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Paullinia cupana Mart var. *sorbilis*, guaraná, reduces cell proliferation and increases apoptosis of B16/F10 melanoma lung metastases in mice

H. Fukumasu[1], J.L. Avanzo[1], M.K. Nagamine[1], J.A. Barbuto[2], K.V. Rao[1] and M.L.Z. Dagli[1]

[1]Laboratório de Oncologia Experimental e Comparada, Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, [2]Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brasil

Correspondence to: M.L.Z. Dagli, Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, USP, Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508-900 São Paulo, SP, Brasil
Fax: +55-11-3091-7829. E-mail: mlzdagli@usp.br

We showed that guaraná (*Paullinia cupana* Mart var. *sorbilis*) had a chemopreventive effect on mouse hepatocarcinogenesis and reduced diethylnitrosamine-induced DNA damage. In the present experiment, we evaluated the effects of guaraná in an experimental metastasis model. Cultured B16/F10 melanoma cells (5 x 10^5 cells/animal) were injected into the tail vein of mice on the 7th day of guaraná treatment (2.0 mg *P. cupana*/g body weight, per gavage) and the animals were treated with guaraná daily up to 14 days until euthanasia (total treatment time: 21 days). Lung sections were obtained for morphometric analysis, apoptotic bodies were counted to calculate the apoptotic index and proliferating cell nuclear antigen-positive cells were counted to determine the proliferation index. Guaraná-treated (GUA) animals presented a 68.6% reduction in tumor burden area compared to control (CO) animals which were not treated with guaraná (CO: 0.84 ± 0.26, N = 6; GUA: 0.27 ± 0.24, N = 6; P = 0.0043), a 57.9% reduction in tumor proliferation index (CO: 23.75 ± 20.54, N = 6; GUA: 9.99 ± 3.93, N = 6; P = 0.026) and a 4.85-fold increase in apoptotic index (CO: 66.95 ± 22.95, N = 6; GUA: 324.37 ± 266.74 AB/mm^2, N = 6; P = 0.0152). In this mouse model, guaraná treatment decreased proliferation and increased apoptosis of tumor cells, consequently reducing the tumor burden area. We are currently investigating the molecular pathways of the effects of guaraná in cultured melanoma cells, regarding principally the cell cycle inhibitors and cyclins.

Key words: Melanoma; Guaraná; Metastases; Apoptosis; Growth control

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Despite the advances in cancer treatment, there continues to be a need for intervention strategies and one alternative is the use of chemopreventive agents that could act by preventing, delaying or reversing the cancer's malignant phenotype, and affect secondary or recurrent cancer lesions. Chemopreventive agents are known to have low side effects and toxicity and are involved in the neutralization of carcinogens and/or their effects on cells. In recent years, considerable effort has been devoted to identifying naturally occurring chemopreventive agents, particularly those present in dietary and medicinal plants. Several studies have reported a remarkable chemopreventive effect of plants, especially green tea (1,2).

Paullinia cupana Mart var. *sorbilis*, known as guaraná, is a Brazilian native plant originally growing at Maués, an area situated in the Amazon rain forest (3). The Sateré-Maués Indians have used guaraná for centuries as a stimulant, aphrodisiac and for headaches (3). Several

effects have been recently proved experimentally, such as protection against diethylnitrosamine-induced DNA damage (4), against gastric lesions induced by ethanol and indomethacin (5) and antioxidant and antibacterial activity (6). More recently, it was reported that guaraná administration reduced the incidence and multiplicity of preneoplastic lesions induced by diethylnitrosamine in mouse liver (7). This effect was attributed to reduced cell proliferation determined by analysis of proliferating cell nuclear antigen (PCNA) (7).

In view of these interesting effects of guaraná, the study of its effects on cell proliferation and apoptosis in a metastasis model, where cells already show a malignant phenotype, seemed appropriate. Thus, in the present study, we used the experimental melanoma B16/F10 metastasis model, a widely used experimental model (8,9). Metastases arise by cancer spread from a primary site, becoming able to form new tumors in distant organs. Lung metastases are considered to be the most important cause of death in cancer patients (10). This is a process that involves shedding cells from the primary tumor into the circulation, promoting cell survival, localization in a new organ, extravasation and accommodation in tissues, initiation and maintenance of growth, and vascularization of the metastatic tumor (10,11). When cancer is detected after it has metastasized, treatment of metastases is, in general, unsuccessful.

The main objective of the present study was to evaluate the growth inhibition effect of daily guaraná administration (2.0 mg/g body weight) on the experimental melanoma B16/F10 metastasis model. We report here the growth control effect of guaraná based on reduced tumor cell proliferation and increased apoptosis and suggest that this plant could be a potential chemopreventive agent for cancer.

The experiments were carried out with 2-month-old female C57Bl/6 mice which were maintained at the animal facility of the Department of Pathology, Faculty of Veterinary Medicine and Animal Science, University of São Paulo (FMVZ, USP). Twelve animals were divided into 2 groups of six each: guaraná-treated (GUA) and non-guaraná-treated or control (CO). Animals were maintained on a 12-h light/dark cycle under controlled conditions of temperature (20 ± 4°C), and relative humidity (55 ± 10%). The mice had *ad libitum* access to a standard diet (Nuvilab-CR1®, Nuvital Nutrientes Ltda., Brazil) and water (pH ±7.0). All procedures using animals were performed according to the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised in 1985) and were reviewed and approved by the Bioethics Committee of FMVZ-USP (953/2006).

Powdered *Paullinia cupana* seed was supplied by EMBRAPA, Amazônia Ocidental (Amazônia, Brazil), and stored under dry conditions at ±4°C until administration. Guaraná (2.0 mg *P. cupana*/g body weight) was diluted in 0.2 mL filtered water and administered by gavage once a day. The control group received only water by gavage daily (0.2 mL per animal). We chose this dose based on two previous studies from our laboratory (4,7) which already showed chemopreventive and DNA protective effects. The chemical composition of the guaraná sample was the same as published elsewhere (4,7). The caffeine content of the sample was determined by the method of Pagliarussi and co-workers (12) and was 1.02% of the dry matter. In addition, mouse B16/F10 melanoma cells were grown in RPMI-10 1640 medium supplemented with penicillin (50 IU/mL), streptomycin (50 mg/mL), and L-glutamine (2 mM) under standard conditions (37°C and a 5% CO_2 humidified atmosphere). Approximately 5 x 10^5 B16/F10 cells were diluted in 0.2 mL 0.9% saline before injection in mice and Trypan blue dye exclusion staining was used for cell quantitation and viability determination. The *P. cupana* preparation (2.0 mg/g body weight) or water was administered by gavage once a day throughout the experimental period. On the 7th day, mice from both groups received an intravenous injection of 5 x 10^5 B16/F10 cells into the tail vein and were euthanized at day 21. The lungs were exposed and carefully insufflated with metacarn (60:30:10 methanol, chloroform, and acetic acid, v/v) and the number of macroscopic metastases was counted. All organs were fixed in metacarn for a maximum of 8 h and routinely processed for paraffin embedding for further analysis. The histopathological analyses were performed using representative lung sections (5 µm) stained with hematoxylin and eosin (H&E). Next, all lesions were circled with an electronic pen and the software calculated each area automatically (Image Pro-Plus, version 4.5, Media Cybernetics, Bethesda, USA). The melanoma colonies and total area of the lung sections were measured for each animal. Tumor burden was calculated by dividing the total area occupied by melanoma colonies (µm^2) by the total area of a representative lung section (mm^2) and reported as percent. In addition, immunohistochemistry for the PCNA (PC 10, diluted 1:3200, Dako, Denmark) was performed in representative 5-µm lung sections from each animal. Briefly, lung sections were deparaffinized and rehydrated with consecutive baths in xylene and ethanol at different concentrations and endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 5 min. Non-specific protein-binding sites were then blocked with 5% skimmed milk (Dako) for 2 h and slides were treated with PCNA antibody overnight at 4°C. In order to detect PCNA, slides were treated by the streptavidin-biotin-peroxidase method according to manu-

facturer instructions (LSAB, Dako) and PCNA protein was detected after staining. Finally, hematoxylin was used to counter-stain the slides. The nuclei of PCNA-positive cells have a brown color due to 3'3 diaminobenzidine staining and at least 1000 cells per animal were counted. The proliferation index was obtained by dividing the number of brown-stained nuclear cells by the total number of cells counted for each animal and multiplied by 100. Also, apoptosis was evaluated by the presence of melanoma apoptotic bodies (AB) by fluorescence microscopy as described by Stinchcombe et al. (13) using a Nikon microscope (E-800, Tokyo, Japan) equipped with an epi-fluorescence unit. This method is based on the strong eosin fluorescence of AB in H&E-stained tissues detected under UV light (465-495 nm). Identification of AB was confirmed by switching the microscope system from fluorescent to transmitted light and using the morphological criteria established by Kerr et al. (14). ABs were represented by acidophilic bodies with fragmentation or lack of chromatin accompanied by cytoplasmic condensation and/or fragmentation. If single cells or clusters of directly neighboring cells contained multiple AB, these were assumed to be derived from the same apoptotic cell and were counted as only one event. At least 1000 apoptotic cells from all lesions of each animal were counted. The apoptotic index was reported as the number of melanoma AB/mm^2 of melanoma lesions.

Statistical analysis was performed using the GraphPad Prism software (version 3.0, GraphPad Software Inc., USA). Two-way ANOVA was used to evaluate body weight variation throughout the experiment. Also, the Mann-Whitney test was applied to all other data because they did not follow normal distribution. The level of significance was set at $P < 0.05$.

The macroscopic evaluation was performed immediately after euthanasia. No pathological alterations were observed in the thoracic or peritoneal cavity. In addition, no relevant effects were observed in spleen, liver, brain, intestines, or kidneys after guaraná treatment. Also, guaraná did not alter body weight gain throughout the experiment (Table 1). When gross lung examination was performed, visible melanoma nodules were counted and the results are shown in Table 1. No significant differences were noted regarding the total macroscopic lesions when the groups were compared (CO: 38.16 ± 23.77, GUA: 30.33 ± 16.19 lesion/animal; P = 0.56). Additionally, no significant reduction in the number of microscopic lesions (CO: 0.15 ± 0.08, GUA: 0.11 ± 0.06 lesion/mm^2; P = 0.59) was observed after guaraná treatment (Table 1). We also showed that guaraná promoted a significant three times reduction (CO: 0.84 ± 0.26, GUA: 0.27 ± 0.24%; P = 0.0043) of tumor burden (Table 1) compared to animals that received only water. This interesting effect was due to the decreased expression of PCNA in melanoma colonies after treatment with guaraná.

In this experiment we counted PCNA-labeled cells of all melanoma colonies in each animal from both groups, and the results showed that guaraná reduced melanoma proliferation 2.4 times (CO: 23.75 ± 20.55, GUA: 9.99 ± 3.94%; P = 0.026) compared to control group (Table 1 and Figure 1). In addition to decreased cell proliferation, guaraná also increased apoptosis in melanoma colonies. Apoptotic tumor cells were seen randomly and singly throughout the melanoma lesions, as shown in Figure 1. The apoptosis index for control and guaraná-treated animals is shown in Table 1, and was significantly increased by guaraná treatment (CO: 66.94 ± 22.94, GUA: 324.37 ± 266.74 AB/mm^2; P = 0.0152).

Metastases are defined as tumor implants discontinuous with the primary tumor, and are unequivocally defined as malignant because benign neoplasms do not metastasize (15). Spread metastases occur generally at late stages of the carcinogenic process, characterized by the progression phase of carcinogenesis (16). Additionally, metastases can spread in three major ways: seeding of body cavities and surfaces and lymphatic or hematogenous spread (15). The intravenous injection of melanoma B16/F10 cells in C57Bl/6 mice used here is considered to be a model of experimental metastasis since melanoma cells enter the venous system and grow primarily in lungs (9). Depending on the concentration of injected cells, the mouse lifespan is altered. Therefore, in our experiment we injected 5 x 10^5 cells per animal, and sacrificed mice after 14 days. At the dose tested here, 2.0 mg/g guaraná for 21 days showed no toxic effects on four major organs: spleen, liver, cerebrum, and kidney, or body weight alterations, in agreement with early published studies (4,7). Macroscopic

Table 1. Analysis of total macroscopic lesions, microscopic lesions, microscopic tumor burden, and proliferating cell nuclear antigen (PCNA)-positive cells in animals submitted to the experimental metastasis model.

Groups	Control (N = 6)	Guaraná (N = 6)
Total macroscopic lesions	38.16 ± 23.77	30.33 ± 16.19
Microscopic lesions per mm^2	0.15 ± 0.08	0.11 ± 0.06
Microscopic tumor burden (%)	0.84 ± 0.26	0.27 ± 0.24*
PCNA-positive cells (%)	23.75 ± 20.55	9.99 ± 3.94*
Apoptosis index	66.94 ± 22.94	324.37 ± 266.74*

Data are reported as means ± SD.
*P < 0.05 compared to control (Mann-Whitney test).

evaluation of the lung revealed the presence of melanoma colonies in both groups, varying in number and size. The gross results (Table 1) led us to conclude that guaraná treatment has no effect on melanoma cell colonization and establishment in the lungs. In agreement with this result, the number of microscopic lesions (Table 1) was the same for the control and guaraná-treated groups, supporting the view that there is a lack of effect on some of the major steps of the metastasis cascade (16) in this experimental model.

However, histological analysis of the lung brought new information after the evaluation of the number and area of melanoma colonies in order to quantify tumor burden. Guaraná-treated animals presented a smaller tumor burden than control animals (P = 0.0043) and this interesting result led us to determine the balance between the proliferation and death of these tumor cells by evaluating growth

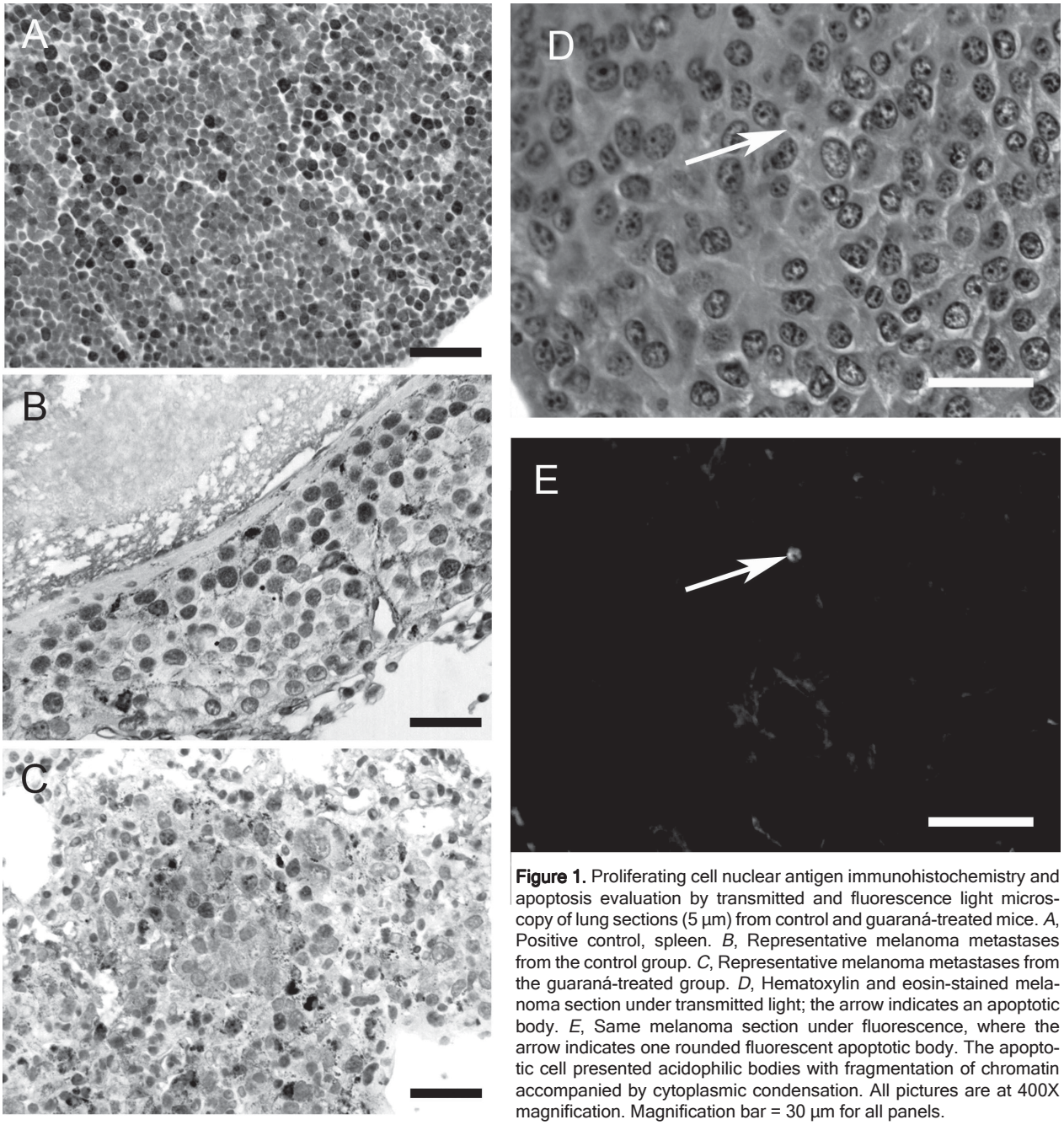

Figure 1. Proliferating cell nuclear antigen immunohistochemistry and apoptosis evaluation by transmitted and fluorescence light microscopy of lung sections (5 µm) from control and guaraná-treated mice. *A*, Positive control, spleen. *B*, Representative melanoma metastases from the control group. *C*, Representative melanoma metastases from the guaraná-treated group. *D*, Hematoxylin and eosin-stained melanoma section under transmitted light; the arrow indicates an apoptotic body. *E*, Same melanoma section under fluorescence, where the arrow indicates one rounded fluorescent apoptotic body. The apoptotic cell presented acidophilic bodies with fragmentation of chromatin accompanied by cytoplasmic condensation. All pictures are at 400X magnification. Magnification bar = 30 µm for all panels.

control by determining PCNA expression. The PCNA protein starts to accumulate in late G1, reaches a peak at the S-phase, and decreases in the G2 and M phases. Therefore, quantitation of PCNA-labeled cells in a given tissue is useful for the evaluation of tissue proliferation index (17). Surprisingly, in the present mouse model, tumor cells from guaraná-treated animals showed reduced PCNA expression (P = 0.026), which was correlated with a lower cell proliferation state. These results agree with our previously published data since we showed that guaraná reduced the incidence and multiplicity of liver preneoplastic foci (7) by diminishing cell proliferation. Both models demonstrate that guaraná can effectively control the growth of altered cells from early transformed (liver preneoplastic foci) to highly malignant cells (melanoma B16/F10) in *in vivo* experiments.

It is known that failure of normal apoptotic mechanisms in cancer cells underlies the process of carcinogenesis. Neoplastic cells undergo changes that decrease their susceptibility to apoptosis and it is considered that reversal of such a process is a hallmark of cancer chemotherapy. The technique used in the present study is useful to evaluate the number of apoptotic bodies in H&E sections based on the eosin-stained property of apoptotic bodies, which fluoresce under defined conditions of UV light. In addition, to be sure about this quantitation, we confirmed all apoptotic bodies under transmitted light (13), where the classical apoptosis signals could be noted (14). The principal advantages of this method are: quick recognition of apoptotic bodies which sometimes are poorly visible under transmitted light, and approximately three times more sensitivity than the standard method, causing this to be an excellent technique for comparing apoptosis between different groups. Following these lines, we quantitated the apoptotic index by dividing the total number of apoptotic bodies by the total area of melanoma lesion. The guaraná treatment promoted a 5-fold increase in apoptotic index, contributing to an extensive reduction of tumor burden. This effect, associated with slower cell proliferation, indicates that guaraná has a strong growth control effect in this model.

Following the re-orientation of emphasis on cancer research that will direct greater resources towards prevention of new disease rather than treatment of end-stage disease (18), studies are in progress to isolate the substances responsible for this growth control effect of guaraná. It is known that guaraná is rich in caffeine and tannins (4,7), and caffeine has been shown to play a key role in controlling the metastasis process (see Ref. 19 and references therein), mostly due to up-regulation of several genes related to the extracellular matrix such as *Fbln1*, *Bgn*, *Sparc*, *Fbn1*, *Loxl1*, *Col1a1*, *Col3a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col6a1*, *Col6a2*, and *Col6a3*. On the other hand, the anti-cancer effects of catechins (tannins) from green tea are world recognized as other plant-derived tannins (1,2). Guaraná contains catechin, epicatechin, ent-epicatechin, and procyanidins B1, B2, B3, B4, A2, and C1 (20), and some of these substances are also present in the composition of green tea. Possibly, the substances mentioned above could be at least partially responsible for the guaraná effects observed in the present study. To our knowledge, this is the first study showing that guaraná can control melanoma growth *in vivo* by two distinct cancer hallmarks: reducing cell proliferation and increasing cell death through apoptosis.

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