Analysis of the Antioxidant & Antimutagenic Potential of CGA, a phenolic component of Coffee, in Inhibiting Mutations in *Salmonella typhimurium*

Ambreen Niaman*, J.F. Treml*, and R. Logan*†

Excess cellular radical oxygen species are associated with an increase in cellular, including genetic damage. In somatic cells, this may lead to cancer when genes associated with cell growth and reproduction are impacted. Opposing radical oxygen species are antioxidants which can quench free radicals that otherwise may damage a cell's DNA. The body produces some antioxidants naturally, such as glutathione, while others are obtained through diet, including vitamins C and E. Chlorogenic Acid (CGA) is one example of a polyphenolic compound in coffee with the potential to exhibit antioxidant and antimutagenic properties. The concentration of CGA in a commercially available coffee was measured and its potential to exhibit antioxidant properties was assessed. An extract was prepared in which the spectral properties of CGA were exploited to quantitate its concentration in green coffee extract via HPLC. The antioxidant activity of CGA was characterized by measuring its relative ability to neutralize free radicals using a colorimetric assay vs. a pure vitamin C standard. The antimutagenic activity of CGA was to be assessed via an Ames test using mutant Salmonella strains incapable of producing the amino acid histidine. The results demonstrated a considerable amount of CGA in green coffee extract, that exhibited antioxidant properties. Future work will assess antimutagenic effects of CGA in the extract compared to the antimutagenic effect of vitamin C and pure CGA standards. Overall, these results suggested that coffee beverages may serve as potent antioxidants with the potential to protect consumers from the harmful effects of mutagenic free radicals.

Coffee is a beverage, prepared as an extract from roasted coffee beans, widely consumed across the world. It has been regarded as an ancient wonder drug due to the presence of a variety of Phyto biomolecules with therapeutic potential1. Seeds of the coffee plants are separated and dried to produce green coffee beans which are then roasted and ground into fine granules that are boiled and steeped in hot water.[1,2,3] Many of the polyphenols in coffee are also found in other plant-based foods. These polyphenols are believed to have antioxidant as well as antimutagenic properties.[4] The antioxidant property of polyphenols[5] is exhibited by their ability to neutralize reactive oxygen species(ROS) in the body which cause oxidative stress in normal, healthy cells by damaging their constituent proteins, lipids, and DNA and subsequently leading to oncogenesis.[6,7] Thus, these compounds provide health benefits to people who consume these foods on daily basis.[8]

Reactive oxygen species (ROS) are associated with an increase in cellular, including genetic damage. These are highly reactive chemicals formed from diatomic oxygen O_2 such as peroxides, superoxide, and hydroxyl radicals.[5] ROS's are produced endogenously by some biochemical reactions occurring during respiration and photosynthesis inside mitochondria, peroxisomes, and chloroplasts.[5] Whereas exogenous sources include pollution, smoke, drugs, xenobiotics, microplastics, and radiation.[5]

Chlorogenic acid (CGA), one of the polyphenolic compounds in coffee, is claimed to exhibit antioxidant properties.[9] This naturally occurring compound is found in many plant-based foods, including coffee.[10] Its concentration in coffee is documented to be highest in Arabica coffee beans which are the most popular type of beans used worldwide.[2,8]

Coffee made with green coffee beans serves as a dietary vehicle for polyphenolic compounds such as CGA which exert their antioxidant activity on cells, protecting them from free radical attacks and preventing mutations potentially leading to cancers.[7]

While we know that CGA may be beneficial, we do not know to what extent it exerts its effect at the cellular level. Thus, in vitro studies were performed to evaluate the antioxidant and antimutagenic potency of CGA and green coffee extract.[11] Several experimental questions were explored regarding CGA such as the concentration of CGA in commercially available green coffee beans, its ability to neutralize free radicals, and subsequently preventing the mutation in healthy cells.[12]

Overall, this research project seeks to identify compounds in nature that can prevent cancers in humans by neutralizing free radicals in the body.[13] This research may serve to establish a therapeutic use for these compounds that could be incorporated as a preventive measure to reduce the probability of cancer development.[13,14]

*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author rlogan@ku.edu

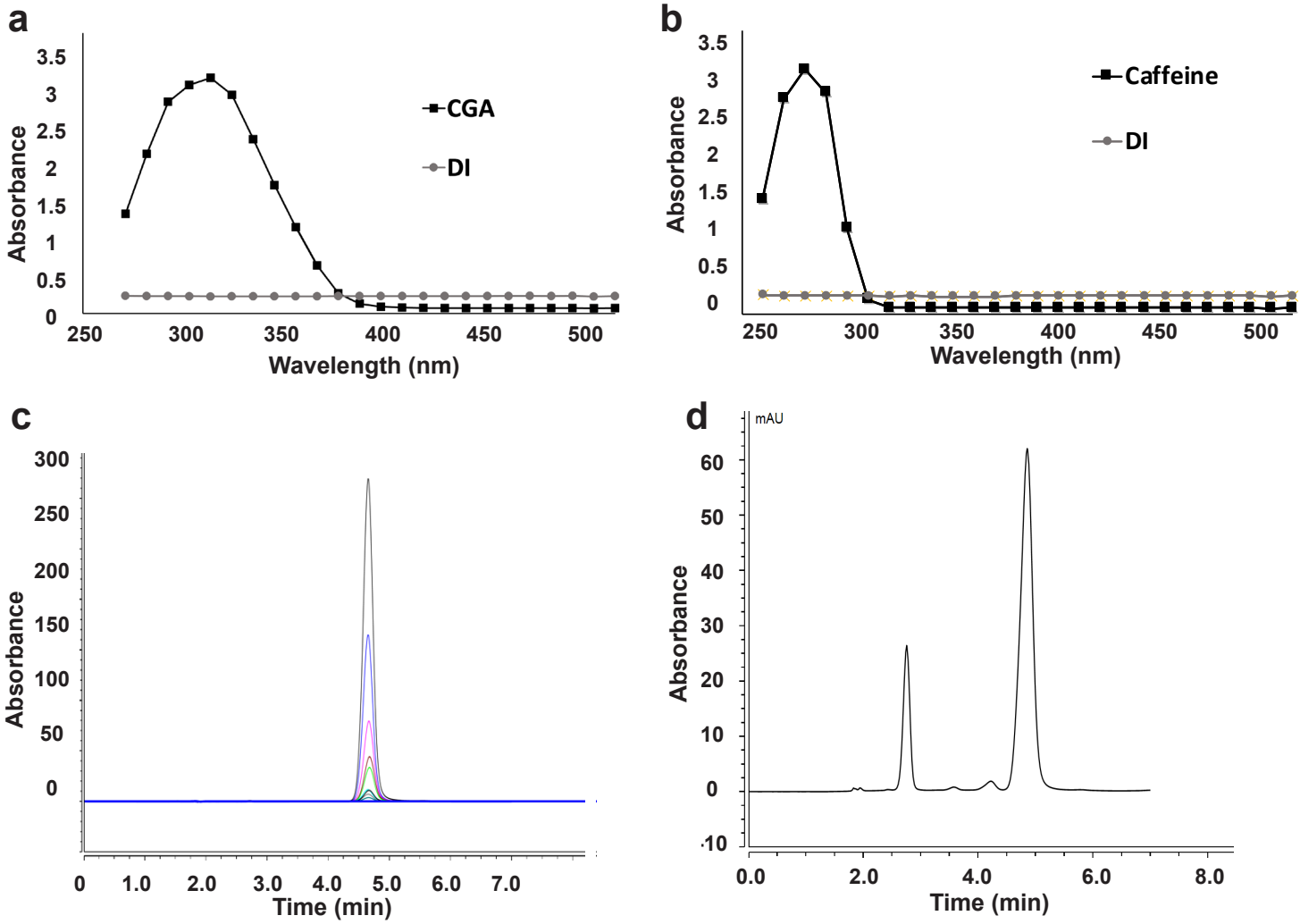

Figure 1 | Quantification of Chlorogenic acid (CGA) via Spectrophotometer and HPLC. CGA standards were prepared in DI matrix with organic crash.
(**a**) Absorbance Spectrum of 70.6μM Chlorogenic acid between 270 and 500nm. (**b**) Absorbance spectrum of 0.32mM Caffeine between 250 and 400nm. (**c**) Overlay of various concentrations of CGA ranging from 250 μM to 0.975 μM were run on HPLC at 20% B (Methanol + 0.1% Formic acid) + 80% A (DI + 0.1% Formic acid) on a reverse-phase, BDS Hypersil C 18 column. (**d**) CGA Calibration curve for HPLC Method.

Materials & Methods

Generation of the Green Coffee Bean Extract

Coffee was extracted by grinding the green coffee beans (Unroasted Green Coffee Beans, Primos Coffee Company) into a medium-ground powder. in order to optimize the extraction of the polar CGA molecule, the ground powder was weighed & boiled in deionized (DI) water at 94°C for 5 minutes for maximum extraction. DI water was used because of its potential as an efficient solvent for the extraction of polar molecules as well as its correlation to human consumption. The mixture was filtered using a Q8 filter paper (Fisher Scientific; Cat No: 09-790-B). The filtrate was stored at 4°C.

Quantification of CGA

The quantification of CGA in green coffee bean extract was carried out using "High Performance Liquid Chromatography" (HPLC). The CGA filtrate was analyzed using a C-18 column (Thermo-Scientific Cat No.28105-154630) and was run at 20% B [Methanol(Fisher Scientific: Cat # A452-4) + 0.1% Formic acid(Honeywell Fluka: Cat # 94318)] for 7 minutes using an isocratic method. The absorbance spectrum of standard CGA(Sigma Aldrich: C3878-1G) was run on a spectrophotometer (SpectraMax M3) and an optimal detection wavelength of 310 nm was determined. A standard curve was generated on the HPLC using various concentrations of pure CGA with 20% B. Green coffee extract was run on the HPLC & its average area under the curve (peak) was substituted into the equation derived to fit the standard curve. CGA concentration was determined by the following equation:

$$[\text{CGAmM}]=(\text{OD310nm}-1.2213)/0.2492$$

Assessment of Antioxidant Activity

A colorimetric assay using an ABTS (Cat No: KF01002) kit was performed using a 1:30 concentration of reagents A & B. A bar graph was generated depicting the antioxidant activity exhibited by DI water, Vitamin C (Ascorbic acid, Sigma Aldrich: 47863), CGA(Sigma Aldrich: C3878-1G), and coffee extract. DI was used as a negative control while Vitamin C (Ascorbic acid), being a well-known potent antioxidant, was used as a positive control. The antioxidant potential of the coffee extract was established by the equation:

Antioxidant potency = 1/(Abs 734nm)

Verification of rfa mutation in Salmonella strains

CGA's potential to block mutation in cells caused by free radicals was to be assessed via the Ames test. For this purpose, mutant (His-) strains of Salmonella typhi TA 1535 (Cat No: 29629) & TA 1538(Cat No: 29631) were used. The rfa mutation in these strains was confirmed via a crystal violet sensitivity test. For this purpose, 520 mg of nutrient broth (MP Biomedicals: Cat # 1007917) was mixed in 40 ml of sterile DI water. This broth was divided in half and dispensed to two sterile 50 ml Erlenmeyer flasks and labeled as TA 1535 & TA 1538. Then, using aseptic technique, both strains were inoculated into the Erlenmeyer flasks containing the broth. Those flasks were secured with wax tape and incubated for (10-12 hours) at 37 C° & 120 rpm in an incubator (ThermoScientific: Cat # SHKE4000). Nutrient agar plates were made by dissolving 7.5 g of nutrient agar (Fisher Scientific: Cat # S71614A) in 500 ml of sterile DI water. This mixture was autoclaved at 121 C° for 20 minutes and plates were poured. 100 µL of a fresh culture of each strain was added onto plates. 0.1% Crystal Violet solution was prepared by dissolving 1mg of Crystal Violet powder (Millipore Sigma: Cat # C6158) in 1 ml sterile DI water. The sterile paper disks 6mm in diameter (Becton Dickinson & Company: Cat # 231039) were soaked in 100 µL of this 0.1% Crystal violet solution and ~ 5 discs were placed on Nutrient agar plates labeled with both strains. These plates were incubated at 37 C° for 24 hours.

Results

Green Coffee Extraction and determination of CGA content

Green coffee extract was made by heating ground coffee beans in DI to 94 C° for 5 minutes and then steeping for 24 hours. The absorbance spectrum of pure CGA was measured from 250-750 nm at 10 nm intervals on the Spectrophotometer. The wavelength at which pure CGA exhibited the highest absorbance of light was selected as the optimum for detecting CGA in the coffee extract (**Figure1a**). A set of CGA standards was prepared and mixed 1:2 with methanol. CGA standards ranging from 0.975 µM to 250 µM were run in triplicates on the HPLC and detected by absorbance of light at 310 nm. Chromatograms of the standards were superimposed (**Figure 1c**, 250, 125, and 62.5µM results are highlighted). Using the average areas under the curve of these standards, a standard curve was created (data not shown). Coffee samples were similarly prepared with different dilutions in a 1:2 methanol mixture and run on the HPLC column. The chromatogram revealed two major peaks (**Figure 1d**). The peak representing CGA was confirmed by making a superimposed image of the standards and coffee extract (data not shown). We briefly considered the possibility that the smaller peak appearing on the chromatogram could be caffeine, but this was ruled out after the absorption spectrum of caffeine demonstrated no absorption at 310nm (**Figure 1b**). The concentration of CGA in the diluted coffee sample was calculated to be 57.7µM by using the linear equation shown in (**Figure 1d**). The CGA concentration in the undiluted coffee extract was calculated as 769 µM or 272.5 mg/L.

The Antioxidant Assessment of Green Coffee Bean Extract

The antioxidant activity of CGA was determined by a colorimetric assay using an ABTS kit. The results reveal that pure CGA has less antioxidant activity compared to

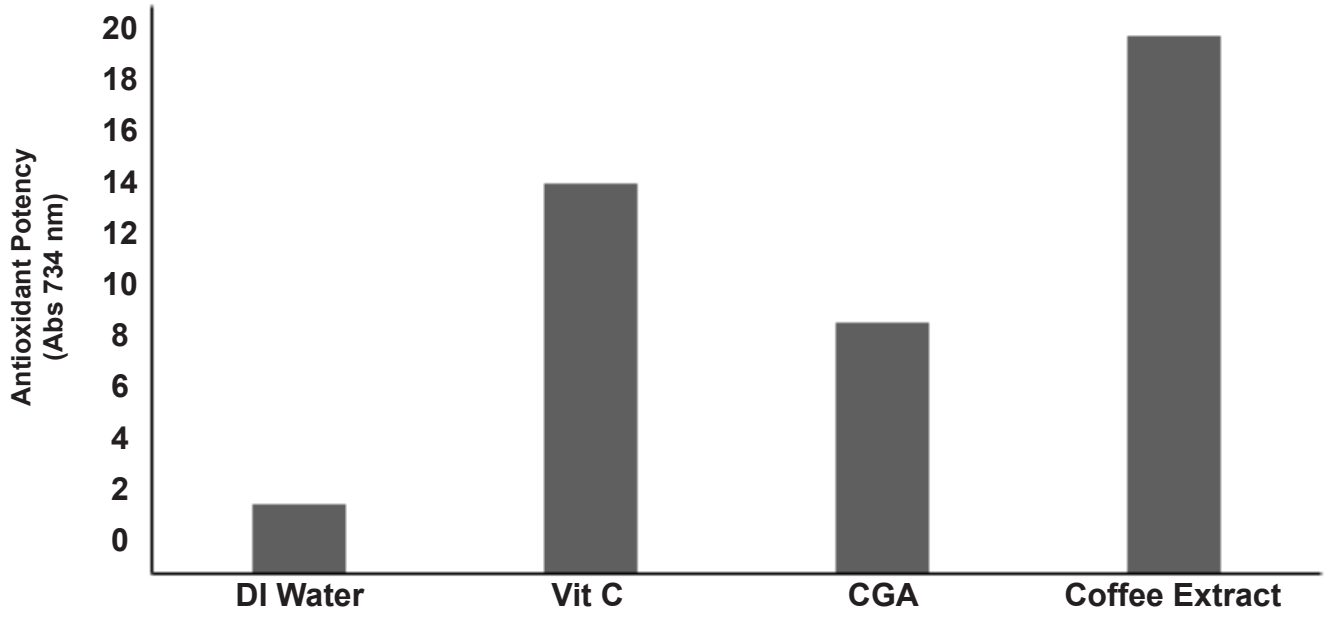

Figure 2 | Comparing the antioxidant potency of Green Coffee bean extract to the standard Vitamin C (Ascorbic acid) & Pure CGA.

Five µL of Vitamin C (769 µM), pure CGA (769 µM), Coffee extract and DI Water were added to 96-well plate. Then, 200 µL of colorimetric substrate (A & B) in the ratio of 1:30 was added to each well and incubated for 5 minutes at 27°C. Absorbance measured at 734 nm using spectrophotometer.

the known antioxidant Vitamin C. However, the green coffee bean extract exhibited almost double the antioxidant activity of Pure CGA when normalized for molar concentration (**Figure 2**). This suggests the presence of additional, possibly phenolic, compounds present in the coffee that could be exerting antioxidant effects independent of CGA.

Determination of Antimutagenic property of CGA

The question of whether CGA in green coffee beans exhibits antimutagenic effects was to be determined using mutant bacterial strains. In preparation to execute Ames tests[15] on these compounds, histidine mutations in each of two mutant strains Salmonella (TA 1535 & 1538) were verified by a crystal violet sensitivity test. Salmonella TA 1535 has a missense mutation, while TA1538 has a frameshift mutation. Additionally, both strains exhibit a rfa mutation in genes responsible for LPS synthesis that are disrupted leading to truncated LPS formation.[16] This mutation serves as a marker to ensure that these bacteria cultures have not been overgrown by wild, or other lab strains of bacteria. Crystal violet, being a large molecule was able to penetrate the disrupted LPS-containing membranes and lysed those cells. The crystal violet sensitivity test revealed that both strains were equally sensitive to the compound.[17] This is clearly visible on the plates exhibited by zones of inhibition around the discs soaked in crystal violet solution (**Figure 3**).

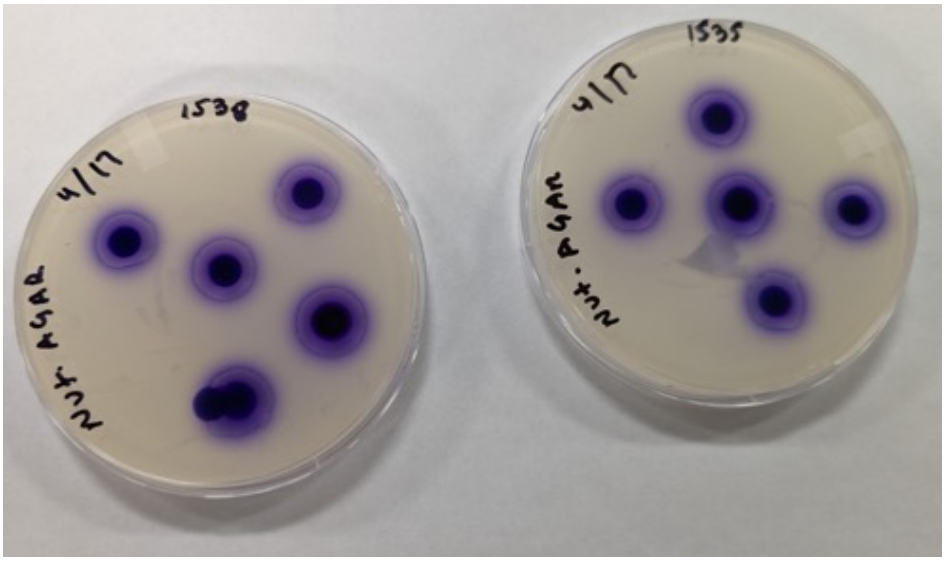

Figure 3 | Crystal Violet Sensitivity Test.
Salmonella typhimurium strains TA 1535 & TA 1538 exposed to 0.1% Crystal Violet solution (1 mg/ml) using 6mm sterile paper disks (Disk diffusion method). Zones of inhibition observed after incubation at 37° C for 12 hours, confirming rfa mutation.

Discussion

Unroasted Arabica green coffee beans were obtained from a Nicaraguan source. These mutant/hybrid arabica coffee beans originated from the combination of Maragogype (known as "elephant beans") and Catura beans, famous for excellent flavor.[18] These "Maracatu" hybrids have CGA concentrations calculated to be 272.5 mg/L which is relatively lower compared to the concentration in unroasted raw coffee beans as examined by Awwad et al.[19] On the other hand, they possess CGA concentrations comparable to those measured in light-roasted beans identified in the same study. The variation in CGA concentration might be due to the difference in extract preparation or variable storage conditions. It could also be due to the fact that these mutant beans are derived from the combination of other varieties, which might contribute towards the lower concentration of CGA.

The hypothesis that CGA in green coffee beans exerts its antioxidant effect was supported by their ability to neutralize free radicals in the colorimetric assay.[20,21] According to Tang et al., there was a strong correlation between antioxidant capacity and total phenolic compounds in Pitahaya fruit peel, which highlighted the contribution of each phenolic compound to the overall antioxidant capacity.[22] Similar results were obtained when the green coffee bean extract was exposed to free radicals in a colorimetric assay. According to Figure 2, the antioxidant capacity exhibited by the extract was almost double the antioxidant potency of Vitamin C and pure CGA on a molar basis. These results suggest the copresence of other compounds in coffee beans besides CGA, which might contribute towards the total antioxidant capacity exerted by the extract.

The crystal violet sensitivity test revealed that two Salmonella strains (TA 1535 & 1538) possess rfa mutation which renders them sensitive to penetration of large molecules. Crystal violet, being a large molecule and highly toxic, kills the cells causing the appearance of zones of inhibition around each disc soaked in it.

Future Directions

Future direction includes evaluating the antimutagenic potential exhibited by CGA via Ames test. The mutant strains (TA 1535 & 1538) don't have the ability to grow on media lacking histidine because of missense and frameshift mutations in their genome, respectively. These strains, when exposed to mutagens would grow on media without histidine confirming the reversion of mutation. Conversely, CGA and Vitamin C would be expected to prevent this reversion and spontaneous colonies won't be observed. Prevention of mutation is a promising aspect of utilizing coffee as a therapeutic for preventing cancer and opens a possibility for CGA to be considered as a potential cancer-preventive drug.

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Author's Biography

Ambreen Niaman is a senior in Biotechnology BAS program at KU Edwards Campus. She received an associate degree in Biotechnology from the Johnson County community college. She plans to graduate

in Spring 2023 and is looking forward to work in the science industry to expand her knowledge and experience.

Author's Contributions

A.N. contributed to the design, experimental work, and writing of this work; J.F.T. contributed to the editing and design of this work; R.L.L. contributed to the editing and design of this work.

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