these molecules on antitumor drug efficacy. Therefore, a complementary investigation is required to evaluate whether guarana exerts an effect on chemotherapeutic drug action associated with the effect of catechins on apoptosis and antitumor gene modulation.

Since all the investigated chemotherapeutic drugs were affected by the addition of guarana, mostly by improving the antiproliferative activity after 72 h of exposure, the therapeutic use of guarana in the treatment of CRF apparently does not compromise the effect of chemotherapy. However, *in vitro* protocols present with methodological limitations that require consideration in the interpretations of the results. Complementary *in vitro* investigations evaluating the gene modulation of the metabolic routes involved in carcinogenesis, as well as studies using animal models are required to verify our results.

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