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Sangre de grado *Croton palanostigma* induces apoptosis in human gastrointestinal cancer cells

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Abstract

Sangre de grado is an ethnomedicinal red tree sap obtained from *Croton* spp. that is used to treat gastrointestinal ulcers, cancer and to promote wound healing. To evaluate the potential role of sangre de grado (SdG) in cancer we examined its effects on human cancer cells, AGS (stomach), HT29 and T84 (colon). Viability of cells treated with SdG (10–200 µg/ml) decreased ($P < 0.01$) in a dose dependent manner measured over a 24-h period. Cell proliferation at 48 h decreased ($P < 0.01$) in all cells treated with SdG (> 100 µg/ml). When cells in suspension were treated with SdG (100 µg/ml) cell adherence was severely compromised ($> 85\%$). Cells treated with SdG (100 µg/ml) underwent apoptosis as detected by nucleus condensation and DNA fragmentation determined by ELISA, and flow cytometry. Morphological changes as assessed by acridine orange. These effects were similar to that observed with Taxol (30 µM). A significant alteration of microtubular architecture was equally observed in both stomach and colon cancer cells exposed to SdG (100 µg/ml). The induction of apoptosis and microtubule damage in AGS, HT29 and T84 cells suggest that sangre de grado should be evaluated further as a potential source of anti-cancer agents. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Croton; Apoptosis; Cancer cells

1. Introduction

Sangre de grado (*Croton palanostigma*), a member of the family Euphorbiaceae, is one of the most widely used medicinal plants of the South American tropics. The *Croton* genus includes several species (*Croton lechleri*, *C. palanostigma*, and *C. dracooides*) all of which contain a red viscous sap that is obtained from making cuts in the bark (Duke and Vasquez, 1994). The sap is reported to have a wide range of pharmacological and physiological actions (Chen et al., 1994; Desmarchelier et al., 1997; Phillipson 1995). It is used for wound healing (Porrás-Reyes et al., 1993), controlling diarrhea (Gabriel et al., 1999; Holodniy et al., 1999), gastric ulcer

healing and as a treatment for intestinal inflammation (Miller et al., 2000).

Several compounds have been isolated previously from sangre de grado (Cai et al., 1991, 1993a,b). It has been reported that the bark sap contains only one alkaloid, Taspine, (Bettolo and Scarpati, 1979) which has been claimed to be the active principle responsible for the anti-inflammatory and wound healing properties (Perdue et al., 1979; Vaisberg et al., 1989). However, the wound healing activity has also been attributed to the lignan 3'4'-*O*-dimethylcedrusin (Pieters et al., 1993).

Recent findings from our laboratory showed the ability of sangre de grado promotes healing of gastric ulcers in rats and impairs the intestinal secretory response to capsaicin in guinea pig ileum (Miller et al., 2000). Thus, it appears that SdG has generalized benefits in gastrointestinal function. However, an evaluation of its ethnomedical application in cancer is

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lacking. A number plants of the family Euphorbiaceae have been used to treat cancers and tumors from at least the time of Hippocrates, and scientific references have been published in several countries (Iancu et al., 2000; Itokawa et al., 1991; Kupchan et al., 1976).

In our continuing efforts to evaluate the anti-cancer activity of medicinal plants from the Amazon, we collected ethnomedical information from tropical regions of South America and that led us to investigate the *Croton* spp., sangre de grado, which is used to treat certain forms of gastrointestinal cancers. It has been claimed that the growth inhibitory or antiproliferative effects of sangre de grado are associated with its cytotoxic and anti-tumor activity (Chen et al., 1994). However, the mechanisms of this beneficial effect have not been elucidated.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all reagent grade chemicals and the rabbit polyclonal antitubulin antibody were purchased from Sigma Chemical Co (St. Louis, MO). All cellular reagents and culture medium were from Gibco BRL (Gaithersburg, MD).

2.2. Preparation of plant material

The red sap of sangre de grado (*Croton palanostigma*) was collected from the tropical region of Upper Huallaga Valley (Tingo Maria, Peru) and was provided by Rainforest Phytoceuticals (Delmar, NY). The sangre de grado (SdG) sap was centrifuged at 4000 rpm for 15 min at room temperature then filtered at 20 μm using a Whatman paper no. 4. The filtrate was freeze-dried using a Freezemobile 6 concentrator (Virtis, Gardiner, NY). For cell culture experiments, the freeze-dried SdG was reconstituted in water to a 1% solution and filtered at 0.2 μm . Aliquots of the extracts were kept at 4 °C until further use.

2.3. Cell cultures

Cancer cells [AGS (stomach), HT29 and T84 (colon)] were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in DMEM (high glucose), 10% FCS and supplemented with 25 mM HEPES, pH 7.4; 4 mM L-glutamine; 40 $\mu\text{g}/\text{ml}$ penicillin; 90 $\mu\text{g}/\text{ml}$ streptomycin and 1.2 g/l NaHCO_3 . Cell cultures were maintained in a humidified 5% CO_2 incubator at 37 °C. At confluence cells were detached with trypsin–EDTA. Harvested cells were grown in 6-well tissue culture plates (1×10^6 cells/well) for viability, adhesion and apoptosis experiments. Cells were allowed

to grow to confluence over 24 h before use. For proliferation studies, cells were plated in 96-well plates (5×10^3 cells/well).

2.4. Viability assay

Cells were plated in medium containing SdG (10–200 $\mu\text{g}/\text{ml}$) for 24 h. The number of surviving cells are presented relative to the number initially plated (1×10^6 , designated 100%). After the cells were treated with SdG, the cells were harvested by trypsinization, and the viability of the cells was assessed immediately by trypan blue exclusion assay. Stained and unstained cells were counted in a hemocytometer as previously reported (Sandoval et al., 1997a).

2.5. MTT assay

The amount of viable cells was determined by examining cell number with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) dye-reduction assay measuring mitochondrial respiratory function (Mosmann, 1983). Cancer cells (5×10^3 /well) were plated in 96-well plates and treated with SdG at various concentrations and for different periods of time. Cells were incubated with medium containing MTT (0.5 mg/ml) for 4 h and solubilized with acidified isopropanol (0.04 N HCl in isopropanol). Absorbance was determined in a V_{max} kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 550 nm. The absorbance is directly related to viable cells.

2.6. Cell adhesion

AGS, HT29 and T84 cells were plated in six-well plates and immediately were treated with SdG (10–200 $\mu\text{g}/\text{ml}$) for 6 h. The number of floating and attached cells was counted using a hemocytometer.

2.7. Detection of apoptosis by ELISA

AGS, HT29 and T84 cells were treated with SdG (100 $\mu\text{g}/\text{ml}$) and incubated for 12 h. The assay is based on the photometric sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against cytosolic DNA fragments and histones, as described previously (Sandoval et al., 1997b). Three incubation steps were performed: (a) the anti-histone antibody was fixed on the wall of a microtiter plate, (b) the nucleosomes contained in the sample were bound via their histone components to the anti-histone antibody, and (c) anti-DNA-peroxidase was added to react with the DNA-part of the nucleosome. The amount of low molecular weight DNA present in the cytosolic fraction was determined using a V_{max} kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 405/490 nm.

Assays were performed using a detection kit and following the recommendations of the manufacturer (Boehringer–Mannheim, Indianapolis, IN).

2.8. Fluorescence microscopy

After treatment with SdG (100 µg/ml) for 12 h, adherent cells (AGS, HT29 and T84) were trypsinized from the plates, and collected for analysis. Another set of experiments with AGS cells were conducted to evaluate the effect of Taxol (30 µM = 25.6 µg/ml) on apoptosis and elucidate if Taxol also elicits similar effects as observed with sangre de grado. Acridine orange staining was performed as previously described (Sato et al., 1995). Briefly, cell suspensions (100 µl) were mixed with acridine orange (5 µg/ml); from this mixture an aliquot of 25 µl was dropped on to a microscope slide. Cells were visualized for nuclear fragmentation under blue–green fluorescence using a phase-contrast inverted microscope DMIRB (Leica Mikroskopie und, Germany).

2.9. Detection of apoptosis by flow cytometry

To detect breaks in DNA, HT29 and T84 cells were treated with SdG (100 µg/ml) and incubated for 12 h. A one-step TUNEL assay was performed using the in situ cell death detection kit, Fluorescein (Boehringer–Mannheim, Indianapolis, IN) as described previously (Sato et al., 1995). In brief, after the induction of apoptosis, cells were fixed by 4% paraformaldehyde in PBS for 30 min at 4 °C. Then samples were processed as indicated by the manufacturer. Cells were gated by forward and side light scatters. Samples were analyzed using an Epics Elite flow cytometer (Coulter Corp., Hialeah, FL). The TUNEL technique permits discrimination between cell death by apoptosis or necrosis.

2.10. Immunofluorescent detection of microtubules

HT29 cells were plated (1×10^5 cells/well) onto Lab-Tek 8 well glass chamber slides (Nunc Inc., Naperville, IL) and incubated at 37 °C to allow them to adhere to the well glass chamber. After 12 h, the adherent HT29 cells were cultured for 12 h in fresh media with or without SdG (100 µg/ml). The cells were then fixed in 10% buffered formalin, washed in PBS (pH 7.4) and permeabilized for 10 min in PBS containing 0.05% Triton X 100 and 1% BSA (Manthey et al., 1992). Determination of microtubule morphology was assayed using a microscope Microphot-SA EPI-FL3 (Nikon Corporation, Tokyo, Japan). Photographs were taken using Kodak film TMAX 400 (Rochester, NY) as described previously.

Table 1

Effect of sangre de grado (*C. palanostigma*) on cell viability of gastrointestinal cancer cells

Group	Viability (%)		
	AGS	HT29	T84
CTRL	98.3±3.2	96.7±2.8	98.1±2.3
SdG, 10 µg/ml	96.1±4.7	97.8±4.2	97.9±7.4
SdG, 100 µg/ml	45.3±1.1*	53.8±4.4*	54.7±7.1*
SdG, 200 µg/ml	23.6±1.0*	29.0±2.6*	30.1±2.4*

Cells (1×10^6 cells/well) were treated with sangre de grado (SdG) for 24 h and incubated at 37 °C. Values are mean±S.E. of two experiments, each with three replicates.

* Significant decreases ($P < 0.01$) compared to control (CTRL) group.

Table 2

Effect of sangre de grado (*C. palanostigma*) on proliferation of gastrointestinal cancer cells

Group	Cell proliferation, OD 515 nm		
	AGS	HT29	T84
CTRL	0.351±0.06	0.22±0.02	0.199±0.05
SdG, 10 µg/ml	0.365±0.04	0.22±0.02	0.219±0.01
SdG, 100 µg/ml	0.197±0.01*	0.122±0.018*	0.028±0.002*
SdG, 200 µg/ml	0.157±0.01*	0.11±0.01*	0.016±0.001*

Cells (5×10^3 cells/well) were treated with sangre de grado (SdG) for 48 h and incubated at 37 °C. Values are mean±S.E. of two experiments, each with three replicates.

* Significant decreases ($P < 0.01$) compared to control (CTRL) group.

2.11. Statistical analysis

Each experiment was performed at least two times and results are presented as the mean±S.E.M. Statistical analyses were performed using one-way ANOVA. Post hoc comparison of means was done by the least significant difference test. A probability of < 0.05 was considered significant.

3. Results

3.1. Cell viability and proliferation

The trypan blue exclusion assay demonstrated that SdG (10–200, µg/ml) treatment resulted in a dose-dependent inhibition of cell viability (Table 1). Similarly, SdG treatment of AGS, HT29 and T84 cells resulted in maximum loss of cell viability at concentrations > 200 µg/ml at 48 h. Sangre de grado treatment also resulted in a time-dependent inhibition of cell growth in the three cell lines (AGS, HT29 and T84). The earliest effect of SdG (> 100 µg/ml) on cell

Table 3

Effect of sangre de grado (*C. palanostigma*) on adhesion of gastrointestinal cancer cells

Group	Adhesion index (%)		
	AGS	HT29	T84
CTRL	46.5±2.3	45.2±2.3	66.5±1.6
SdG, 10 µg/ml	42.5±1.3	43.2±1.6	61.3±1.8
SdG, 100 µg/ml	8.5±1.3*	6.8±0.6*	14.2±1.0*
SdG, 200 µg/ml	7.4±0.7*	4.8±0.5*	9.5±0.9*

Cells (1×10^6 cells/well) were treated with sangre de grado (SdG) and incubated for 6 h at 37 °C, and floating cells collected and counted. Values are mean±S.E. of two experiments, each with three replicates.

* Significant decreases ($P < 0.01$) compared to control (CTRL) group, and low levels of SdG.

proliferation was detected at 24 h, and the effect was more pronounced at 48 h (Table 2).

3.2. Cell adhesion

To evaluate the effect of SdG on cell adhesion, cells in suspension were treated with SdG (10–200 µg/ml). After a 6 h incubation, floating AGS, HT29 and T84 cells were collected and counted; results indicated that SdG

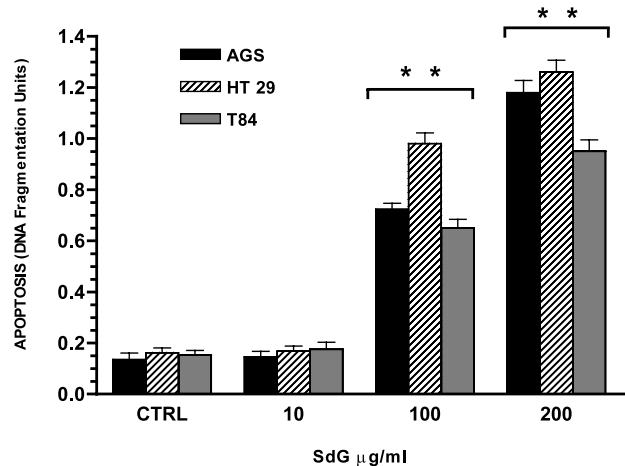


Fig. 1. Apoptosis in AGS, HT29 and T84 cells after treatment with sangre de grado (SdG, 10–200 µg/ml). Cells were seeded at a density of 1×10^6 cells/well. Bars represent enrichment of the cytosol with low molecular weight DNA fragments of cells incubated for 16 h. Details of the experiments are described in Section 2. Values are mean±S.E.M. of two experiments, each with three samples. **Significant increase ($P < 0.001$) in apoptosis compared to control.

treatment decreased ($P < 0.01$) attachment when compared to the untreated control group (Table 3). Floating cells excluded trypan blue with this exposure (6 h)

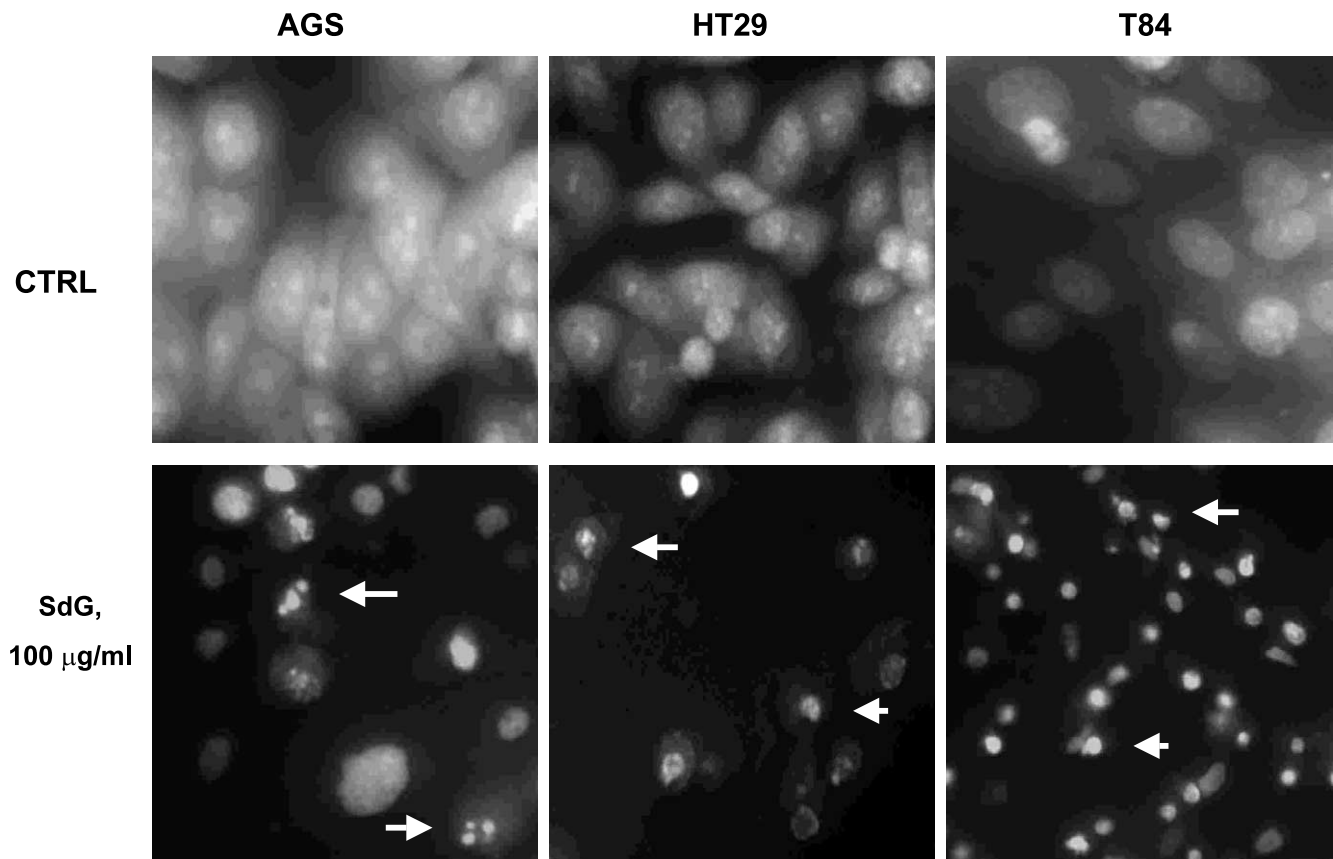


Fig. 2. Sangre de grado-induced apoptosis in human cancer cells. AGS, HT29 and T84 cells, stained with acridine orange, from untreated control cultures or cells exposed to sangre de grado (SdG, 100 µg/ml) for 12 h as described in Section 2. Apoptotic cells show chromatin condensation and nuclear fragmentation.

indicating that necrosis was not the cause of the failure of cells to adhere to the surface of the dish. In separate experiments, we evaluated the ability of the floating AGS, HT29 and T84 cells treated with SdG (100 $\mu\text{g}/\text{ml}$) to reattach when transferred to another flask containing medium without SdG. Results indicated that the three cell lines failed to regain their capacity to adhere to the bottom of the flask after an additional 24 h incubation.

3.3. Assessment of apoptosis

We evaluated the effect of SdG on apoptosis in various gastrointestinal cancer cells by several means. As shown in Fig. 1, compared to the control group, SdG (> 100 $\mu\text{g}/\text{ml}$ for 12 h) resulted in the formation of DNA fragments in AGS, HT29 and T84 cells typical of apoptosis. This assay utilizes a kit based on the photometric sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against cytosolic DNA fragments and histones. The induction of apoptosis by SdG was also evident from the morphologic alterations as shown by fluorescence microscopy (Fig. 2). The untreated control as well as concentrations of SdG < 20 $\mu\text{g}/\text{ml}$ did not cause any alterations in the nuclear morphology in these three cell lines. However, at 100 $\mu\text{g}/\text{ml}$ of SdG the cells showed significant apoptosis as evidenced from advanced chromatin condensation, nuclear condensation, and formation of apoptotic bodies (Fig. 2). To compare the effect of SdG (100 $\mu\text{g}/\text{ml}$) with Taxol (30 μM), another set of experiments was conducted and similar effect on cell morphology were observed on exposure to both compounds (Fig. 3). Flow cytometric analysis of AGS, HT29 and T84 cells treated with SdG (100 $\mu\text{g}/\text{ml}$) and labeled with Fluorescein, confirmed apoptosis as had previous experiments with acridine orange (Fig. 4). The TUNEL technique used for the flow cytometry experiments allowed us to assess DNA strand breaks generated during apoptosis.

3.4. Microtubule morphology

Fixed preparations of HT29 cells were examined by indirect immunofluorescence on a light microscope using a β -tubulin-specific monoclonal antibody. In HT29 cells, SdG (100 $\mu\text{g}/\text{ml}$) caused a marked loss of microtubule structure and the formation of intensively stained, prominent amorphous clumps (Fig. 5). Similar observations were recorded with AGS and T84 cells where following SdG treatment of cells at 100 $\mu\text{g}/\text{ml}$ caused microtubules to stain more intensely and to form clusters. Based on the degree of microtubule depolymerization, T84 cells appeared more susceptible than HT29 cells, and the earliest signs were observed after a 6 h incubation with SdG.

4. Discussion

The finding of new agents that could inhibit cell proliferation or induce programmed cell death could be some of the potential mechanisms for controlling the growth of malignant cancer cells. Here we discuss the therapeutic potential of sangre de grado to inhibit proliferation of malignant cells (AGS, HT29 and T84) by a mechanism that involves interference with the cell capacity to adhere, and in turn induce cell death via apoptosis. In this context, the medicinal plant sangre de grado (*C. palanostigma*) could represent an important ethnomedicine in the treatment of gastrointestinal disorders.

Although colorectal cancer is more prevalent in developed countries, an increasing number of colon cancer cases are also being reported where Western dietary habits have taken hold. Data from recent

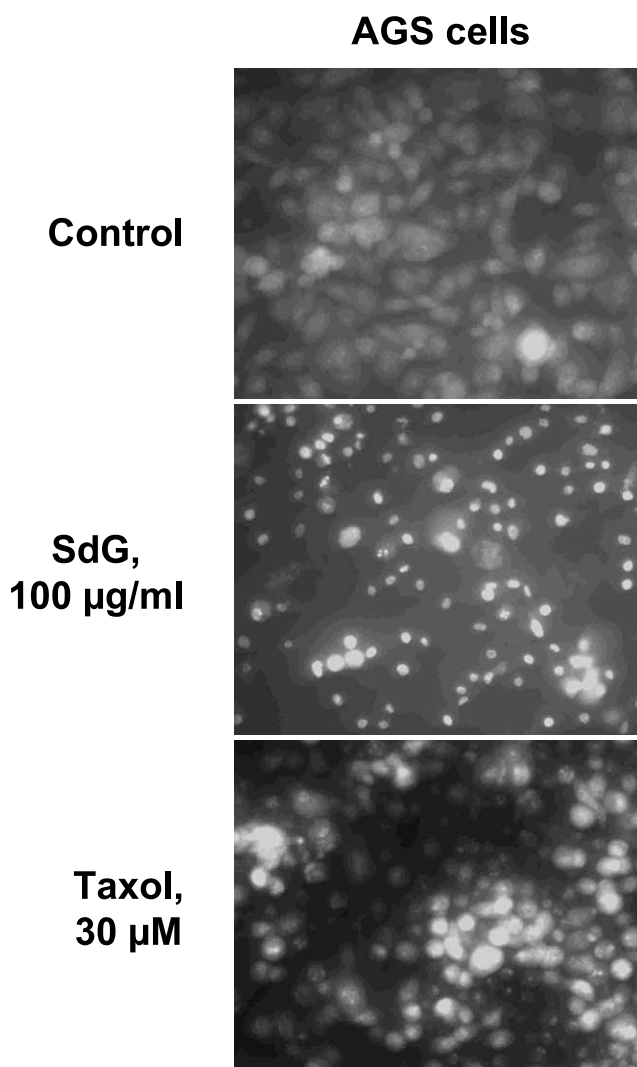


Fig. 3. The morphology of AGS cells, stained with acridine orange, from untreated control cultures or cultures exposed to sangre de grado (SdG, 100 $\mu\text{g}/\text{ml}$) or Taxol (30 μM) for 16 h as described in Section 2.

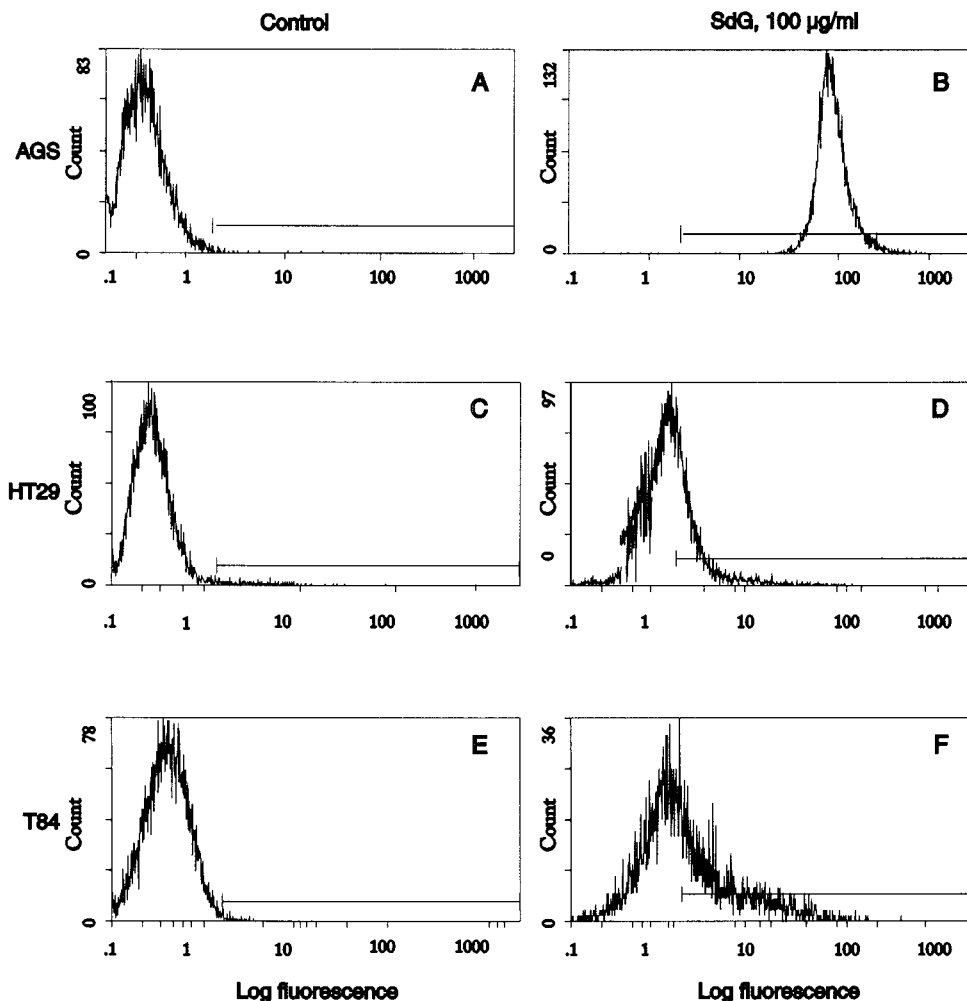


Fig. 4. Flow cytometric analysis of Fluorescein D-uridine triphosphate in AGS, HT29 and T84 cells. Control cells (A, C, E) were incubated in medium without sangre de grado. Cells (B, D, F) were treated with sangre de grado (SdG, 100 µg/ml). After incubation at 37 °C for 12 h, the cells were harvested, washed, and permeabilized. Prior to flow cytometric examination, the cells in suspension were labeled with TUNEL as described in Section 2. In the presence of sangre de grado, an increase in the shrunken population of cells was observed compared to the control cells when gated by forward and slight scatters.

investigations suggests that a range of compounds such as isoflavones and, monoterpenes derived from the diet modulate apoptosis in cell culture *in vitro*. These observations have important implications concerning the mechanisms whereby dietary components affect health (Watson et al., 2000). In addition, many plant-derived substances, such as phenolic compounds, have demonstrated significant efficacy in tumor prevention (Stoner and Mukhtar, 1995).

Non-steroidal anti-inflammatory agents (NSAIDs) are currently being used to lower the incidence of gastrointestinal cancer (Tomozawa et al., 2000). Rodent models involving carcinogen-induced tumors and treatment of adenomas produced by germ-line *Apc* mutations, have provided useful data concerning the relative efficacy of many potential chemopreventive agents (Bertagnolli, 1999; Weyant et al., 2000). Despite these clues the mechanism by which NSAIDs and other

chemopreventive agents reduce the risk of colon carcinogenesis remains poorly understood. We have shown that SdG decrease gastric COX2 expression associated with ulcers but that may reflect an anti-inflammatory action versus its chemotherapeutic role (Miller et al., 2000).

Our studies demonstrated that 6 h exposure to SdG (> 100 µg/ml) caused a significant reduction in cell adhesion (> 85% remained in suspension) in the three cancer cell lines. When the cancer cells that were in suspension were collected and transferred to another medium without SdG it was observed that they were unable to restore their ability to adhere. This indicated that the degree of damage exerted by SdG was irreversible making this ethnomedicine a potent source of compounds that may control replication of cancer cells. The mode of cell death observed indicated a slow process, and cells exhibited shrinkage of the nucleus,

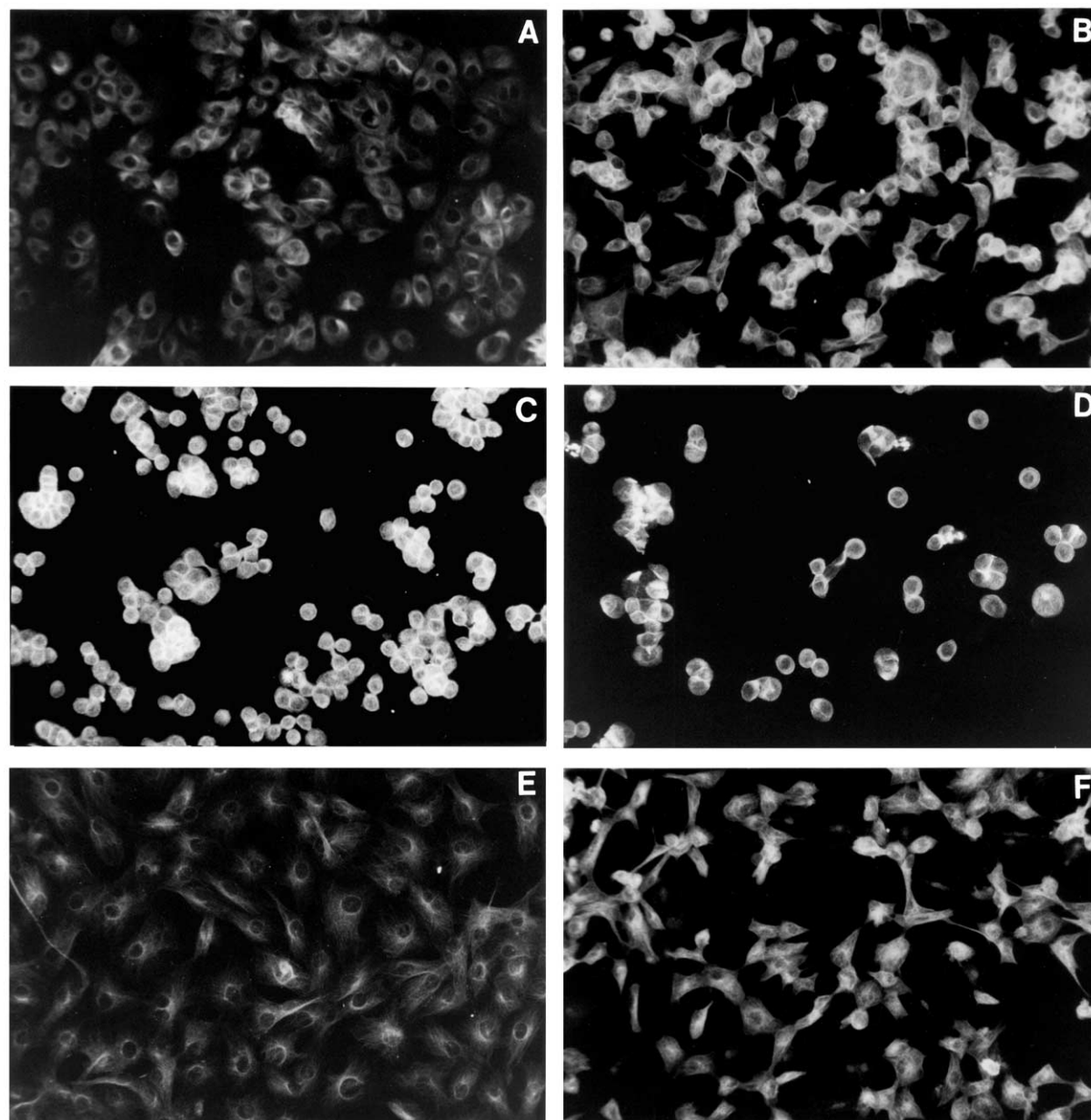


Fig. 5. Sangre de grado induces microtubular bundles in AGS, HT29 and T84 cancer cells. Control cells (A, C, E) were cultured for 12 h and cells (B, D, E) were treated with sangre de grado (SdG, 100 µg/ml). The cells were then fixed and the microtubules were stained and visualized by indirect immunofluorescent microscopy as described in Section 2. Original magnification was 200 × .

chromatin condensation, and blebbing, typical signs of apoptosis (Hacker, 2000).

We believe that one mechanism by which SdG induces apoptosis in cancer cells is the perturbation of cell adhesion and de-arrangement in the structure of microtubules. In relation to the depolymerization of the microtubules, similar effects have been observed with other anti-cancer agents such as Taxol (Manthey et al., 1992). Taxol, a botanical-derived antimetabolic agent, has shown significant cell-killing activity in a variety of tumor cells including human gastric cancer cells (Chang

et al., 1996; Huang et al., 2000; Rowinsky et al., 1992). It has been claimed that Taxol affects cell replication by blocking the cells in the G2 and M phase of the cell cycle and stabilizing cytoplasmic microtubules (Schiff and Horwitz, 1980). Our studies demonstrate that SdG (100 µg/ml) has the ability to induce a Taxol-like effect at comparative doses (30 µM = 25.6 µg/ml). Shrinkage of the nucleus and, chromatin condensation were evident after a short time exposure (6 h). These observations imply that SdG, like Taxol, exerts its action at the cellular level and stabilizes the microtubules, resulting in

nonfunctional microtubules, crippling the cell and rendering it apoptotic and unable to adhere (Iancu et al., 2000).

The action of SdG in severely compromising cancer cell replication provides an opportunity to discover novel anti-cancer plant-derived compounds that can be isolated from *C. palanostigma* which grows abundantly in the Amazon rainforest of Peru. Sangre de grado (*Croton* spp.) contains a wide number of components (Pieters et al., 1993; Ubillas, 1994; Phillipson, 1995) and based on those compounds, anti-inflammatory, anti-bacterial and anti-tumor properties have been reported. Vanilloids also have been reported to induce apoptosis in several cell lines (Biro et al., 1998; Lee and Surh, 1998). We have already confirmed that SdG has vanilloid-receptor antagonist properties (Miller et al., 2000), suggesting that these structures may contribute to the chemotherapeutic actions. Vanilloid-induced apoptosis has been claimed to occur via a mechanism that involves inhibition of plasma membrane NADH-oxidase (Wolvetang et al., 1996), and oxidation of mitochondrial megachannel pores that allows for the disruption of mitochondrial transmembrane potential (Macho et al., 1998). Sangre de grado seems undoubtedly to contain biologically active components that have anti-tumor and anti-bacterial agents (Chen et al., 1994), vanilloid compounds (Miller et al., 2000), and complex molecular structures with functions similar to Taxol. These characteristics make SdG also a potential source of new anti-cancer agents.

Besides the anti-cancer function, the experimental observations obtained in our laboratory with sangre de grado, indicate that inclusion of SdG (20–200 µg/ml) in the drinking water of rats accelerated the healing of stomach ulcers initiated by acetic acid (Miller et al., 2000). Associated with this healing response was a decrease in gastric mucosal inflammation, bacterial content of the ulcers, and a decreased expression of TNF α , IL-1, IL-6 and iNOS. Although in vitro experiments with SdG (> 100 µg/ml) demonstrates induction of apoptosis in cancer cells, rats drinking water containing SdG (200 µg/ml) did not show any signs of toxicity, rather their gastrointestinal health improved. This is consistent with the clinical study conducted by Holodniy et al. (1999) where SdG was evaluated as an anti-diarrheal agent. Thus it appears that the concentrations of SdG that are effective in killing cancer cells in vitro are devoid of deleterious effects on the gut in the in vivo setting. Another clinical study with SdG in the form of a commercial preparation, SP-303 (Provir), when given orally was safe and well tolerated by patients with AIDS and diarrhea.

Collectively our results demonstrate that the traditional medicine, sangre de grado, has potential for providing biologically active compounds to deter proliferation of malignant cells, and it deserves additional

evaluation as a chemotherapeutic agent. Indeed there is a current need for availability of new plant-derived anti-cancer agents; thus sangre de grado, *Croton* spp., may be a great natural source for the development of new chemotherapeutic drugs and may be a cost effective mean of treating gastrointestinal cancer in the developing world.

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