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Antioxidant Activity and Inhibition of Human Cancer Cells by the Herbal Product, ARCOMIG

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To assess the potential efficacy of this product, we investigated two relevant biological activities of the ARCOMIG preparation. Specifically, ARCOMIG was evaluated for inhibition of a representation of human cancer cell-lines, including colon (HT29), ovarian (SK-OV-3), mammary (SK-BR-3), renal (A498) and lung adenocarcinoma, as well as glioblastoma (U251) and lymphocytic leukemia (CEM), lines. In addition, ARCOMIG was assessed by spectrophotometric techniques for both *in vitro* antioxidant activity, specifically using the model free-radical, 1,1,-diphenyl-2-picrylhydrazyl (DPPH) and for inhibition of intracellular reactive oxygen species (ROSs), specifically using the fluorescent indicator, 2',7'-dichlorofluorescin (DCFH). Results of these assays demonstrate that ARCOMIG moderately inhibited all cancer lines tested and was particularly active with respect to a lymphocytic leukemia line. Moreover, at concentrations well below those for which cytotoxicity was observed, ARCOMIG rapidly and potently reduced the free radical, DPPH, as well as inhibiting the intracellular production of ROSs in human colon cells. When compared to the well-recognized dietary antioxidant, resveratrol, the results suggest that the antioxidant activity of ARCOMIG may equal or even surpass the activity of this compound. Taken together, these findings are consistent with purported clinical efficacy of ARCOMIG, however, further studies are clearly needed to elucidate the putative health benefits of this herbal product.

Key words: Herbal, anticancer, antioxidant, dietary supplement, ARCOMIG

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INTRODUCTION

The herbal preparation, ARCOMIG, is marketed and used clinically throughout Latin America as a dietary supplement and treatment for chronic and degenerative diseases, including cancer, diabetes, arthritis, high blood pressure and high cholesterol. Included among the botanical and other natural ingredients are chamomile, angelica, shaved grass, alfalfa and garlic that have been documented to contain compounds with a variety of pharmacological activities (O'Hara *et al.*, 1998; Hernandez-Ceruelos *et al.*, 2002; Kimura, 2005; Sarkar and Nahar, 2004; Banerjee *et al.*, 2003; Khanum *et al.*, 2004; Tattelman, 2005) In particular, a number of these have been suggested to have efficacy as antioxidants and potential in treatment and prevention of cancer (O'Hara *et al.*, 1998; Hernandez-Ceruelos *et al.*, 2002; Kimura, 2005; Sarkar and Nahar, 2004; Banerjee *et al.*, 2003; Khanum *et al.*, 2004; Tattelman, 2005).

To-date no published reports have detailed biological activity of ARCOMIG despite its wide use. Here we specifically report on the cytotoxicity of ARCOMIG toward human cancer cells and its *in vitro* and intracellular antioxidant activity.

MATERIALS AND METHODS

ARCOMIG preparation: For evaluation of biological activity, ARCOMIG was prepared as a "tea" (water extract) as described for oral administration. The undiluted tea was syringe-filtered (0.2 µm pore size) to sterilize and remove any sediment from, the preparation. Aliquots of the filtered preparation were lyophilized and an approximate concentration (w/v), specifically of 95.4 mg "extracted material" per mL of tea, was determined. Data are therefore expressed as either concentration (w/v) based on this determination, or alternatively per dilution (i.e., x-fold) of the undiluted tea.

Inhibition of cancer cells: Cytotoxicity was evaluated for human cancer cell-lines, specifically including glioblastoma (U251), lung adenocarcinoma (H460), colon adenocarcinoma (HT29), ovarian adenocarcinoma (SK-OV-3), melanoma (SK-MEL-28), renal adenocarcinoma (A498), lymphocytic leukemia (CEM) and mammary adenocarcinoma (SKBR3). All cell-lines were obtained from the US National Cancer Institute Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD), except SKBR3 that was supplied by Dr. Maria Laux of Department of Clinical Sciences at Cornell University College of Veterinary Medicine (Ithaca, NY). Cells were cultured in RPMI 1640 medium with L-glutamine (Cambrex Bio Science Walkersville, Inc.,

Walkersville, MD), supplemented with either 10% fetal bovine serum or newborn calf serum and antibiotics/antimycotics (Sigma-Aldrich, St. Louis, MO) and sub-cultured twice weekly by trypsinization (except non-adherent CEM).

Cytotoxicity was evaluated for cells grown in 96-well plates and treated with serially diluted ARCOMIG. Inhibition of cell-viability was qualitatively determined based on reduction the colorimetric indicator, alamarBlue, as previously described (Berry *et al.*, 2002, 2004; Wagner *et al.*, 1999). Minimum inhibitory concentrations (MICs) of ARCOMIG, as a measure of relative cytotoxicity, were determined for each cell-line based on the color change from blue to red resulting from reduction of the indicator by viable cells. As appropriate, percent inhibition was determined based on the fluorescence (EX 530 nm, EM 590 nm) of the reduced indicator relative to an untreated control, as described previously (Berry *et al.*, 2002, 2004). Fluorescence was measured using a BioTek® Synergy™ HT Multi detection Microplate Reader. Median inhibitory concentration (IC_{50}), when determined, was calculated by the "least squares method" for linear fits ($R^2 \geq 0.90$) between percent inhibition (relative to the untreated control) and log of the concentration as described previously (Berry *et al.*, 2004).

***In vitro* antioxidant activity:** Antioxidant activity of ARCOMIG was measured based on the ability of the preparation to scavenge the free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), as has been previously described (Miller *et al.*, 2001; Koleva *et al.*, 2002; Tarozzi *et al.*, 2004). Briefly, 50 µL aliquots of serially diluted ARCOMIG (in water), as well as negative control of water alone, were mixed with 200 µL of 100 µM DPPH (in 80% MeOH in water) in a 96-well plate. Absorbance at 515 nm was measured every 1 minute for 30 minutes, using a Bio-Tek® Synergy™ HT Multi detection Microplate Reader (Bio-Tek® Instruments, Inc., Winooski, VT), as a measure of the reduction of DPPH. Antioxidant activity was calculated as the percent reduction of DPPH relative to the water control. A "blank" of the preparation without DPPH (i.e., 80% MeOH only) was used to subtract the background absorbance due to the color of the preparation. The well described (Aggarwal *et al.*, 2004; Bhat *et al.*, 2001) antioxidant, resveratrol (Sigma-Aldrich, St. Louis, MO), along with the appropriate solvent control (i.e., MeOH), was tested identically as a positive control for antioxidant activity. Median effective concentration (EC_{50}, i.e. the dilution at which DPPH is 50% reduced) was calculated for ARCOMIG using the "least squares method".

Intracellular antioxidant activity: The ability of ARCOMIG to inhibit intracellular reactive oxygen species (ROSs) was evaluated using 2',7'-dichlorofluorescin diacetate (DCFH-DA) as per a previously developed method (Wang and Joseph, 1999). DCFH-DA crosses cell membranes and enters cells where it is converted by intracellular esterases to DCFH. In the presence of ROSs, DCFH is oxidized to the fluorescent Dichlorofluorescein (DCF). Intracellular ROSs are measured as fluorescence of cells following treatment with an oxidizing agent (Wang and Joseph, 1999).

Specifically, inhibition of intracellular ROSs was measured using colon cells (HT29). HT29 cells were grown in a 96-well plate, as described above, until completely differentiated. Cells were treated for 24 h with serially diluted ARCOMIG, or resveratrol (as a positive control), at concentrations below those found to be cytotoxic (as discussed above). Subsequently, cells were washed with Phosphate Buffered Saline (PBS) and incubated with 5 μM DCFH-DA for 30 min. Following further washing with PBS, cells were incubated for 1 h with the *tert*-butyl hydrogen peroxide (*t*-BuOOH) which induces oxidative stress. Fluorescence of DCF in cells (EX 485 nm, EM 528 nm) was measured with a BioTek® Synergy™ HT Multi detection Microplate Reader. Cells treated identically, but without DCFH-DA, were used as blanks to subtract background fluorescence. Inhibition of the formation of ROSs was measured as the adjusted fluorescence of treated cells (F_x) relative to that of the untreated controls (F_0), specifically using the formula $1-(F_x/F_0) \times 100\%$. The median inhibitory concentration (IC_{50}) was calculated based on the relationship between percent inhibition and log concentration.

RESULTS

The highest inhibition was observed for the lymphocytic leukemia line (CEM) which was inhibited at concentrations as low as 1.5 mg mL^{-1}, equivalent to a 64-fold dilution of the undiluted tea (Table 1). Cytotoxicity for other lines tested was relatively modest. In contrast, for instance, renal (A498) and ovarian (SK-OV-3) lines were only inhibited at dilutions as high as 4-fold, or approximately 23.9 mg mL^{-1} (Table 1).

On the other hand, ARCOMIG was found to be a relatively potent antioxidant. For example, the tea rapidly reduced the free radical, DPPH, as a measure of antioxidant activity. The EC_{50} of ARCOMIG after 30 min was calculated as 90.8 μg mL^{-1}, equivalent to a 1052-fold dilution of the undiluted tea (Fig. 1). The rate of reduction was concentration dependent. At a concentration of ARCOMIG which most closely

Table 1: Cytotoxicity of ARCOMIG against human cancer cell-lines. Inhibition of cells, based on reduced viability, was determined calorimetrically by the alamarBlue method. Given are the minimum inhibitory concentrations (MICs) of the prepared "tea."

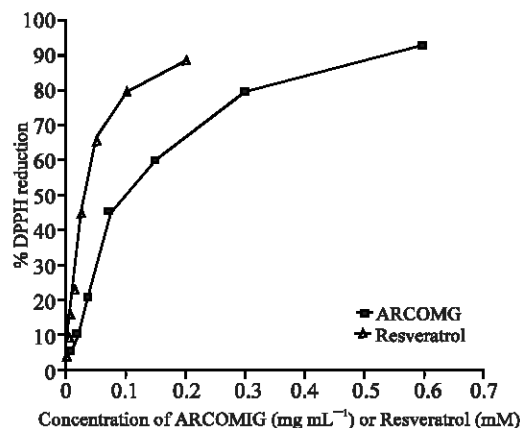| Cell Line	Cell Type	Histology	MIC (mg mL^{-1})
SK-BR-3	Mammary	Adenocarcinoma	11.9
SK-OV-3	Ovarian	Adenocarcinoma	23.9
NCI-H460	Lung	Large-cell carcinoma	6.0
U251	CNS	Glioblastoma	11.9
HT29	Colon	Adenocarcinoma, GIII	11.9
A498	Renal	Adenocarcinoma	23.9
SK-MEL-28	Melanoma	Malignant melanoma	6.0
CEM	Leukemia	Acute lymphoblastic	1.5

Fig. 1: Percent reduction of the model free-radical, DPPH, as measure of antioxidant activity, relative to concentration of either ARCOMIG or resveratrol, after 30 min. Concentration of ARCOMIG is given as dry weight (mg) of crude extract per mL, whereas concentration of the pure resveratrol is given here in mM. Reduction of DPPH was measured calorimetrically based on absorbance (515 nm) as per previously described methods (Materials and Methods)

approximates the EC_{50} (i.e., 1/1280-fold dilution, or 74.5 μg mL^{-1}), DPPH was reduced approximately 14% within five minutes of incubation reaching a maximum inhibition of nearly 45% within 30 min at the same concentration (Fig. 2). That said, at higher concentrations, antioxidant activity is much more rapid. For example, at a concentration of 1.2 mg mL^{-1} (i.e., 1/80-fold dilution) which is below the lowest MIC for cytotoxicity (i.e., 1.5 mg mL^{-1} for CEM, discussed above), DPPH is reduced approximately 63% within 1 min and up to 94% within 30 min (Fig. 2).

Antioxidant activity of ARCOMIG was compared to the well-described antioxidant, resveratrol (Aggarwal *et al.*, 2004; Bhat *et al.*, 2001). Resveratrol was found to reduce DPPH after 30 min, with an EC_{50} of
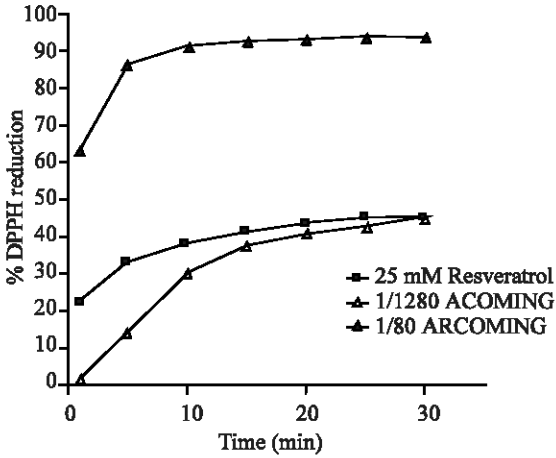
Fig. 2: Percent reduction of DPPH, as a measure of antioxidant activity, relative to time of incubation. Reduction was measured every minute for 30 min; data shown for 5 min increments only for purposes of clarity. Shown is rate of reduction for concentrations (i.e., 1/1280-fold dilution and 25 μM, respectively) which most closely approximate the calculated IC_{50} (after 30 min) of ARCOMIG (i.e., 90.8 mg mL^{-1}, or 1052-fold dilution) and resveratrol (27.3 μM). Also shown is rate of reduction at the highest concentration of ARCOMIG tested below the lowest MIC value for cytotoxicity
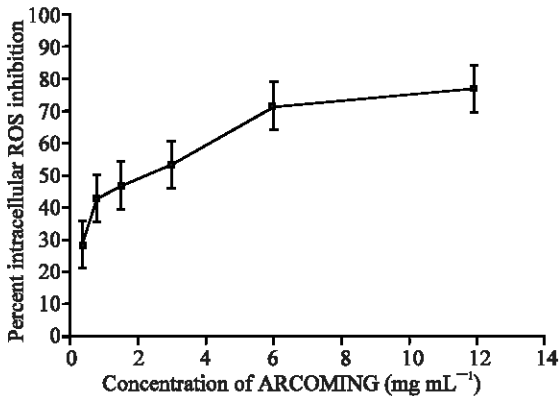

Fig. 3: Inhibition of intracellular ROSs in colon, in response to the oxidizer, t-BuOOH, by pretreatment with ARCOMIG. Intracellular ROSs were specifically measured for colon (HT29) cells using the fluorescent indicator, DCF (Materials and Methods), as per a previously described method (Wang and Joseph, 1999), after 1 h exposure to t-BuOOH.

approximately 27.3 8 μM (Fig. 1), or about 6.2 μg mL^{-1}. The free-radical scavenging activity of resveratrol observed in the present study was comparable to that

previously reported by Wang *et al.* (1999) who measured, using the same method, approximately 30% reduction of DPPH at a concentration of 20 μM resveratrol. At a concentration (i.e., 25 μM) approximating this EC_{50}, reduction of DPPH was more rapid than ARCOMIG, reaching more than 22% reduction within 1 min (Fig. 2). However, after 30 minutes, an almost identical percent reduction (i.e., 45%) of DPPH was observed for both resveratrol and ARCOMIG, equally tested at a concentration approximating the EC_{50} (i.e., 1/1280-fold dilution, or 74.5 mg mL^{-1}; Fig. 2).

In addition to scavenging free radicals *in vitro*, ARCOMIG inhibited the intracellular production of ROSs (Fig. 3) in response to the oxidizer, t-BuOOH. Effect of ARCOMIG on intracellular ROSs was specifically tested using the colon adenocarcinoma line (HT29) at concentrations equal, or less than, the determined MIC (i.e., 11.9 mg mL^{-1}) for this line (Table 1). Cytotoxicity to (i.e., percent inhibition of) cells at this highest concentration was less than 20% and generally negligible. Inhibition of intracellular ROSs was dose-dependent with an IC_{50} of approximately 1.7 mg mL^{-1}, however, up to approximately 77% inhibition was observed for the highest concentration tested (i.e., 11.9 mg mL^{-1}, or 8-fold dilution). This activity was again compared to resveratrol as a known antioxidant. However, inhibition of intracellular ROSs was only observed for the highest concentrations of resveratrol (i.e., 200 and 100 μM) tested, with an approximate IC_{50} of 174 μM.

DISCUSSION

The herbal product, ARCOMIG, is widely sold and used as a natural dietary supplement, particularly in parts of South and Central America where it is prescribed clinically for an array of preventive and curative purposes. Purported health benefits include prevention and even therapeutic treatment, of chronic and degenerative diseases including various forms of cancer, diabetes and arthritis, as well as maintenance of general systemic and cellular health including immuno-stimulation, anticholesterolemic and antioxidant activity. Prepared from various herbs, no single plant or derived compound has been yet identified as the active component and health benefits of the tea are generally considered to be related to the cumulative contribution of these various components of the complex mixture. That said, preliminary chemical studies (unpublished data) have identified compounds, including bisabolol, zingerone and herniarin, with known pharmacological activity (Isaac, 1979; Silvan *et al.*, 1996; Cavalieri *et al.*, 2004; Kabuto *et al.*, 2005; Kuo *et al.*, 2005). To date, however, no published laboratory studies have described any biological activity related to the health benefits of

ARCOMIG. The present study is the first report of an evaluation of apparent pharmacological activity consistent with these purported health benefits.

Specifically, we evaluated the cytotoxicity of ARCOMIG to various human cancer cell-lines. As shown in Table 1, ARCOMIG was at least marginally cytotoxic to all eight lines tested. These lines were specifically chosen, as they approximate the breadth of cell-types (specifically 8 of the 9 cancer types) routinely employed by the US National Cancer Institute for evaluation of potential anticancer drugs (Shoemaker *et al.*, 1988). The highest inhibition was observed for the lymphocytic leukemia cell-line, CEM. This is particularly relevant, as ARCOMIG has been, indeed, recommended by physicians as a natural treatment for leukemia (Dr. Carlos Ibarra de la Toba, personal communication). On the other hand, cytotoxicity to most other lines tested was generally minimal, although these results are representative of only acute, short-term (i.e., 24-48 h) exposure of cancer cells to the crude tea. To fully understand the possible effects of administration of ARCOMIG on cancer, further studies would be obviously required, including long-term *in vivo* studies, particularly with respect to evaluation of individual metabolites that may be accumulated following administration of the mixture.

On the other hand, considerable antioxidant activity was demonstrated for ARCOMIG, both *in vitro* and in a cell-based system (Fig. 1-3). ARCOMIG effectively scavenged the model free-radical, DPPH, at concentrations as low as 5 μg mL^{-1} (Fig. 1) within 30 min and within 1 min at concentrations well below levels at which it was cytotoxic to cells (Fig. 2). Furthermore, ARCOMIG was shown to inhibit the formation of ROSs in colon cells exposed to the oxidizer, *t*-BuOOH, by up to 77% and at concentrations as low as 47 μg mL^{-1} (Fig. 3). As these concentrations are, indeed, below the cytotoxic MICs determined for all cell-lines, it is suggested that these effects could occur *in vivo* without presenting additional toxicity to cells.

The antioxidant activity of ARCOMIG was further compared to the known antioxidant, resveratrol, which is generally considered to be responsible for the observed health benefits (e.g., anticancer, cardioprotective properties) associated with consumption of red wine and various other foods and dietary supplements (Aggarwal *et al.*, 2004; Bhat *et al.*, 2001). Comparison of this pure compound to the complex mixture of ARCOMIG is not possible on a "per concentration" basis, however, the results suggest that antioxidant potential of the (as of yet) unidentified active principle(s) of ARCOMIG is/are likely at least as potent as resveratrol. In terms of active concentration, the EC$_{50}$ for the reduction of DPPH by ARCOMIG was measured as 90.8 μg mL^{-1}, compared

to an EC$_{50}$ of 27.3 μM, or approximately 6.2 mg mL^{-1}, for resveratrol after 30 min (Fig. 1). Assuming that the antioxidant component of ARCOMIG represents no more than 7% of the crude mixture, the free-radical scavenging activity would be at least equal to that of resveratrol. Considering a single compound generally represents much less than one percent of any crude extract such as this, it is conceivable that the antioxidant potential the active principle may, in fact, be one or more order of magnitude greater than resveratrol. Likewise, though comparison of the rates of free-radical scavenging by ARCOMIG were initially lower than resveratrol, specifically when measured at concentrations approximating the EC$_{50}$, nearly equal percent reduction was observed after 30 min (Fig. 2). Moreover, at concentrations well below the cytotoxic MIC for HT29, similarly rapid reduction of the indicator was observed. For example, at concentrations of 0.6 mg mL^{-1} (or 160-fold dilution) of ARCOMIG, an approximately 44% reduction was observed within 1 min, or nearly double the rate of reduction (i.e., 22.5% in 1 min) for the EC$_{50}$ of resveratrol. Again, considering this is a comparison of a crude mixture versus a pure compound, it is quite likely that the active component(s) of ARCOMIG is equally active as, or perhaps even considerably more active than, resveratrol.

Similarly, when the ability of ARCOMIG to inhibit intracellular ROSs was compared to resveratrol, the calculated IC$_{50}$ (w/v) was significantly less for the latter. However, adjusting for presumptive contribution of the active principle to the total composition of the crude extract, it is very likely that the antioxidant activity of the purified principle could be at least equal and likely higher than, that of resveratrol. Specifically, the IC$_{50}$ of ARCOMIG was approximately 43-times that calculated for resveratrol (i.e., 1.7 mg mL^{-1} for ARCOMIG vs. 0.04 mg mL^{-1} for resveratrol). If the active principle is equal or less than 2.3% of the total crude extract, it would translate to an, at least, equipotent antioxidant activity relative to resveratrol. Again, typically any single compound contributes considerably less than 1% of most crude extracts, suggesting that the antioxidant activity may very well surpass that of resveratrol.

In conclusion, though levels of cytotoxicity of ARCOMIG to cancer cells in this study were found to be relatively modest, further studies are clearly needed to elucidate any possible efficacy in relation to its purported anticancer benefits. On the other hand, it is clear that ARCOMIG is a seemingly potent antioxidant. Again, however, further studies particular focusing on isolated components of the crude herbal product, are necessary to definitively characterize this activity and its associated health benefits.

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