

The Impact of the Roast Levels of Coffee Extracts on their Potential Anticancer Activities

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Abstract: Coffee is one of the most widely consumed beverages in the world and contains numerous phytochemicals that are beneficial to consumer health. The phytochemical profile of coffee, however, can be affected by the roast level. In this study, we compared the effect of roasting level on the growth inhibitory activity of HT-29 (colon) and SCC-25 (oral) cancer cell lines. The different roasting stages selected for this study were green, cinnamon/blonde, city/medium, full city/medium-dark, and full city plus/dark. Cancer cells were treated with various concentrations of coffee extracts for 72 hr. Cell viability was quantified using the thiazolyl blue tetrazolium bromide assay. It was found that the lighter roast extracts, Cinnamon in particular, reduced cell growth more than darker roast extracts. The Cinnamon extract had the greatest amount of total phenolic content and antioxidant activity. Relative levels of gallic, caffeic, and chlorogenic acid in the extracts were also compared. The Cinnamon coffee extract had the highest levels of gallic and caffeic acids, which have both been widely-regarded as bioactive phytochemicals. In conclusion, the consumption of lighter roasted coffee, may contribute to the prevention of certain types of cancer such as oral and colon.

Keywords: cancer, coffee, phenolic compounds, roasting

Practical Application: Chemical compounds in coffee may reduce the risk for certain types of cancers. These compounds may be particularly abundant in lighter roasted coffee. Therefore, lighter roasted coffee could contribute to the prevention of cancer through a healthy diet.

Introduction

According to the American Cancer Society, colon cancer is the 3rd most common type of cancer in the United States for both men and women. Oral cancer is far less prevalent, yet incidence rates are more than double in men compared to women (Society, 2016). Coffee is a widely consumed beverage that has many beneficial health effects including prevention of certain types of cancers such as colon (Sugiyama et al., 2010) and oral cancer (Li, Peng, & Li, 2016).

Brewed coffee contains numerous phytochemicals that are beneficial to consumer health. Of those phytochemicals found in coffee, some exert potent anticancer effects by inhibiting phosphorylation of ERKs, suppressing activity of MEK1 and TOPK, and inhibiting transcription factors such as AP-1 and NF- κ B in colon cancer cells (Kang et al., 2011). Caffeic and chlorogenic acid are types of hydroxycinnamic acids that are particularly abundant in coffee (Clifford, 2000; Duarte, Pereira, & Farah, 2010). Caffeic acid has been found to inhibit proliferation of breast cancer cells (Kampa et al., 2003) and fibrosarcoma cells (Prasad, Karthikeyan, Karthikeyan, & Reddy, 2011). Chlorogenic acid has been found to inhibit hepatoma cell invasion (Yagasaki, Miura, Okauchi, & Furuse, 2000) and inhibit growth of bladder cancer cells (Cherng, Shieh, Chiang, Chang, & Chiang, 2007). Gallic acid is also present in small yet potent levels. The levels of these specific phenolic compounds may play an important role in the prevention of oral and colon cancers by coffee.

Coffee is traditionally prepared by roasting coffee beans which are then ground and brewed in hot water. The concentration of the already mentioned phenolic compounds (Mills, Oruna-Concha, Mottram, Gibson, & Spencer, 2013) and aroma compounds (Gonzalez-Rios et al., 2007a,b) are dependent on roast levels of coffee. The amount of total beneficial compounds present in coffee can be affected at any point from the harvest to the actual preparation of the beverage, including fermentation (Lee, Cheong, Curran, Yu, & Liu, 2015, 2016; Nunes & Coimbra, 2007; Wei et al., 2012). Because the levels of bioactive compounds in coffee are dependent upon the degree of roasting, then the antiproliferative activity of coffee should also be dependent upon roasting level.

The connection between roasting level on the antioxidant capacity of coffee has been studied (Cho, Park, Kim, Kim, & Han, 2014), where the lighter roasted coffee had the highest antioxidant activity compared to greater roasted coffee and also unroasted coffee. Although this finding has relevance to human health, the connection to cancer prevention needs to be clarified.

In this study, we compared the anticancer activities of various freeze-dried coffee beverages, differing only in their roast treatments. We also compared the bioactivity with total phenolic content and antioxidant capacity. In addition, high-performance liquid chromatography (HPLC) was used to determine the concentrations of gallic acid, caffeic acid, and chlorogenic acid (Figure 1) in the freeze-dried coffee extracts.

Materials and Methods

Chemicals

Coffee beans were donated by Covenant Coffee Co. (Bakersfield, CA, U.S.A.). All other chemicals were of the highest purity from Sigma (St. Louis, MO, U.S.A.).

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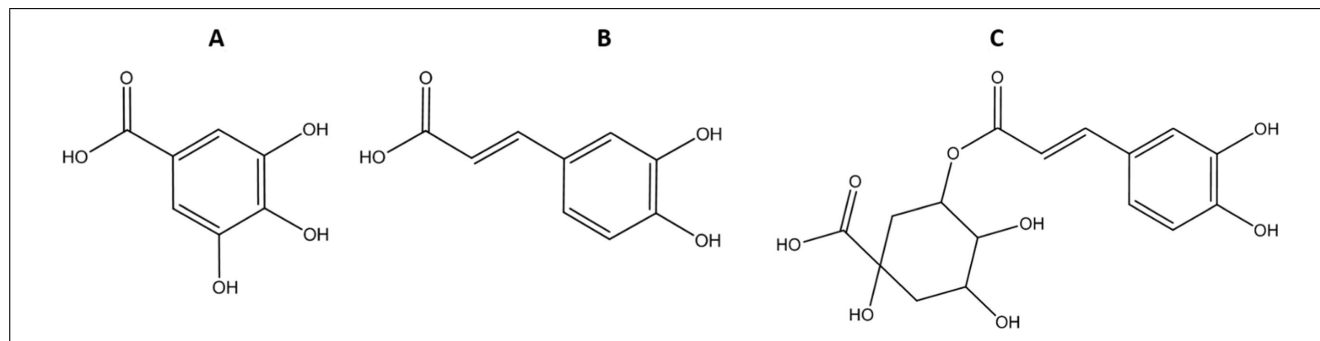


Figure 1—Structures relevant to this study, including (A) gallic acid, (B) caffeic acid, and (C) chlorogenic acid.

Preparation of coffee beans

A total of 1.5 pounds of Green beans were of a single variety and origin were supplied (Bean type: Columbia Supremo), and were roasted in-house at Covenant Coffee Co. The in-house roaster (apparatus brand Primo Roasting Eq.) was loaded with 1.25 pounds of the Green coffee beans, where 0.25 pounds were set aside for the designated Green coffee sample. The roasting apparatus was set to an initial temperature of 420 °F, measured within the roasting drum. The beans were roasted at different temperatures and times depending on the roast level. The Cinnamon roasted beans were heated at 437 °F for 13:20 min. The City roasted beans were heated at 443 °F for 13:52 min. The Full City roasted beans were heated at 455 °F for 14:32 min. Finally, the Full City Plus roasted beans were heated at 463 °F for 15:04 min. The beans were then allowed to cool, vacuum sealed, and stored in a cool dry place until ready for use.

Preparation of coffee extracts

Before the preparation of coffee samples was to begin, the beans were allowed to degas in their respective vented bags for a period of 72 hr. The ratio of whole coffee beans to water was measured as 59 mL of whole coffee bean to 355 mL of water. The coffee was then ground and brewed with purified boiling water for 5 min, utilizing a pour-over technique for the brew method. The coffee was then allowed to cool, lyophilized for a total of 48 h, and either resuspended in the same volume of cell culture media to make stock solutions or left as a solid extract. Once complete, the samples were stored at −80° C until ready to use.

Cell culture and incubations

The HT-29 (colon), SCC-25 (oral), and Caco-2 (colon) human cancer cells were maintained with McCoy's, DMEM:F-12, and MEM media, respectively (American Type Culture Collection, Manassas, VA, U.S.A.). Media was supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, U.S.A.), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma) under 5% CO₂ atmosphere and 37 °C at 95% humidity. The cells were subcultured by trypsinization to maintain growth in the log phase.

Cell viability assay

Cells were seeded (5000 cells per well) in 96-well plates and allowed to attach overnight. Cells were treated with either 1 to 50× dilutions of the coffee stock solutions or treated with solid extracts that were weighed and dissolved in cell culture media. In a separate experiment, Caco-2 cells were differentiated into a suitable normal cell model. Caco-2 cells were allowed to differentiate for 2 wk after confluence, and then treated with 500 and 1000 ppm of each of the coffee extracts. Finally, all three cell

lines were treated with various concentrations of acrylamide (0 to 1000 ppm). Cell viability was determined spectrophotometrically after 72 hr of incubation using the thiazolyl blue tetrazolium bromide (MTT) assay ($\lambda = 540$ nm). For the assay, treatment media was removed and cells were washed with fresh media. Cells were then incubated with MTT media (1 mg/mL) for 30 min. After 30 min, the MTT media was replaced with dimethyl sulfoxide (DMSO) and absorbances were measured. Blanks were prepared by removing fresh media from wells with no cells, adding MTT media, and finally replacing it with DMSO prior to absorbance measurement.

Folin-Ciocalteu assay

The total polyphenol content (TPC) was determined spectrophotometrically in a 96-well plate, using a gallic acid standard. The diluted sample extract or standard was transferred at a volume of 2 µL to wells containing 158 µL of water and 10 µL of Folin-Ciocalteu's reagent. After 5 min, 30 µL of a sodium carbonate solution was added. The plates were then allowed to stand at room temperature for 2 hr before absorbance at 620 nm was measured against water blanks. The TPC was expressed as gallic acid equivalents (GAE) in mg/g extract. The concentration of polyphenols in samples were derived from a standard curve of gallic acid ranging from 50 to 500 µg/mL.

Oxygen radical absorbance capacity assay

The coffee extracts were freeze dried and resuspended in media. The diluted samples were combined with 5.3 nM fluorescein in a 75 mM phosphate buffer (pH 7.4). Samples were equilibrated for 15 min at 37 °C followed by the addition of 18.75 mM 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH). Fluorescence signal (485 nm excitation, 520 nm emission) intensities were monitored until baseline and integrated. A standard curve generated from trolox peak areas was used to quantify the antioxidant capacities of the samples. Oxygen radical absorbance capacity (ORAC) values were expressed as µmol TE (trolox equivalents) per mg of extract.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) separation of phenolic compounds in gradient mode utilized solvent A (3.5% formic acid in water) and solvent B (3.5% in methanol) at a flow rate of 1.0 mL/min. The column selected was C-18 Luna (150 × 4.6 mm, 5 µm packing; Phenomenex, Torrance, Calif., U.S.A.) protected by a C-18 guard column. Samples were run on a UFLC (Shimadazu, Kyoto, Japan) HPLC system. Coffee extract samples were dissolved in 50% methanol in purified water, and then filtered using a 0.45 µm pore size PTFE membrane into

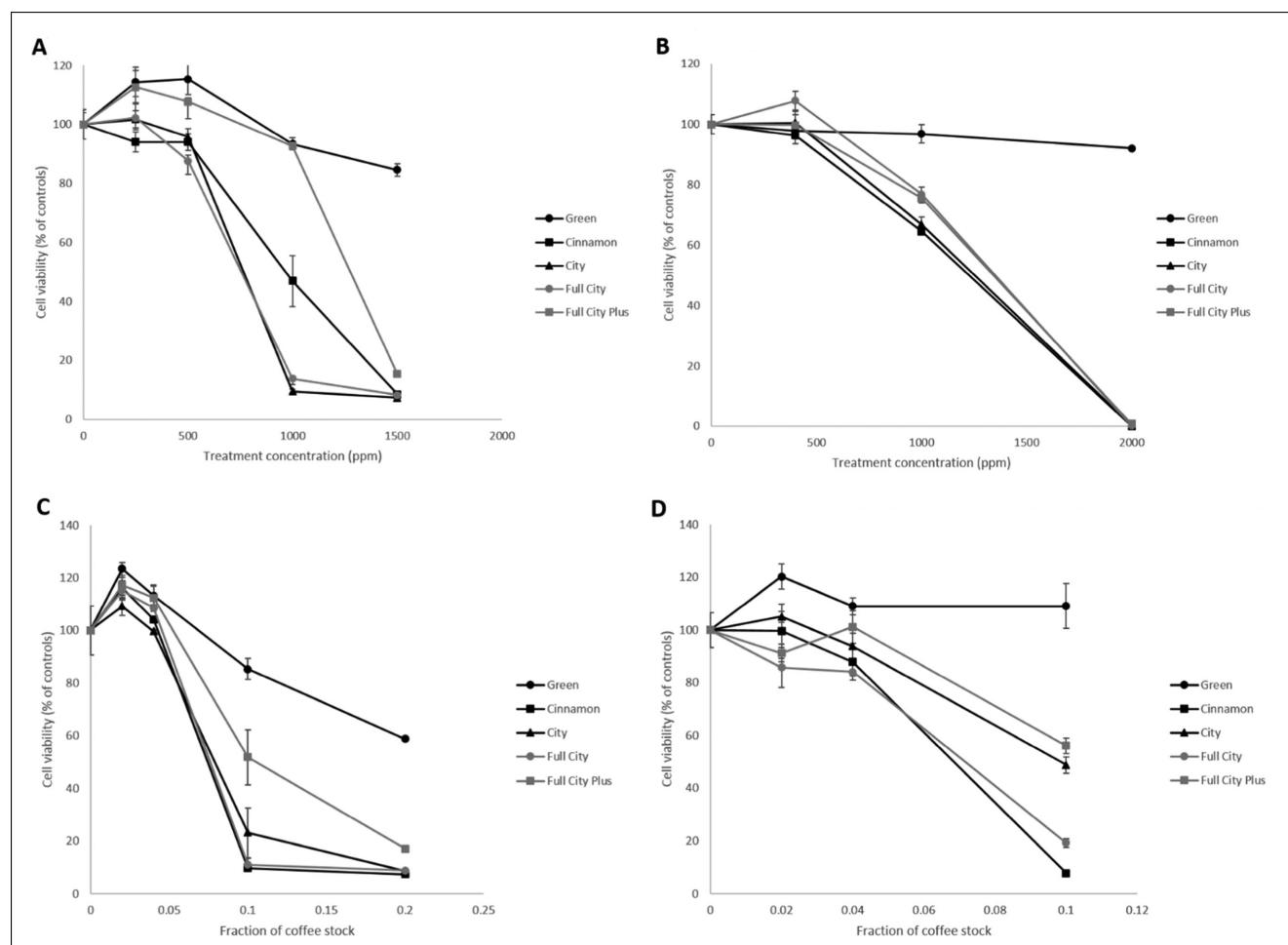


Figure 2—Antiproliferative effects of the coffee extracts. Cell viability of (A) HT-29 colon cells and (B) SCC-25 oral cancer cells treated with coffee extract samples; and cell viability of (C) HT-29 colon cells and (D) SCC-25 oral cancer cells treated with coffee samples prepared from diluted stock solutions (x-axis represents the concentration of coffee sample as a fraction of stock coffee solution concentration). Results are shown as means \pm SEM ($n = 3$ to 5).

an HPLC vial. Trace collected was 280 nm. Gallic, caffeic, and chlorogenic acid standards were used to confirm identity of sample peaks.

Statistical analysis

All values are reported as means \pm SEM. Two-tailed Student's *t* tests were performed to determine statistical significance. The value $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Cell viability assay

The cell lines were treated in two different ways. In one approach, coffee was brewed, freeze dried, and then used to treat the cells by resuspending weighed extracts in cell culture media to various concentrations (ppm). In this approach, greater differences between the roast levels were observed in the HT-29 cells compared to the SCC-25 cells. In the HT-29 cells, the Full City Plus treatment caused the least effect in reducing cell proliferation as compared to the Cinnamon, City, and Full City roast treatments (Figure 2A). In the SCC-25 cells, the Full City and Full City Plus treatments were not as effective compared with the Cinnamon and City treatments (Figure 2B). In both cell lines, the

Green treatment did have some activity, but it reduced cell proliferation to a lesser extent as compared to the other roasted coffee treatments.

In another approach, coffee was brewed, a set volume was freeze dried, and then resuspended in the same volume of cell culture media to make a stock. That stock was then diluted to several fractions (by volume) of the original stock. This method accounts for extractability of the beans, making it more relevant to traditional coffee consumption, whereas the other method may be more relevant to taking an extract in pill form. Of the different roast levels, the Cinnamon was the most effective and Full City plus the least effective in reducing cell proliferation of both cell lines (Figure 2C and D). As in the first set of cell viability experiments, the Green treatment had much lower effect on reducing cell viability compared to the other treatments. From Figure 2A to D, a general trend of lighter roasted coffee having greater anticancer effects was observed, particularly the Cinnamon roasted treatment. Despite the Green coffee treatments having some antiproliferative activity, they were not as potent as compared to the other roasted coffee treatments.

For comparison, Caco-2 cells were differentiated into a suitable normal cell model. The after differentiation, the cells were treated with 500 and 1000 ppm of each of the coffee extracts. Even at these

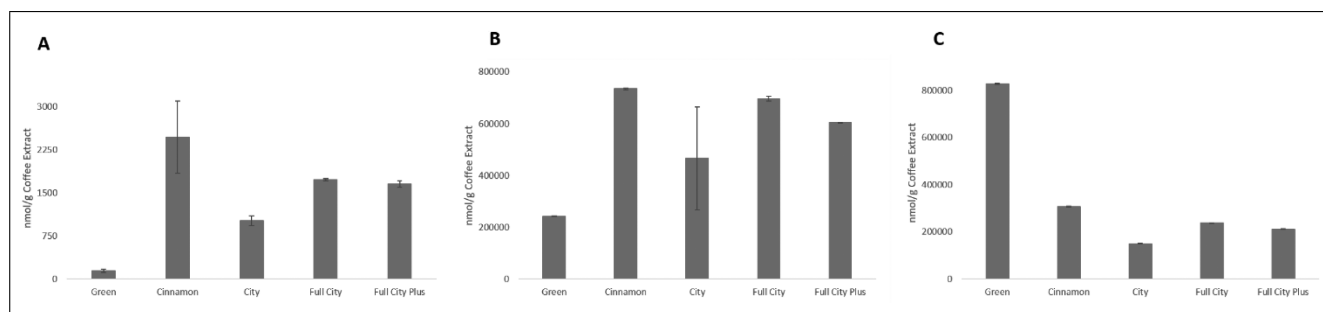
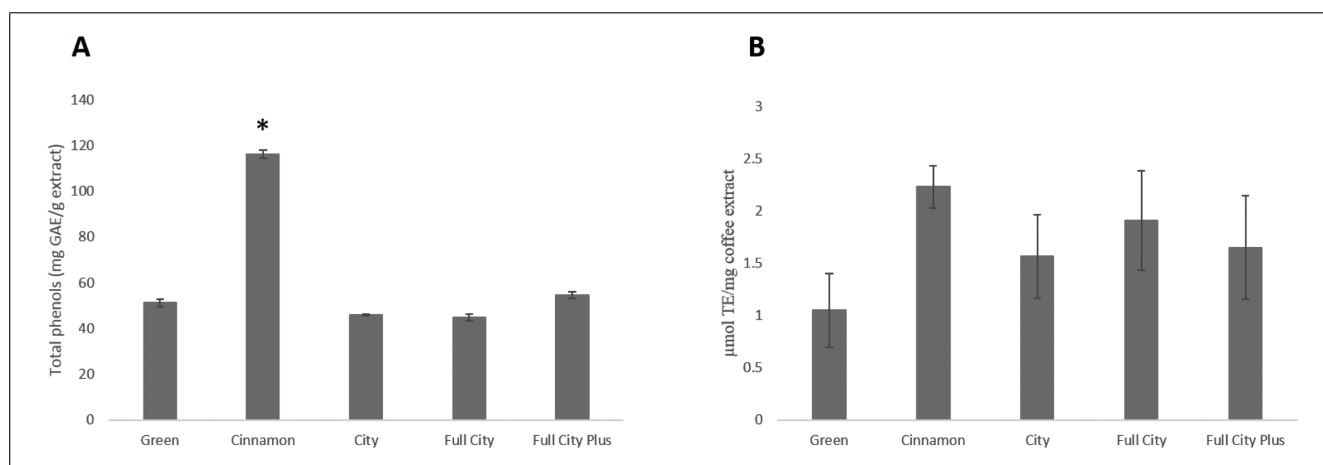
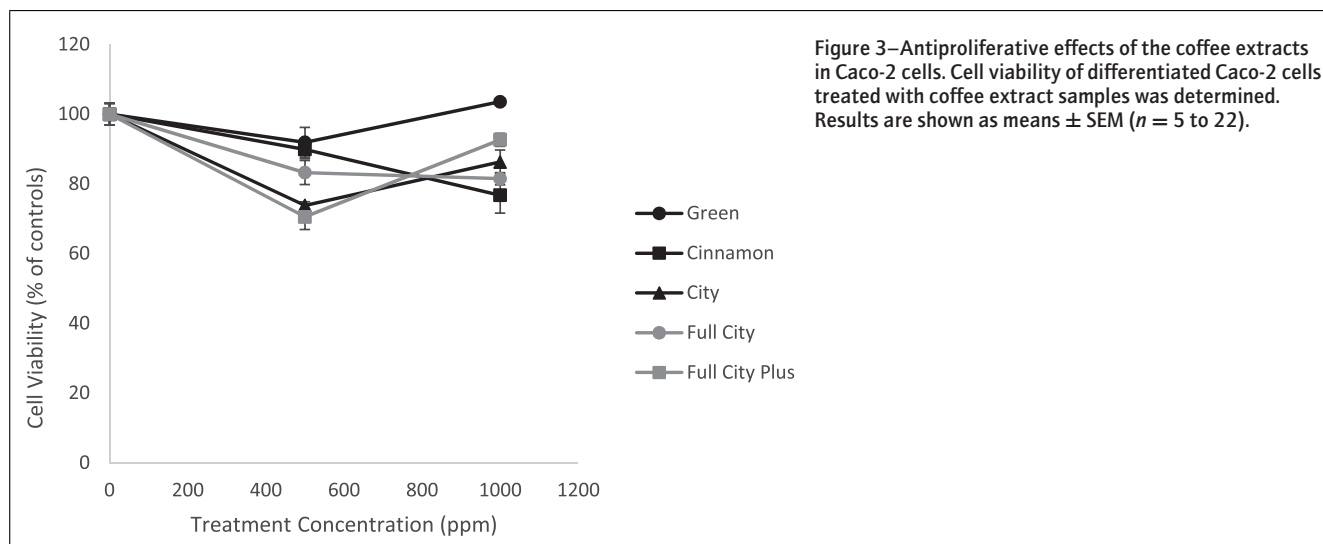


Figure 5—Concentrations of (A) gallic, (B) caffeic, and (C) chlorogenic acids in the coffee extracts. Results are shown as means \pm SEM ($n = 3$).

high treatment concentrations, the cell viability was minimally affected by the coffee extracts as compared to the cancer cell lines (Figure 3).

Total phenols, antioxidant capacity, and individual phenolic acid measurement

The Cinnamon roasted extract had statistically higher total phenols (116.1 mg GAE/g extract) and higher antioxidant capac-

ity (2.2 μ mol TE/mg extract) compared to the other treatments (Figure 4A and B).

Using HPLC, the relative levels of gallic acid, caffeic acid, and chlorogenic acid were determined. The relative levels of gallic acid and caffeic acid were higher in the cinnamon roasted extract, although this difference was not significant (Figure 5A and B). The relative levels of chlorogenic acid were the highest in the Green coffee extract (Figure 5C).

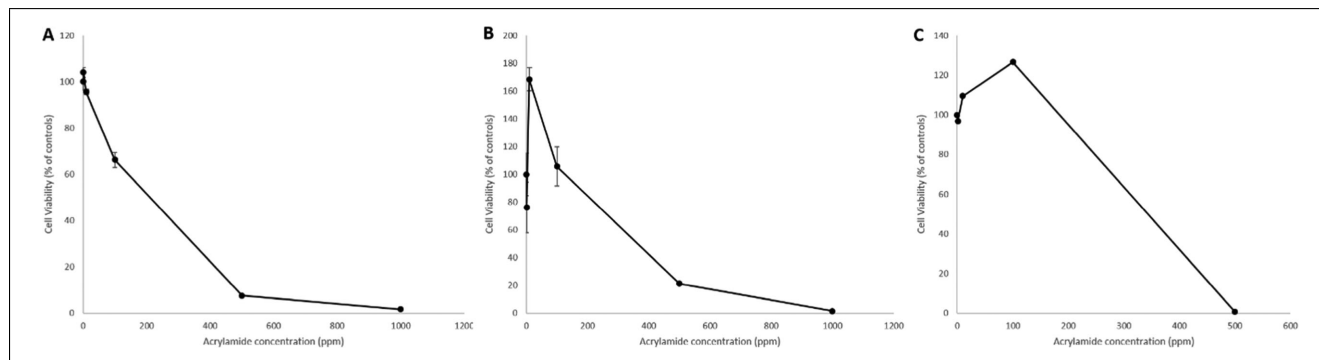


Figure 6—Antiproliferative effects of acrylamide in (A) HT-29 colon cells, (B) SCC-25 oral cancer cells, and (C) Caco-2 cells. Results are shown as means \pm SEM ($n = 3$ to 6).

In this study, the observed trend was that the lighter roast extracts, Cinnamon in particular, had greater growth inhibitory effects, phenolic content, antioxidant activity, and levels of caffeic and gallic acid. Other studies have investigated the role of roasting level in the health-related properties of coffee. Paur et al. (2010) demonstrated that darker roasted coffees have greater inhibitory effects on inflammation markers of the liver compared to lighter roasted coffees (Paur, Balstad, & Blomhoff, 2010). Although numerous connections have been made between inflammation and cancer, the study by Paur et al. (2010) focused on measurement of inflammatory markers rather than markers of cell proliferation. This may account for the inconsistent results between their study and our study.

This study examined the antiproliferative effects of various roasted coffee treatments in two cell lines that represent tissues that are exposed to relatively high levels of coffee-derived phytochemicals. Even though numerous phenolic compounds like those found in coffee survive the digestive tract and come into direct contact with the intestines (Crozier, Del Rio, & Clifford, 2010), the anticancer effects of these coffee extracts are expected to be enhanced in the oral cavity, where concentrations of phenolic compounds are high and persist for relatively long periods of time. This has previously been shown with green and black tea compounds, where μM levels of catechins and theaflavins were detected in the saliva of human subjects after holding either green tea leaves or black tea in the mouth followed by rinsing with water (Lee et al., 2004). Therefore, the results from the SCC-25 cells are potentially more relevant and comparable to a clinical study.

In addition to testing the effects of various roast levels in two cell lines, the coffee extract treatments were prepared in two ways. The differences in antiproliferative activity of the various extracts were clearer when coffee was brewed, freeze-dried, and then immediately resuspended in the same volume of cell culture media. This method most closely models the traditional approach to coffee consumption, as opposed to taking extract in pill form.

Despite having some activity, it was not surprising that the Green treatment was the least effective when prepared in the more traditional approach, as not much was extracted due to the beans being resistant to grinding. The Green beans were simply harder and more difficult to break when ground at the same setting used for the other roasted coffee beans. In addition, the relatively large chunks of Green coffee beans produced after grinding, were more resistant to extraction with hot water as compared to the roasted coffee beans. The extractability of Green coffee beans could be enhanced increasing the surface area through grinding at higher speed and longer time, yet this would also be true for

the other roasted coffee beans. In this study we treated all coffee bean samples the same, so that their relative activities could be compared. Green bean coffee phenols have been shown to be quite bioavailable in humans (Farah, Monteiro, Donangelo, & Lafay, 2008), yet these compounds were found to be poorly extracted in our study. It was surprising, however, that the Green treatment had the least activity, as compared to the other roasted coffee treatments, when cells were treated with ppm concentrations. On a weight basis, we expected that the Green treatment would have the greatest levels of bioactive compounds, and therefore have the greatest antiproliferative activity. This expectation was based on previous studies that compared the activities of green and black teas. One previous study found that green tea was associated with reduced risk of colon cancer yet not black tea (Sun, Yuan, Koh, & Yu, 2006). Maillard reaction products formed during the roasting process, however, may have their own biological activity (Moreira, Nunes, Domingues, & Coimbra, 2012), and antioxidant activity (Kirigaya, Kato, & Fujimaki, 1968; Yen & Hsieh, 1995) which may have greater potency than the Green coffee derived compounds. From this study, it appears that lighter roast coffee contains optimal levels of both phenolic compounds (as supported by the folin, ORAC, and HPLC data) and Maillard reaction products in order to slow the growth of cancer cells. It is clear from this study that the activity of the Cinnamon roasted coffee in particular can be attributed, in part, to its relatively higher levels of phenols (Figure 4A).

Although it is not clear if dietary levels of acrylamide plays a role in colon cancer development in humans (Larsson, Åkesson, Bergkvist, & Wolk, 2009; Mucci, Adami, & Wolk, 2006; Mucci, Dickman, Steineck, Adami, & Augustsson, 2003), the antiproliferative effects of acrylamide have not been studied *in vitro*. We found that acrylamide did inhibit growth of all three cell lines, yet only at concentrations that are not present in coffee. Interestingly, acrylamide stimulated proliferation of SCC-25 cells and differentiated Caco-2 cells at lower concentrations (Figure 6). From these results, it is likely that acrylamide does not contribute to the growth inhibitory properties of coffee. Although, it is expected that the acrylamide levels increase with roasting levels, one source did report concentrations of various coffee samples not exceeding 10 ppb (Granby & Fagt, 2004).

Despite the possible involvement of Maillard reaction products, our data clearly shows that the lighter roasted coffee contains more phenolic compounds, specifically gallic and caffeic acids. In addition to the already described effects of caffeic acid, gallic acid has been shown to inhibit the growth of colon cancer cells (Forester & Waterhouse, 2010) potentially through

inhibition of transcription factors NF- κ B, AP-1, STAT-1, and OCT-1 (Forester, Choy, Waterhouse, & Oteiza, 2014). Gallic acid was also found to inhibit metastatic properties of SCC-4 human oral cancer cells by targeting NF- κ B, Ras, and matrix metalloproteinase-2 and -9 (Kuo et al., 2014). Therefore, the enhanced activity of the Cinnamon extract may be due, in part, to increased levels of gallic and caffeic acids.

Conclusion

In conclusion, the consumption of lighter roasted coffee, along with a healthy and balanced diet, may contribute to the prevention of certain types of cancer such as oral and colon. Future research will involve characterization of bioactive Maillard reaction products other than acrylamide.

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Authors' Contributions

Benigno E. Mojica and Sarah C. Forester designed the study. Benigno E. Mojica, Lisa E. Fong, Denny Biju, Alfeah Muharram, Isabel M. Davis, Klarisse O. Vela, Diana Rios, Elena Osorio-Camacena, Baljit Kaur, and Sebastian M. Rojas collected data and interpreted the results. Sarah C. Forester designed the study, interpreted the results, and drafted the manuscript.

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