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Total Cranberry Extract versus Its Phytochemical Constituents: Antiproliferative and Synergistic Effects against Human Tumor Cell Lines

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Cranberries (*Vaccinium macrocarpon* Ait.) are an excellent dietary source of phytochemicals that include flavonol glycosides, anthocyanins, proanthocyanidins (condensed tannins), and organic and phenolic acids. Using C-18 and Sephadex Lipophilic LH-20 column chromatography, HPLC, and tandem LC-ES/MS, the total cranberry extract (TCE) has been analyzed, quantified, and separated into fractions enriched in sugars, organic acids, total polyphenols, proanthocyanidins, and anthocyanins (39.4, 30.0, 10.6, 5.5, and 1.2% composition, respectively). Using a luminescent ATP cell viability assay, the antiproliferative effects of TCE (200 μg/mL) versus all fractions were evaluated against human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620), and prostate (RWPE-1, RWPE-2, 22Rv1) cancer cell lines. The total polyphenol fraction was the most active fraction against all cell lines with 96.1 and 95% inhibition of KB and CAL27 oral cancer cells, respectively. For the colon cancer cells, the antiproliferative activity of this fraction was greater against HCT116 (92.1%) than against HT-29 (61.1%), SW480 (60%), and SW620 (63%). TCE and all fractions showed ≥50% antiproliferative activity against prostate cancer cells with total polyphenols being the most active fraction (RWPE-1, 95%; RWPE-2, 95%; 22Rv1, 99.6%). Cranberry sugars (78.8 μg/mL) did not inhibit the proliferation of any cancer cell lines. The enhanced antiproliferative activity of total polyphenols compared to TCE and its individual phytochemicals suggests synergistic or additive antiproliferative interactions of the anthocyanins, proanthocyanidins, and flavonol glycosides within the cranberry extract.

KEYWORDS: ***Vaccinium macrocarpon*; cranberries; polyphenols; proanthocyanidins; anthocyanins; antiproliferative; colon; prostate; oral; cancer; synergistic**

INTRODUCTION

The American cranberry, *Vaccinium macrocarpon* Ait. (Ericaceae), juice and fruits are reported to exhibit various health benefits including the prevention of microbial adhesion in urinary tract infections and reduction of biofilm formation (*1*, *2*), potent antioxidant action (*3*, *4*), cholesterol reduction (*5*), vasorelaxant effects (*6*), and in vitro anticancer effects (*4*, *7*, *8*). Many of these biological effects have been linked to the presence of a wide variety of diverse compounds in the fruit including anthocyanins (*9*), proanthocyanidins (condensed tannins) (*10*), flavonol glycosides (*11*), low molecular weight phenolic acids (*12*), organic acids (*13*), and sugars (*14*).

The anticancer properties of cranberry and the nature of the compounds that provide protection against tumor promotion and proliferation have not been fully investigated (*4*, *8*). Because most of the biological activity of cranberries has been attributed to its phenolic compounds, many studies have focused on the identification and determination of the structure and activity of particular chemopreventive compounds. However, it has been well established that complex mixtures of phytochemicals in fruits and vegetables can provide protective health benefits mainly through a combination of additive and/or synergistic effects. Phytochemicals in fruits and vegetables can have complementary and overlapping effects on oxidative stress, the immune system, gene expression in cell proliferation and apoptosis, and hormone metabolism, and they can have direct antibacterial and antiviral activities (*15*).

Recently, proanthocyanidin-rich cranberry fractions were reported to inhibit the induction of ornithine decarboxylase (ODC), an enzyme involved in tumor cell proliferation, in epithelial cells (*16*). Among extracts of 11 common fruits studied, total cranberry phenolics showed the highest in vitro inhibitory effect on the growth of $HepG_2$ human liver cancer cells (*7*), and cranberry extracts also exhibited antiangiogenic properties (*17*). It has also been reported that two cranberry extracts inhibited the proliferation of MCF-7 and MDA-MB-435 breast cancer cells (*18*).

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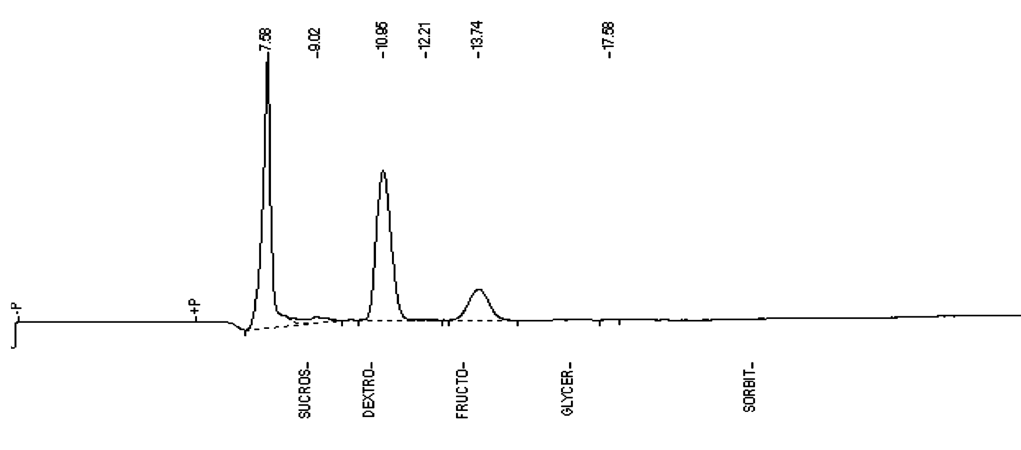

Figure 1. Analytical HPLC chromatogram of sugars isolated from TCE and identified by comparison with commercial standards. Major peaks were identified using a refractive index (RI) detector as sucrose (7.58 min), dextrose (10.95 min), and fructose (13.74 min).

As part of our ongoing research on the biological effects of standardized total extracts versus single purified compounds for investigations pertaining to the health benefits of botanicals, dietary supplements, fruits, and vegetables, we have focused our attention on cranberries. Cranberries contain a diverse range of phytochemicals, and there has been uncertainty as to which group of compounds are responsible for specific biological activities; for example, separate studies have attributed its bacterial antiadhesion effects to different groups of compounds: organic acids (*19*), sugars, for example, fructose (*20*), and, more recently, proanthocyanidins (*1*). In an effort to examine the biological effects resulting from the complex mixture of phytochemicals, we have chemically analyzed, quantified, and separated total cranberry extract (TCE) into its component fractions. We have also evaluated the human cancer cell antiproliferative activities of TCE versus its individual fractions and examined the synergistic effects that result from the combinations of its various phytochemicals. This is the first report of the human tumor cell inhibitory activities of cranberries against this panel of oral, prostate, and colon cancer cell lines in a highly sensitive luminescent ATP cell viability assay.

MATERIALS AND METHODS

Reagents. All solvents were of HPLC grade and purchased from Fisher Scientific Co. (Tustin, CA). Commercially available standards of maltose, sucrose, dextrose, and quinic, citric, malic, tartaric, fumaric, isocitric, and ascorbic acids were purchased from Sigma Aldrich Co. (St. Louis, MO). Anthocyanin, quercetin, and myricetin standards were purchased from Chromadex Inc. (Santa Ana, CA).

Fractionation of Total Cranberry Extract. Cranberry extract powder (60 g) (OceanSpray Cranberries Inc., lot FG 10479, product is >90% cranberry fruit solids with a magnesium hydroxide carrier; 5% moisture) was dissolved in water, filtered, and fractionated onto four RP-Flash 40M C-18 cartridges (Biotage Inc., Charlottesville, VA). Each column was eluted with consecutive 500 mL aliquots of H_2O; H_2O/MeOH (85:15 v/v), and MeOH/CH_3COOH (99:1 v/v) to yield three total combined fractions. Fractions were evaporated in vacuo, reconstituted in water, and freeze-dried to yield fractions I (39.4%, off-white hygroscopic solids, sugars-enriched), II (30%, slightly pink powder, organic acids-enriched), and III (10.6%, dark red powder, polyphenols-enriched). Fraction III (2 g) was dissolved in water (1 mL) and separated on an LH-20 (Sigma Lipophilic Sephadex) column eluted with consecutive 500 mL aliquots of H_2O/EtOH/CH_3COOH (1:1:0.1 v/v/v) and Me_2CO/H_2O (8:2 v/v) to yield two fractions. These fractions were evaporated in vacuo, reconstituted in water, and freeze-dried to yield fractions IV (0.8−1.2%, dark red powder, predominantly anthocyanins and monomeric polyphenols) and V (2.6−5.5%, slightly pink powder, predominantly oligomeric and polymeric polyphenols). Each fraction was further analyzed for phytochemical content as outlined below.

HPLC Analyses of Sugars (Fraction I). The sugar analyses were conducted according to the method U.S. Pharmacoepoeia National Formulary USP 24NF19 *Official Monograph of Cranberry Liquid Preparation*, p 2440. Briefly, an aqueous sample of fraction I (1 g/mL) was loaded onto a resin bed (1 g, 100−200 mesh, Bio-Rex 5), eluted with water (4 mL), filtered (0.45 μm), and analyzed on an Aminex HPX-87C column (Bio-Rad, 300 × 7.8 mm) with a Carbo-C cartridge guard column. The mobile phase was water under isocratic conditions at a flow rate of 0.6 mL/min; an injection volume of 10 μL was used, and peaks were detected with an RI detector (Waters 410 differential refractometer detector with internal temperature = 40 °C). An HP 1050 series LC system controlled by Total Chrom Workstation 6.2 (Perkin-Elmer) was used, and sugars were identified by comparison with commercial standards (**Figure 1**). The method to determine the presence of pectin consisted of solubilizing the sample (in water) and then mixing with acidified ethanol in a 2:3 v/v ratio. After the mixture is shaken, pectin comes out of solution.

HPLC Analyses of Organic Acids (Fraction II). Cranberry organic acids were detected using a Dionex Ion Chromatography 300-mA Suppression ASRS-Ultra 4-mm system controlled by Peak Net software. Acids were separated using an Ion Pac AG11HC-4 mm guard column and an AS11 HC-4 mm column with a gradient system of NaOH at a flow of 1.5 mL/min as follows [time (min)/concentration (mM)]: initial/2, 2/2, 8/7, 17/25, 22/40, 25/66.6, 30/66.6, 30.1/2, 35/2. Organic acids were identified by comparison with pure standards of quinic, citric, malic, tartaric fumaric, isocitric, and ascorbic acids (**Figure 2**).

HPLC Analyses of Total Polyphenols (Fraction III), Anthocyanins (Fraction IV), and Proanthocyanidins (Fraction V). All samples (50 μL injection volume) were filtered (0.22 μm) and analyzed on a Novapak (Waters Corp.) C-18 column, 150 × 3.9 mm i.d., 5 μm. The mobile phase, solvent A (4% aqueous H_3PO_4) and solvent B (CH_3CN), was used under linear gradient conditions starting with 95% A to 75% A over 60 min with a flow rate of 0.75 mL/min. Total polyphenols (fraction III) were observed at 254 nm (**Figure 3A**) using a PDA detector (Waters Corp., Milford, MA) and compared to reference standards of quercetin and myricetin. Fraction IV (anthocyanins) was detected at 520 nm (**Figure 3B**), and retention times were compared to reference standards of commercially available anthocyanins for substantiating identities as (1) cyanidin galactoside, (2) cyanidin arabinoside, (3) peonidin galactoside, and (4) peonidin arabinoside (*21*). Fraction V (proanthocyanidins) was detected at 380 nm (**Figure 3C**), and LC-ES/MS was used for substantiating identities as previously reported (*22*). All fractions were also detected at a wide spectrum window of 210−600 nm to substantiate purity.

LC-ES/MS Analyses of Anthocyanins (Fraction IV) and Proanthocyanidins (Fraction V). HPLC-ES/MS analyses were carried out on an LCQ Classic Finnigan LC-MS/MS system (ThermoFinnigan, San

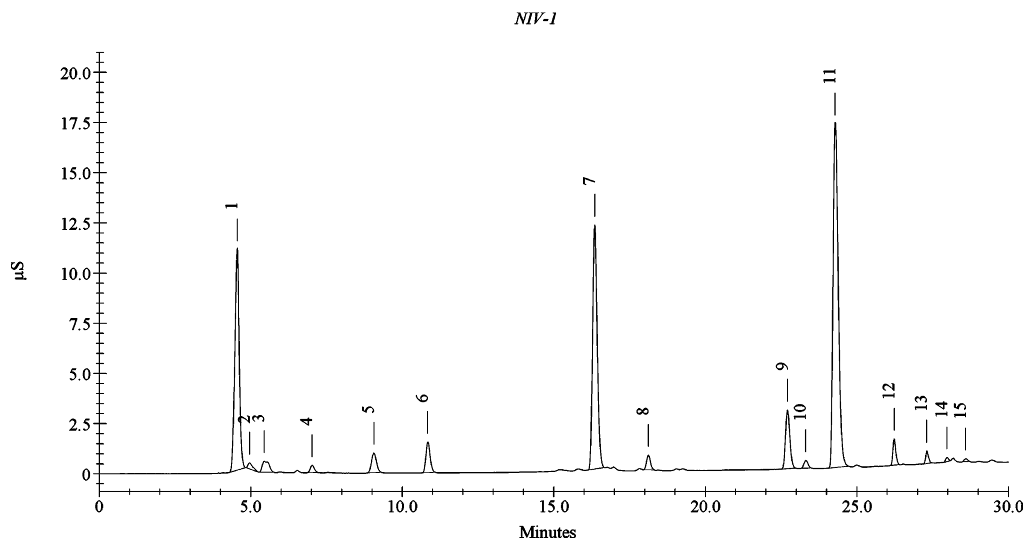

Figure 2. Analytical HPLC chromatogram of organic acids isolated from TCE and identified by comparison with commercial standards as follows: 1, quinic; 7, malic; 8, tartaric; 11, citric; 12, fumaric; 13, isocitric; and 15, ascorbic acid.

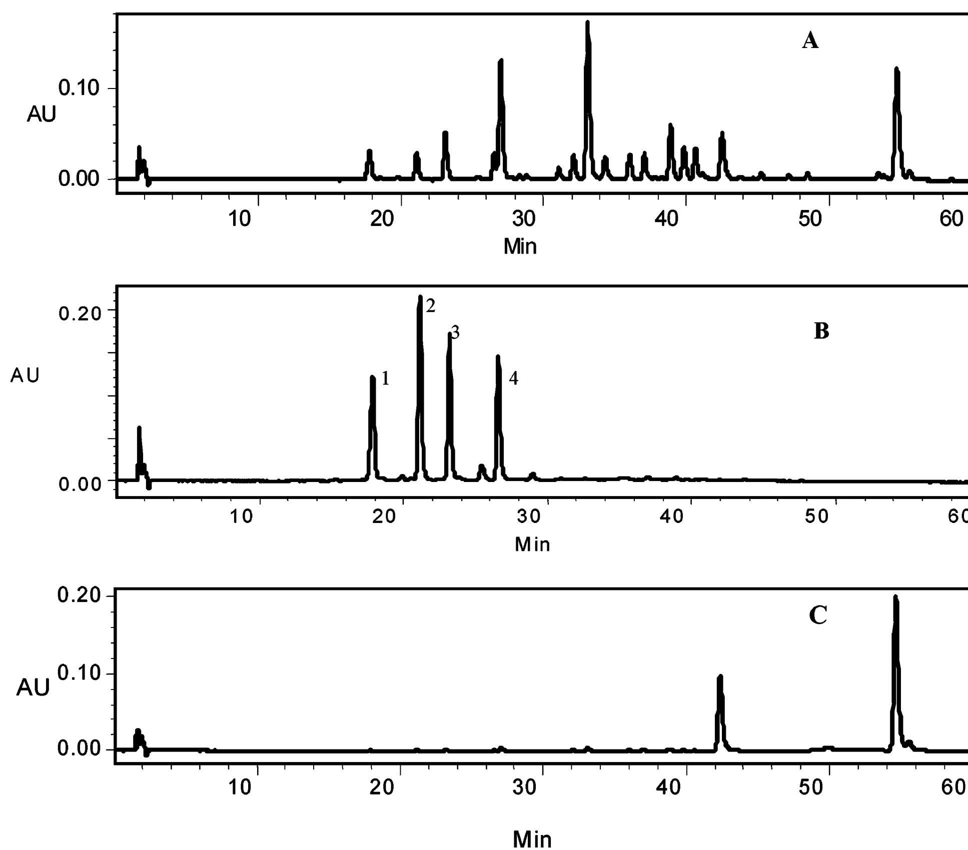

Figure 3. HPLC chromatograms of isolated fractions from TCE: (**A**) total polyphenols at 254 nm; (**B**) anthocyanins at 520 nm identified by comparison to commercial standards and by LC-ES/MS (*m/z* total/aglycon) as (1) cyanidin galactoside (449/287), (2) cyanidin arabinoside (419/287), (3) peonidin galactoside (463/301), and (4) peonidin arabinoside (433/301); (**C**) proanthocyanidins (fraction V) at 380 nm identified by ES/MS spectrum as consisting of epicatechin units with degrees of polymerization (DP) of 4 and 5 at *m/z* 575.1, 863.1, 1151.2, 1439.3, and 1726.4, corroborating the literature (*22*).

Jose, CA), equipped with an HP 1100 series HPLC system consisting of an autosampler/injector, quaternary pump, column heater, and diode array (DAD) detector. Data handling was carried out using Xcalibur 1.2 software (Finnigan Corp). Conditions for detection of anthocyanins were as follows: column, Symmetery C 18, 100 mm × 2.1 i.d., 3.5 µm (Waters Corp., Milford, MA); solvent A, 2% $HCOOH/H_2O$; solvent B, 2% HCOOH/MeOH; gradient % A, initial 99%, 30 min 80%, 45 min 60%, 60 min 5%; run time, 60 min; flow rate, 0.15 mL/min; injection volume, 20 µL; column temperature, 30 °C; DAD range, 210−600 nm, 520 nm as detection wavelength for anthocyanins. MS parameters were as follows: ionization mode ES+; scan range, 200−600 amu; scan rate, 1 scan/s; cone voltage, 17 eV. Peak identities were obtained by matching their molecular ions (M^+) obtained by ES/MS with the expected theoretical molecular weight from literature data at *m/z* total/aglycon: 1, cyanidin hexoside (449/287); 2, cyanidin arabinoside (419/287); 3, peonidin hexoside (463/301); 4, peonidin arabinoside (433/301) (*21*). Proanthocyanidins were identified using negative ion detection by direct infusion, scan range 200−2000 amu, and observed ions at *m/z* 575.1, 863.1, 1151.2, 1439.3, and 1726.4, corresponding to those of the previously published paper (*22*).

Cell Culture Materials. The KB and CAL27 oral cancer, SW480, SW620, HT-29, and HCT116 colon cancer, and RWPE-1 and RWPE-2

prostate cancer cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The 22Rv1 prostate cancer cell line was generously donated by the laboratory of P. Cohen (Division of Pediatric Endocrinology, UCLA Medical Center, Los Angeles, CA). KB oral cancer cells were grown in minimum essential medium (MEM); CAL27 oral cancer cells were grown in Dulbecco's minimum essential medium (DMEM); SW480 and SW620 colon cancer cells and 22Rv1 prostate cancer cells were grown in RPMI 1640; HT-29 and HCT116 colon cancer cells were grown in McCoy's 5A medium, modified. All media contained 10% fetal bovine serum (FBS) in the presence of 100 units/mL penicillin and 0.1 mg/mL streptomycin. RWPE-1 and RWPE-2 prostate cells were grown in defined keratinocyte serum-free medium (DKSFM) containing epidermal growth factor (EGF), insulin, and fibroblast growth factor (FGF). Cells were incubated at 37 °C with 95% air and 5% CO_2. All cells were maintained below passage 20 and used in experiments during the linear phase of growth.

Antiproliferation Assay. Proliferation was measured utilizing the CellTiter-Glo Luminescent Cell Viability Assay (Technical Bulletin 288, Promega Corp., Madison, WI). When added to cells, the assay reagent produces luminescence in the presence of ATP from viable cells. Cells were plated in 96-well plates at a density of 10000 cells/well and incubated for 24 h. Test samples were solubilized in water by sonication, filter sterilized, and diluted with medium to the desired treatment concentration. Cells were treated with 100 μL of control medium, ascorbic acid (100 μM, used as an antioxidant standard), TCE, or separate cranberry fractions and incubated for 48 h drug exposure duration. TCE was tested at 50, 100, and 200 μg/mL, and fractions were isolated from TCE as follows: fraction I (78.8 μg/mL at 39.4% composition), fraction II (60 μg/mL at 30.0% composition), and fraction III (200 μg/mL). Test concentrations of fractions IV (7.1 μg/mL) and V (6.5 μg/mL) were based on an average percentage yield obtained from sequential separations of the total polyphenols (fraction III). At the end of 48 h, plates were equilibrated at room temperature for 30 min, 100 μL of the assay reagent was added to each well, and cell lysis was induced on an orbital shaker for 2 min. Plates were incubated at room temperature for 10 min to stabilize the luminescence signal, and results were read on an Orion Microplate Luminometer (Bertholds Detection Systems, Pforzheim, Germany). All plates had control wells containing medium without cells to obtain a value for background luminescence. Data are expressed as percentage of untreated cells, mean $\pm$ SE, for three replications.

RESULTS AND DISCUSSION

In the mass balance calculation, the 80% material recovery from the cranberry extract powder could be attributed to the presence of water insoluble materials and uncorrected weights due to moisture content (typically 5%). HPLC analyses of fractions I and II revealed that they were enriched in sugars (**Figure 1**) and organic acids (**Figure 2**), respectively. Also, by using a test that exploits the solubility of pectin, the bulk of "unknown" materials in the fractions was determined to be fruit pectins, polymers of sugars. Cranberries contain sugars including fructose and glucose (*14*) and fruit acids, which include quinic, citric, malic, ellagic, shikimic, and oxalic acids (*12*, *13*). The dark red powder obtained from fraction III (**Figure 3A**) was highly enriched in polyphenols including anthocyanins, proanthocyanidins, and quercetin, myricetin, and their glycosides as identified by HPLC. The total polyphenolic fraction III was further separated into fractions IV (anthocyanins and monomers) and V (proanthocyanidins and oligomers). Cranberries have been reported to contain the highest content of total phenolics per serving per weight among 20 fruits (*3*), consisting of anthocyanins, proanthocyanidins, and glycosides of the flavanols quercetin, myricetin, and kaempferol.

The dark red powder obtained from fraction IV was highly enriched in anthocyanins as observed by HPLC-UV detection at 520 nm, the characteristic absorption wavelength of anthocyanins (**Figure 3B**). HPLC detection at a wide spectral window of 210–600 nm and co-injection with authentic samples showed the presence of minor amounts of quercetin and myricetin in this fraction. The four major anthocyanin peaks 1–4 were identified by HPLC as the galactosides and arabinosides of cyanidin and peonidin by comparison of retention times and co-injection with commercially available standards (**Figure 3B**). LC-ES/MS spectra of the cranberry anthocyanins were consistent with published spectra in the literature (*21*), *m/z* total/aglycon: 1, cyanidin hexoside (449/287); 2, cyanidin arabinoside (419/287); 3, peonidin hexoside (463/301); and 4, peonidin arabinoside (433/301). It has been reported that the well-characterized anthocyanins of the American cranberry, *V. macrocarpon*, are the 3-*O*-galactosides and 3-*O*-arabinosides of peonidin and cyanidin; these are distinct from the closely related European cranberry, *V. oxycoccus* L., which contains the 3-*O*-glucosides and 3-*O*-arabinosides of peonidin and cyanidin (*9*). HPLC-UV chromatograms of the slightly pink powder obtained from fraction V showed trace quantities of anthocyanins at 520 nm but prominent proanthocyanidin peaks at 380 nm (**Figure 3C**). This fraction is enriched cranberry proanthocyanidins that are reported to consist of predominantly epicatechin units with degrees of polymerization (DP) of 4 and 5 containing A-type linkage, corresponding with ES/MS ions at *m/z* 575.1, 863.1, 1151.2, 1439.3, and 1726.4 (*22*).

The antiproliferative activities of TCE (200 μg/mL) versus those of fractions I–V isolated from TCE (I, 78.8; II, 60.0; III, 200; IV, 7.1; V, 6.5 μg/mL) were determined using a highly sensitive luminescent ATP viability cell assay (**Figure 4**). This assay is based on the quantitation of ATP that signals the presence of metabolically active viable cells. Of the oral cancer cell lines, TCE inhibited the mouth epidermal carcinoma KB cells (60%) to a larger extent than it did the tongue epithelial CAL 27 cells (8%) (**Figure 4A**). However, the observed antiproliferative activity was significantly enhanced with the enriched fraction containing total polyphenols (KB, 96.1%; CAL27, 95%) compared to the organic acids (KB, 26.7%; CAL27, 29%), anthocyanins (KB, 21.1%; CAL27, 22.4%), and proanthocyanidins (KB, 41%; CAL27, 37.6%) (**Figure 4A**). The cranberry sugar fraction and commercially obtained ascorbic acid (100 μM, used as an antioxidant standard) did not inhibit proliferation of the cancer cells.

Because there is increasing evidence suggesting an association between cancer and cyclooxygenase (COX) enzymes, a COX-expressing cell line, HT-29, was studied (**Figure 4B**). TCE showed the greatest antiproliferative activity against the HT-29 (78%) cells, suggesting a possible mechanistic action via inhibition of COX enzymes. SW480 and SW620 are two colon cancer cell lines obtained from the same patient, with the SW480 being derived from the primary tumor and the SW620 derived from a metastatic site. TCE demonstrated specific antiproliferative activity against the more progressive and metastatic SW620 cells (35%) but did not inhibit SW480 cells. Similarly, fractions enriched in organic acids, anthocyanins, and proanthocyanidins specifically inhibited the SW620 cells (36, 14, and 10%, respectively) but did not inhibit the growth of the SW480 cell lines. The total polyphenol fraction, the most active of the cranberry fractions, showed the highest antiproliferative activity against HCT116 (92.1%) and a similar range of activity against SW620 (63%), HT-29 (61.1%), and SW480 (60.1%) colon cells.

Among the prostate cancer cell lines, 22Rv1 (progressive, 85%) was the more sensitive toward TCE than RWPE-1 (normal prostate, 65%) and RWPE-2 (activated k-*ras* transfected, 65%) (**Figure 4C**). Cranberry total polyphenols was the most active

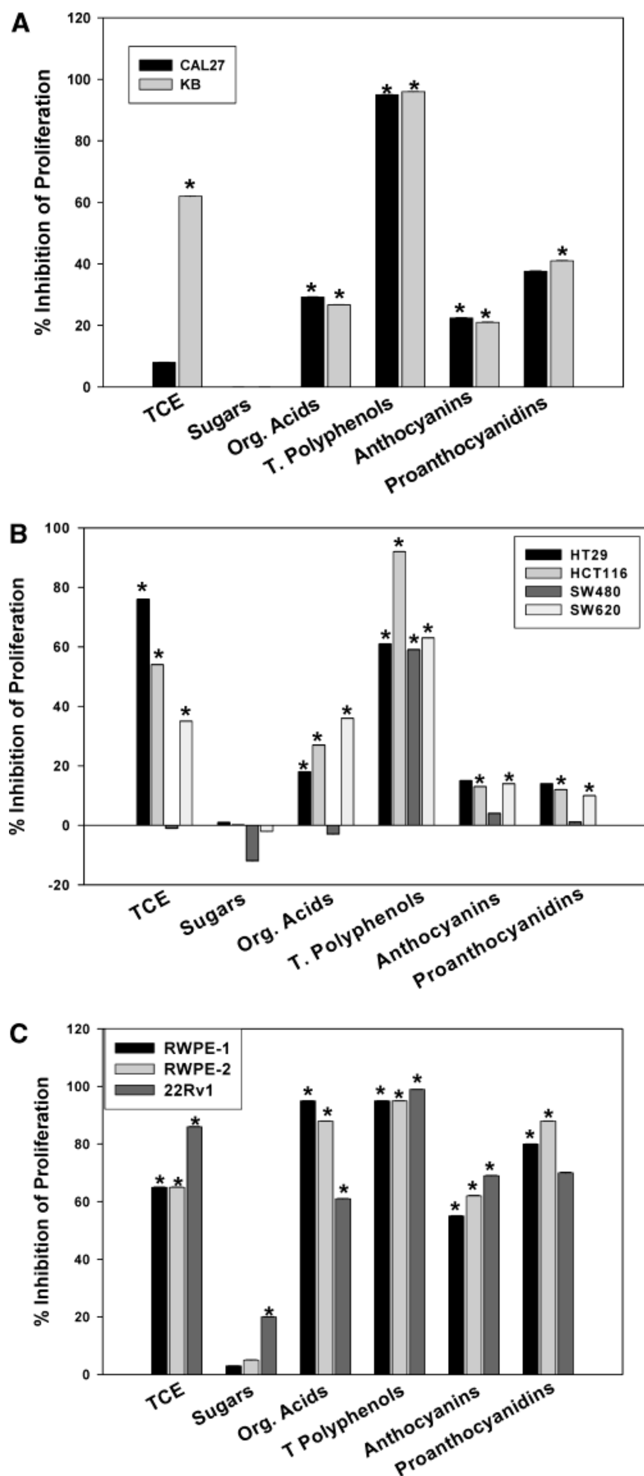

Figure 4. Antiproliferative activities of TCE and its isolated fractions against (**A**) KB and CAL-27 oral cancer cells, (**B**) HT-29, HCT116, SW480, and SW620 colon cancer cells, and (**C**) RWPE-1, RWPE-2, and 22Rv1 prostate cancer cells. Cells were exposed to TCE (200 μg/mL) and purified fractions (concentrations equivalent to percent composition in TCE) for 48 h. Proliferation was measured via the CellTiter-Glo Luminescent Cell Viability Assay. Data are expressed as percentage of untreated cells, mean ± SE ($n = 3$). Asterisks indicate a significant difference compared to untreated controls ($p \leq 0.01$, two-tailed *t* test for oral and prostate cancer cell lines; $p \leq 0.05$, two-tailed *t* test for colon cancer cell lines).

group of phytochemicals against RWPE-1 (95%), RWPE-2 (95%), and 22Rv1 (99.6%). The other cranberry fractions, organic acids (RWPE-1, 95%; RWPE-2, 88%; 22RV-1, 61%), anthocyanins (RWPE-1, 55%; RWPE-2, 62%; 22RV-1, 69%), and proanthocyanidins (RWPE-1, 80%; RWPE-2, 88%; 22RV-1, 70%), also inhibited the proliferation of prostate cancer cells at the concentrations tested.

Cranberry has shown the highest inhibitory effect against HepG2 human liver cancer cells with an EC_{50} of 14.5 mg/mL, followed by lemon, apple, strawberry, red grape, banana, grapefruit, and peach (*7*). Methanol extracts of cranberries have also shown selective inhibition against K562 and HT-29 colon cancer cells in the range of 16−125 μg/mL (*4*). Recently, triterpene hydroxycinnamates isolated from an active ethyl acetate extract of cranberries showed antiproliferative effects against MCF-7 breast, ME180 cervical, and PC3 prostate tumor cell lines (*8*).

It is well established that consumption of fruits and vegetables has been associated with reduced risk of chronic diseases such as cancer and that plant extracts including fruits and berries show antitumor activities (*7*, *17*). Our studies have shown that enhanced antiproliferative activity is obtained when cranberry extract is enriched in polyphenolic content by removing fruit sugars and organic and phenolic acids. Also, there were additive or synergistic antiproliferative effects resulting from the combination of anthocyanins, proanthocyanidins, and flavonol glycosides compared to individual purified phytochemicals. The observed antiproliferative activities of cranberry phytochemicals against tumor cells provide some basic evidence for the potential anticancer effects of cranberry polyphenols and suggest that studies of cranberry extracts should be carried out in appropriate animal models of cancer and ultimately in human cancer prevention trials.

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