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Inhibitory effect of *Camellia sinensis*, *Ilex paraguariensis* and *Ardisia compressa* tea extracts on the proliferation of human head and neck squamous carcinoma cells

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ABSTRACT

In vitro cell proliferation, cell cycle arrest and induction of apoptosis were investigated, using three human head and neck squamous cell carcinoma (HNSCC) cell lines (OSCC-3, SCC-61, and SQ-20B). Aqueous extracts of *Camellia sinensis*, *Ilex paraguariensis*, and *Ardisia compressa* were tested and (−) epigallocatechin-3-gallate (EGCG) was used for comparison. For EGCG the IC_{50} values were between 80 and 166 μM and for the extracts among 75 and 505 μM eq. (+) catechin, with *C. sinensis* demonstrating dominant cytotoxicity. There was not a correlation between antioxidant capacity and cytotoxicity. Flow cytometry analysis revealed similarities in response for EGCG and *C. sinensis*. The *A. compressa* extract altered DNA distribution ($P < 0.05$) and was the most effective in induction of apoptosis *via* caspases ($P < 0.05$). Not all HNSCC cells tested responded to the same preventive agents. The fact that *A. compressa* inhibits HNSCC cell proliferation makes this aqueous extract a potential source of chemopreventive agents.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy and accounts for about 640,000 new cases of cancer worldwide and approximately 355,000 deaths annually [22]. In the United States approximately 45,780 new cases are expected in 2015 with an estimated of 8650 deaths for HNC of the oral cavity, pharynx, and larynx. Both morbidity and mortality rate are higher in men than women [1]. Exposure to tobacco and its smoke, and excessive alcohol consumption are the primary risk factors associated with HNSCC carcinogenesis [17]. Various other factors including infections with Epstein-Barr virus, past infections with human papilloma viruses (HPV16 and HPV18), chewing betel nut or shamma, dietary factors, poor oral hygiene, occupational hazards and possibly infections with human immunodeficiency virus, *etc*., have been also shown to be associated with etiology of HNSCC [25]. The 5-year survival rate (have remained approximately 50% for decades) and quality of life have not been improved significantly because these cancers arise in physically compact and anatomically complex sites (larynx, pharynx, oral cavity and tongue), the so frequent presentation with advanced stage disease (Stage I cancers have an ~80% 5-year survival while Stage IV have an ~20%), the diversity of histologic types of tumors, the high recurrence rate after surgical removal, and additionally the extremely frequent development of multiple additional second primary tumors (STPs) further worsen the prognosis of patients with HNSCC [41,40]. In HNSCC multiple genetic and epigenetic events are highly associated with its pathogenesis, including the dysregulation of the cell growth, cell cycle, apoptosis and angiogenesis. The mutation of the p53 tumor suppressor gene (occur in 47% to 62% of HNSCC), inactivation of the cyclin-dependent kinase 4 (CDK4) inhibitor p16 and overexpression (80–90% of HNSCC) of epidermal growth factor receptor (EGFR), have been considered as critical transforming events of the disease [43,27,2]. The numerous genetic mutations in regulatory genes provide a strong rationale to targeting specific molecular pathways for a chemopreventive approach to the control of the HNSCC carcinogenesis.

Of all strategies involving chemoprevention, the induction of cell cycle arrest and cancer-cell-selective apoptosis by polyphe-

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nols has been receiving considerable attention as an approach to elimination of malignant cells [20]. Polyphenols may derive their preventive effect against HNSCC by coming into direct contact with tissues before being absorbed or metabolized. For instance, *Camellia sinensis* (green tea, GT) contains a high amount of antioxidant polyphenols, most notably (−) epigallocatechin-3-gallate (EGCG), and regular GT consumption is linked to a reduced risk for various types of cancer including colorectal [11], prostate [23] and HNSCC [5,19] cancer. Yerba mate (MT), a tea-like infusion of *Ilex paraguariensis* consumed regularly in many parts of South America, is sold in commercial herbal preparations as antirheumatic with anticancerigen properties [36,38,15]. Aqueous extracts of *Ardisia compressa*, a native plant of the Pacific Coast of Mexico have been used to treat liver cancer [39]. Medicinal properties of ardisia tea (AT) including antigenotoxicity, anticytotoxicity, and antioxidant activities have been previously summarized [24]. Despite the widespread consumption of these herbal extracts, very little is known of their chemopreventive properties against HNSCC. The objective of this investigation was to compare the *in vitro* antitumor capacity of *C. sinensis*, *I. paraguariensis* and *A. compressa* extracts and EGCG against human head and neck squamous carcinoma cells, by assessing their cytotoxicity, cell proliferation, antioxidant capacity, cell cycle distribution and apoptosis induction.

2. Materials and methods

2.1. Preparation of aqueous extracts

A. compressa leaves were collected from pacific coast of Mexico (Michoacan State), while fine dried leaves of *I. paraguariensis* and *C. sinensis* (Romance and Lipton brand, respectively) were obtained from local market. Dry leaves (2.7 g) of AT, MT, and GT were soaked separately in 250 ml boiling water and allowed to stand for 10 min. The mixture of each tea was cooled to room temperature and filtered (0.45 μm nylon filter), freeze-dried and kept at −20 °C in a plastic container sealed with Parafilm and protected from light. Previous to use, the freeze-dried materials (FD) or instant teas were dissolved in double distilled water (ddH_2O) (1 mg/100 μl), filtered with a 0.22 μl syringe top filter and serially diluted in serum-free medium.

2.2. Total polyphenol content of aqueous extracts

All chemicals and reagents used in this study were purchased from Sigma–Aldrich (St. Louis, MO) unless noted otherwise. The total polyphenol content of the aqueous extracts was measured as described by [35]. This method is based on the reduction of Folin Ciocalteu reagent by the electrons from the phenols. Briefly, 1 ml 1 N Folin-Ciocalteu reagent and 1 ml sample were mixed and allowed to stand for 2–5 min, and then ml of 20% Na_2CO_3 solution were added and allowed to stand for 10 min before measuring the absorbance at 730 nm using a Beckman DU® 640 spectrophotometer (Coulter Inc., Fullerton, CA). The total polyphenol content was expressed as μg equivalents of (+) catechin per ml of aqueous extract. The equation of the standard curve used was: $y = 0.027x - 0.50$, $r^2 = 0.98$ (where, y: absorbance at 730 nm; x: polyphenol concentration; r^2: correlation coefficient).

2.3. Antioxidant capacity assay

The oxygen radical absorbance capacity (ORAC) assay [37] was used to assess antioxidant capacity by measuring the protection of the extracts and EGCG on β-phycoerythrin (β-PE) fluorescence in the presence of free radicals generated by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). The assay was carried out in black-walled 96-well plates (Fisher Scientific, Hanover Park, IL). Each well had a final volume of 200 μl. The following reactants were added in the order: 25 μl of 75 mM phosphate buffer pH 7; either 25 μl Trolox standard (1 mM final concentration) or sample (1.0–3.0 μg eq. (+)-catechin/ml); 100 μl of β-PE (1.52 nM final concentration); and 50 μl of AAPH (41.6 mM final concentration). As a blank, 25 μl of 75 mM phosphate buffer pH 7 was added instead of Trolox or samples. Immediately after addition of AAPH, plates were placed in a FL × 800 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT), set with excitation filter 530/25 nm and emission filter 590/35 nm, and then read every 2 min for 2 h to reach a 95% loss of fluorescence. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated based on the differences in the area under the β-PE decay curve between the blank and a sample, and expressed as micromoles of Trolox equivalents (TE)/g dry leaves (DL). Trolox (1–4 μM) was used as a standard ($y = 3.35x + 0.42$, $r^2 = 0.98$).

2.4. In vitro-assays of cytotoxicity and proliferation

OSCC-3 and HaCaT cell lines were cultured in Dulbecco's modified Eagle's medium plus Ham F12 (DME/F12) mixture (1:1) medium containing 10% fetal bovine serum (FBS). SCC-61 and SQ-20B cells were cultured in the same medium with added hydrocortisone (0.4 μg/ml). All cells were maintained at 37 °C in 5% CO_2 atmosphere with 95% humidity. Cultures were passaged as needed, and the culture medium was changed every other day. SQ-20B, HaCaT, SCC-61 and OSCC-3 cells were used because one of our long-term objectives was to determine the role of herbal teas in the prevention of HNSCC. All four different cell types were used as a starting point and emphasis was given to SCC-61, SQ-20B and OSCC-3 for the study of apoptosis.

Cells were plated onto 96-well plates during exponential growth at a density of 5×10^4 cells/ml, 100 μl/well. After 24 h, cells were treated with 100 μl serial concentrations of EGCG (AG Scientific, San Diego, CA), or GT, MT and AT. After incubating for 48 h, the medium containing the extracts or EGCG was discarded and the plates washed twice with phosphate-buffered saline (PBS). PBS was used as a control. One hundred microliters of serum-free medium containing 10 μl of cell counting kit 8 (CCK-8) solution (Dojindo Molecular Technologies, Gaithersburg, MD) were added to each well of the plate. The amount of the formazan dye generated by the activity of dehydrogenases within cells was directly proportional to the number of living cells, ensuring a linear relationship between absorbance at 450 nm and the cell number. The plate was incubated for 2 h and the absorbance measured at 450 nm using a microplate reader interfaced with a computer. At the end of each experiment, cytotoxicity and proliferation were calculated for each extract and EGCG according to the protocol described by [33]. The following parameters were used:

IC_{50}—the concentration of the agent that inhibits growth by 50%, is the concentration at which $(T/C) \times 100 = 50$, where T = number of cells, at time t of treatment; C = control cells at time t of treatment.

GI_{50}—the concentration of the agent that inhibits growth by 50%, relative to untreated cells, is the concentration at which $([T - T_0]/[C - T_0]) \times 100 = 50$, where T and C are the number of treated and control cells, respectively, at time t of treatment and $T > T_0$; T_0 is the number of cells at time zero.

GI_{50} considers cells at time zero and IC_{50} does not.

TGI—the concentration of the agent that completely inhibits cell growth, is the concentration at which $T = T_0$.