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Article

Comparison of total flavonoid content and DPPH• sequestration in Arabica, Robusta, and Liberica coffee beans

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SUMMARY

Coffee beans obtained from the *Coffea* genus of plants are a key source of flavonoids, which are synthesized within many plants as a secondary metabolite. Flavonoids are a group of polyphenolic compounds primarily studied for their antioxidant, anti-tumor, and neuro-therapeutic properties, amongst other properties. Coffee contains a wide variety of flavonoids, such as catechins, quercetin, and myricetin. Given ecological and genomic differences across species of coffee plants, such as the *Coffea arabica*, *Coffea canephora*, and *Coffea liberica* species, variations in their metabolic pathways affect chemical composition of the beans acquired from each plant. In turn, this affects bioavailability and composition of active compounds within different species, with the possibility of rendering one species more pharmacologically favorable. We hypothesized that such differences may significantly affect the flavonoid content within different species, and therefore, their antioxidant capacities within the body. We conducted total flavonoid content assays to quantify holistic composition of flavonoids across different species. We also utilized a DPPH• (2,2-diphenyl-1-picrylhydrazyl) free radical assay to characterize antioxidant capacity by measuring each extract's ROS-sequestering capabilities. Robusta coffee beans obtained from the *C. canephora* plant elicited the highest absorbance value within the flavonoid content assay, signaling greater flavonoid content within Robusta coffee. Moreover, Robusta coffee also presented greater inhibition percentages than other species in the DPPH free radical assay, indicating higher antioxidant capacity within Robusta coffee. Ultimately, these results suggest that the radical sequestering potential of Robusta coffee provides valuable insight into the viability of coffee as a therapeutic agent.

INTRODUCTION

Coffee is one of the most popular drinks worldwide (1). Daily, approximately two billion cups of coffee are consumed according to the British Coffee Association (1). Amongst the variety of coffee bean species obtained from the *Coffea* genus of plants, *C. arabica* constitutes 60% of total sales, with *C. robusta* and *C. liberica* following suit (2). Interestingly, there has been growing evidence that coffee may play a vital role as a therapeutic agent in diseases such as Parkinson's disease, cardiovascular disease, Type 2 Diabetes, and Alzheimer's disease, among others (3).

Much of the therapeutic abilities within coffee are attributed to the abundant presence of flavonoids (4). Flavonoids are a class of polyphenolic secondary metabolites produced by plants which are characterized by two aromatic six-carbon rings connected by a three-carbon bridge, often in the form of a benzo-γ-pyrone ring (5) **(Figure 1a)**. As bioactive compounds, flavonoids have gained recognition for their antioxidant, anti-inflammatory, anti-tumor, and neuroprotective properties (5). In particular, their ability to sequester reactive-oxygen-species (ROS) helps ameliorate oxidative stress and damage, a result of the phenolic hydroxyl groups which readily participate in Sequential Proton-Loss-Electron-Transfer (SPLET) reactions to sequester radicals (6) **(Figure 1b)**. As such, the production of flavonoids within fruits, vegetables, herbs, and nuts represents a key avenue for investigating the potential of dietary therapies in various diseases (5).

Currently, reports demonstrate the existence of the flavan-3-ol, flavonol, and flavone subclasses of flavonoids within coffee (7). Specifically, coffee primarily consists of flavan-3-ols, such as (+)-catechin, (-)-epicatechin, and (-)-epigallocatechin 3-gallate (7). Moreover, flavonols such as quercetin, myricetin, and apigenin are also notable flavonoids with a therapeutic effect in coffee (ibid). The production of these flavonoids, however, may largely depend on species-relevant factors that affect the genome and metabolism of the coffee plant (8). It thus stands to reason that differences in growing conditions and genomes of coffee plants may affect flavonoid content and therapeutic capabilities of the coffee beans produced -- thus, we postulated that there may exist a significant difference in flavonoid content and antioxidant capacity across the beans produced by *C. arabica* (Arabica coffee beans), *C. canephora* (Robusta coffee beans), and *C. liberica* (Liberica coffee beans).

The growing conditions of beans influence the lipid content, amino acid content, sugar content, and mineral content of coffee beans. For example, there exists increased lipid/diterpene content variation amongst Arabica beans grown outside of Ethiopia (8). Moreover, temperature-dependent increases in metabolites such as 2-butoxyethanol, 2,3-butanediol and 1,3-butanediol have also been observed (9). Arabica, Liberica, and Robusta coffee beans are all grown in different environmental conditions **(Table 1)**. As such, the variable conditions of the bean species may reveal key insights into how growth differences affect production of key metabolites such as flavonoids.

Ultimately, our study aimed to explore two key metrics of

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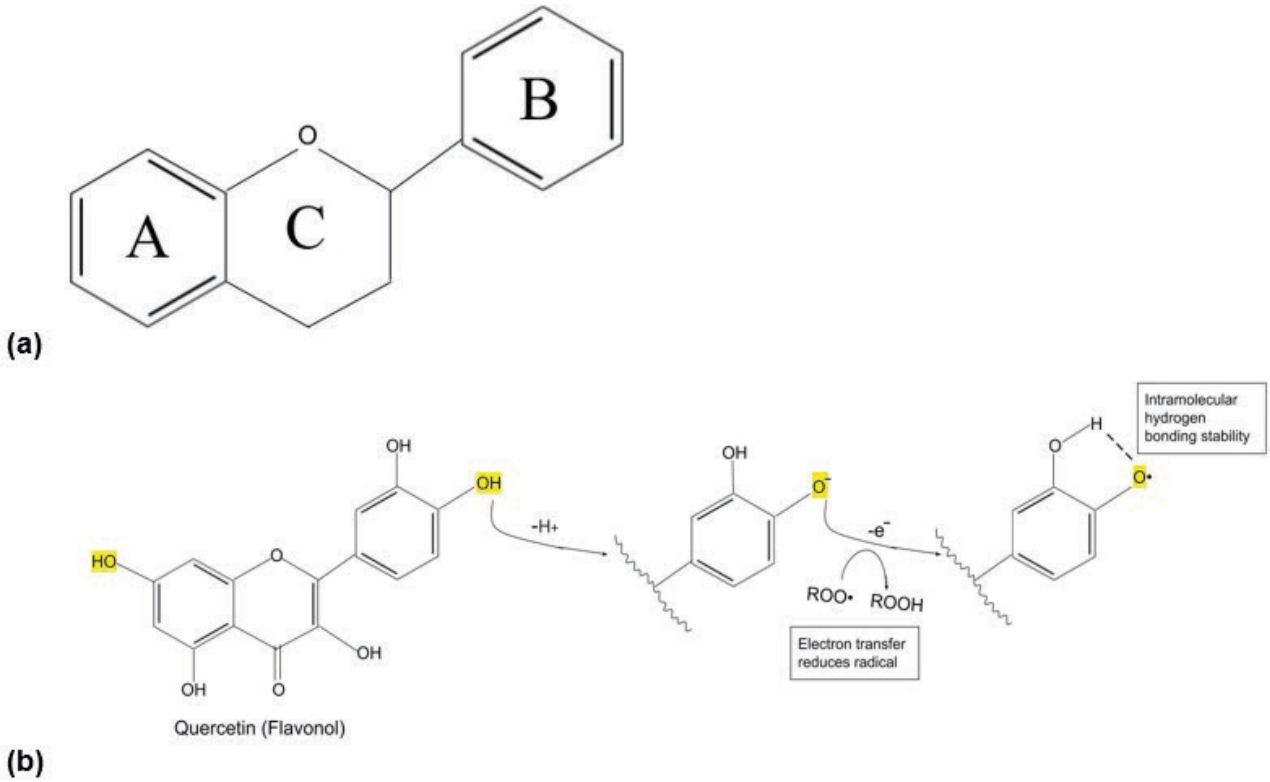

Figure 1: Flavonoids and their mechanism of action. (a) General structure of flavonoids with their two aromatic 6-carbon rings (labeled A and B) as well as 3-carbon bridge (labeled C). The presence of numerous hydroxyl groups in specific flavonoids makes them ready substrates for numerous reactions; most notably, electron transfer reactions to sequester free radicals. **(b)** Mechanism of action for Sequential Proton-Loss-Electron-Transfer (SPLET) reactions involving the flavonoid quercetin. Hydroxyl groups that are preferential substrates (highlighted in yellow) undergo proton loss to form an oxygen anion, which thereafter loses its electron via oxidation to reduce the free radical as a means of sequestration. Finally, intermolecular hydrogen bonding stabilizes the radical oxygen group, therefore completing the SPLET reaction. Image prepared by authors using Google Drawings™.

flavonoid-related therapeutic capability across each coffee bean species – firstly, total flavonoid content (TFC), which was measured by a colorimetric aluminum complexation assay (10). Secondly, we aimed to quantify antioxidant capacity via the DPPH• (2,2-diphenyl-1-picrylhydrazyl) free radical assay. Results from both assays highlighted significant differences in the therapeutic capabilities of Robusta coffee beans compared to other species. As such, the relevant growing conditions Robusta coffee may be a promising avenue in research about agricultural flavonoid production, as well as starting points for dietary pharmacology.

RESULTS

Total Flavonoid Content Across Coffee Species

To investigate differences in the level of flavonoid content across bean species, we performed the TFC assay and verified the results at 510 nm using a UV-Vis spectrophotometer **(Figure 2)**. To place absorbance values in context of (+)-Catechin concentration, we prepared a calibration curve using different concentrations of the flavonoid (+)-Catechin and measuring respective absorbance values. We determined unknown concentration values within the experimental coffee extracts using the linear model formed by the calibration curve (R^2 = 0.979, **Figure 3**).

Of the three coffee bean extracts, absorbance spectroscopy revealed that the Robusta coffee bean extract elicited a significantly higher mean absorbance value, compared to Arabica and Liberica coffee beans ($p < 0.0001$, one-way ANOVA, **Figure 4**). Based on the mean absorbance value of 0.5720 ± 0.017 obtained from the Robusta extract, we found that the Robusta extract contained an average of 8.90 mg Catechin/mL of extract. Meanwhile, mean absorbance values of Arabica and Liberica coffee bean extracts revealed average flavonoid content of 2.98 and 3.00 mg Catechin/mL, respectively. Following the use of a one-way ANOVA test to analyze significance across all groups, Tukey's HSD test was used post-hoc to analyze pairwise significance of results between each species of coffee extract. Ultimately, there existed a significant difference in the concentration of flavonoids between Arabica and Robusta coffee bean extracts ($p < 0.0001$), as well as a significant difference in flavonoid concentration between Liberica and Robusta coffee bean extracts ($p < 0.0001$). However, no significant difference in concentration was found between Arabica and Liberica coffee bean extracts ($p = 0.99489$).

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	Coffea arabica	*Coffea canephora*	*Coffea liberica*
Region of growth	Within inter-tropical Africa: primarily Ethiopia (19). Also grows in Mexico, El Salvador, Colombia, Indonesia, Nicaragua, and Vietnam (19).	Within inter-tropical Africa – ranges from West to Equatorial regions of Africa, as well as Vietnamese highlands (22).	Primarily grows in Southeast Asia – specifically, Malaysia, Indonesia, and the Philippines (25).
Altitude	Ideally between 1200 and 1400 meters above sea level -- full range can extend up to 1700 meters above sea level (20).	Grows at lower altitudes than arabica species – 200 to 800 meters above sea level (23).	No quantification found; general consensus is growth at low altitudes, similar to robusta (25).
Average growth temperature	Optimally grows at mean temperature of 20 degrees Celsius – full range is 14 degrees to 25 degrees Celsius (19).	Grows between 22 degrees Celsius and 30 degrees Celsius (22). Average temperature of growth is 23.7 degrees Celsius (24).	Average temperature of growth is 23.9 degrees Celsius (24).
Soil conditions	Soil pH is acidic, with an average value of 4.0 ± 0.1 (21). Carbon/Nitrogen ratio mean value is 11.33 ± 0.57 (21).	Soil pH is acidic, with an average value of 4.5 ± 0.4 (21). Carbon/Nitrogen ratio mean value is 8.66 ± 1.52. (21)	Soil pH is acidic, ranging from 3.01 to 3.80 (26). General organic carbon availability is around 1.3 to 4.2% while total Nitrogen values are 0.08% to 0.32% (26).

Table 1: Conditions of growth across the three different coffee species. Values of various categories, such as general altitude, temperature, region, and soil conditions (primarily pH and Carbon/Nitrogen content) are summarized across each species of coffee, as a means of comparative analysis. Values reveal higher altitude of growth for the *C. arabica* species, as well as higher Carbon/Nitrogen ratios than *C. canephora* species. Lower availability of Carbon/Nitrogen for the *C. liberica* species is also noticed, which may play a role in resultant chemical composition. Lower comparative temperatures of C. arabica species may indicate lower environmental stress, which may possibly downregulate flavonoid biosynthesis (13).

DPPH Assay

To quantify antioxidant capacity, the absorbance value of each extract's reaction with the DPPH nitrogen radical was measured at 516 nm, wherein higher antioxidant capacity of an extract was correlated with lower absorbance values. Experimental measurements were determined by calculating percentage of reduction in absorbance value relative to the control sample, and the results were expressed in % inhibition of DPPH radical.

Ultimately, Robusta coffee bean extract presented higher inhibition percentages than either Arabica or Liberica coffee bean extract ($p < 0.0001$, one-way ANOVA, **Figure 5**). A mean absorbance value of 0.92 ± 0.025 obtained from measurements of the Robusta coffee bean sample indicated a 21.71% average inhibition, which was significantly greater than the 8.05% average inhibition of the Arabica coffee bean extract ($p < 0.0001$, Tukey HSD test) as well as the 14.41% average inhibition of Liberica coffee bean extract ($p = 0.00012$, Tukey HSD test). Statistical significance from the control group of non-sequestered DPPH solution was conducted using post-hoc Tukey's HSD test following one-way ANOVA. Each extract demonstrated statistically significant results compared to the control value ($p < 0.0001$, Tukey's HSD test).

DISCUSSION

To address the primary question of how differences in species-related growth across coffee beans may affect therapeutic capabilities, we conducted a total flavonoid content assay, as well as the DPPH assay. The results illuminated key insights about the quantity of flavonoids and antioxidant capacities in the Arabica, Liberica, and Robusta coffee beans. Such quantifications act as crucial first steps in gaining insight into coffee's possible benefits in acting as a therapy against oxidation-related diseases.

Throughout the assays, we established a significant difference in the antioxidant power and flavonoid content between Robusta coffee and the other species tested. Analysis of absorbance values in the TFC assay at 510 nm ultimately revealed that the Robusta coffee bean extract contained a higher concentration of flavonoids (using (+)-Catechin as a reference), while analysis of absorbance values in the DPPH assay at 516 nm revealed that the Robusta coffee bean extract was able to sequester far greater amounts of the DPPH radical compared to other species of coffee. Given the positive correlation in literature between concentration of Catechin-based flavonoids and antioxidant capacity, it is possible that much of this antioxidant

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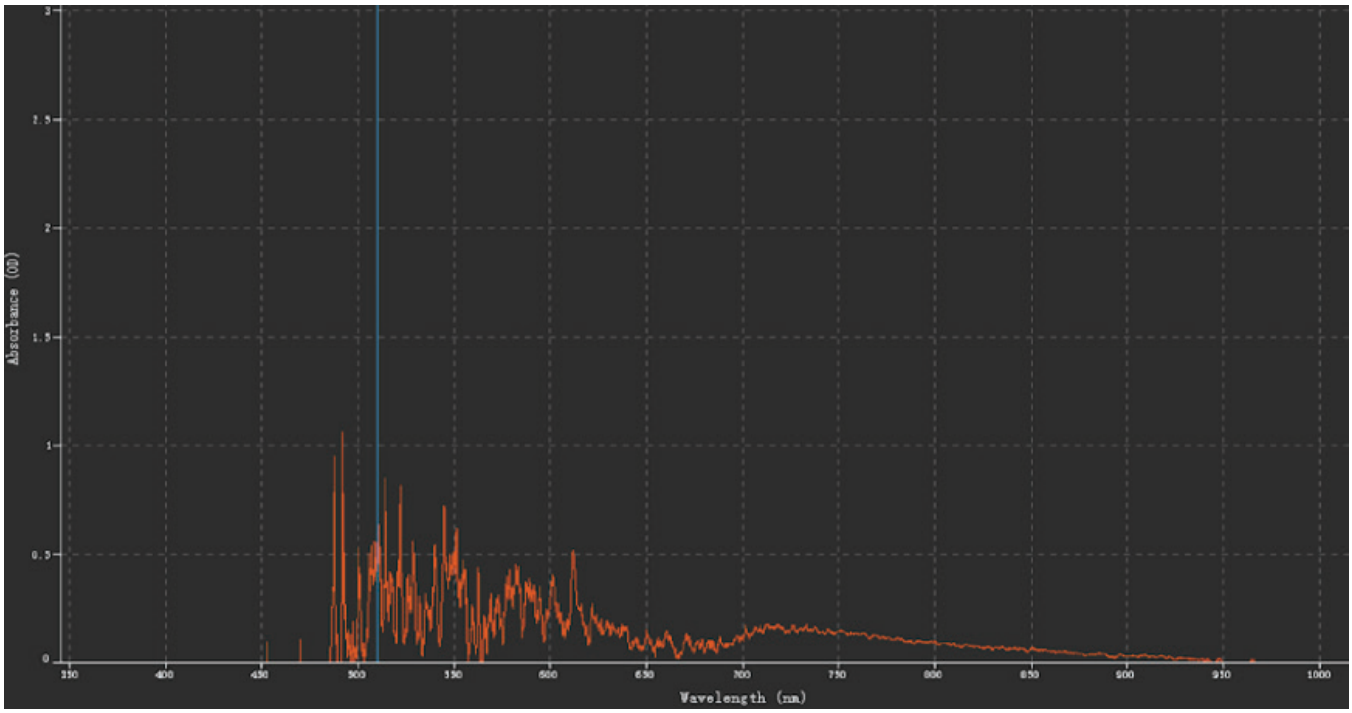

Figure 2: Example absorbance window from OceanView™ spectroscopy software. Readings were taken by inputting a wavelength value into the software and collecting absorbance value in OD, after using a blank reference measurement and dark measurement for calibration. In this specific reading, for instance, the absorbance spectrum of a (+)-Catechin calibration curve solution with 10 mg/mL concentration is shown.

ability can be attributed to the higher content of Catechin-based flavonoids within Robusta coffee beans– however, establishing such a connection may require further studies (12). It is also important to note that using Catechin as a basis for flavonoid measurement primarily explores one of multiple sub-classes of flavonoids (namely, flavan-3-ols). Therefore, it may be useful to also conduct assays using flavonoids from the flavone, flavonol, and other such subclasses, to further develop a holistic analysis of flavonoid content.

Growing conditions of coffee differ across the three species of coffee beans **(Table 1)**. For instance, lower average temperatures and higher altitude of Arabica coffee compared

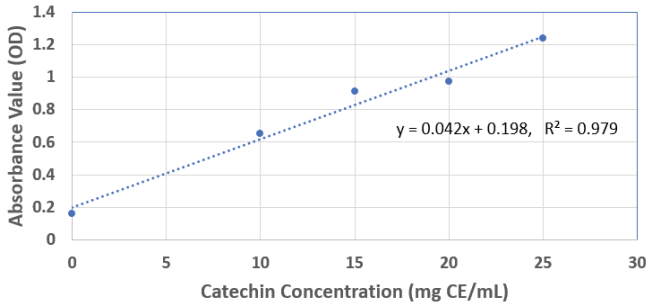

Figure 3: Calibration curve prepared with various concentrations of (+)-Catechin. Absorbance values of each catechin solution were measured by optical density (OD) and compared to catechin concentration with a linear regression eliciting the equation $y = 0.042x + 0.198$ ($R^2 = 0.979$). The linear model derived from the calibration curve was used to determine unknown flavonoid content values in each coffee extract by substituting absorbance value for y within the equation and solving for x.

to the other two species may be a possible factor in its lower antioxidant and flavonoid values, given the establishment in literature that flavonoid biosynthesis primarily occurs in plants with more environmental stress, such as higher temperatures and UV-radiation (13). Whether or not these conditions are a significant cause of differences in flavonoid content across the three coffee species requires further study. However, the significant differences we observed in flavonoid production and antioxidant capacity suggest that exploring this avenue of physio-agronomy may aid in further understanding the synthesis of flavonoids in certain environmental conditions. In turn, analyzing the effect of growing conditions on flavonoid production may establish key sources of flavonoid extraction for use in pharmaceuticals. Moreover, it may provide valuable insight into the role of agricultural and dietary differences in flavonoid intake across different regions. In this context, one key limitation may be the region of growth for each of the coffee beans -- the use of Arabica coffee beans from Colombia (as used in this study), for instance, may differ slightly from Arabica beans obtained from countries such as Ethiopia or Nicaragua. While this study still provides insight into species-related differences between the flavonoid content and antioxidant capacities of coffee beans, utilizing coffee beans from different regions may also facilitate thorough analyses of physio-agronomic differences.

Moreover, genomic differences across the individual species may also highlight the role of certain genes in increasing flavonoid biosynthesis – for instance, current literature already states that genes such as *Ca03809-F3H, Ca95013-CYP75A1*, and *Ca42029-CYP75A2* are key factors in coffee flavonoid production (14). As such, the results obtained within this study may suggest further avenues

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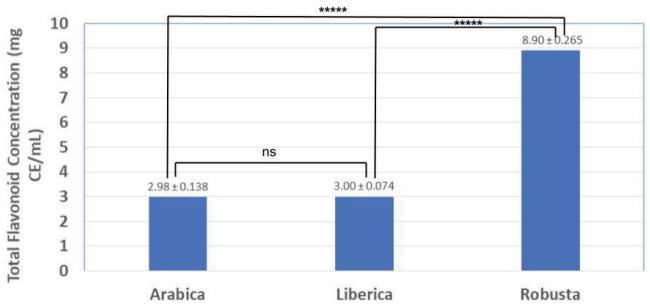

Figure 4: Total flavonoid content quantification based on coffee species. Bar graph showing mean ± SD percentages of total flavonoid quantification for expressed in mg of Catechin/mL (n=3). Absorbance values of each coffee species' assay solution were obtained following the addition of NaOH, and substituted into the linear model derived from the calibration curve to determine concentration **(Figure 3)**. Statistical significance was determined by one-way ANOVA and Post-hoc Tukey HSD; ***** *p*-value < 0.00001.

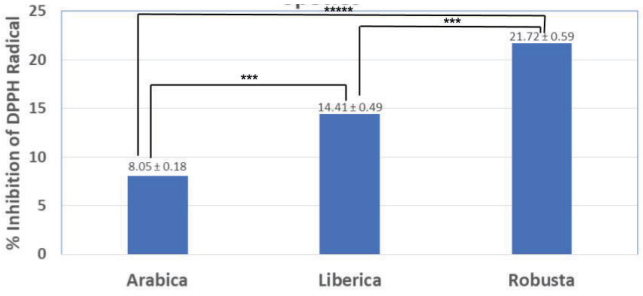

Figure 5: Percentages of DPPH Inhibition based on coffee species. Bar graph showing mean ± SD percentages of DPPH inhibition as a quantification for antioxidant capacity (n=3). Absorbance values of each coffee species' assay solution were subtracted from the mean control absorbance value (mean = 1.18 ± .026) and divided by 1.18 to find total percentage of absorbance inhibition relative to the non-sequestered control group. Statistical significance was determined by one-way ANOVA and Post-hoc Tukey HSD; *** *p*-value < 0.001, ***** *p*-value < 0.00001..

of study into isolating which specific genes may increase flavonoid biosynthesis within Robusta, for instance.

Currently, coffee diets have been proposed by numerous studies as a means of therapy for diseases such as Alzheimer's disease, cancer, and Parkinson's disease (3). Further elucidation of the coffee species that possess superior therapeutic capabilities may provide more benefit in dietary therapy. As such, this study provides a key roadmap to further understanding the role of Robusta coffee beans as a potential dietary supplement in order to treat such diseases. Moreover, the creation of health-based coffee extracts, or even the isolation of specific flavonoids in drug design to increase bioavailability, can be better approached by isolating the differences between coffee species' therapeutic capabilities. This may inform the sourcing of various flavonoids to create flavonoid-centered drug strategies and delivery for various diseases. Further studies with high-performance-liquid-chromatography (HPLC) may help characterize the exact profile of flavonoids within coffee species. Furthermore, nuclear magnetic resonance (NMR) monitoring could be valuable in understanding the structure-activity relationship of various functional groups within coffee species throughout anti-oxidation assays, to better quantify radical sequestration capabilities. Ultimately, the analysis of Robusta coffee beans as a more pharmacologically relevant species of coffee beans may inform pharmacological evaluation of coffee as a starting point for both dietary therapies and drug design.

MATERIALS AND METHODS

Obtaining Extracts for Coffee Species

Dry, medium-roasted beans of the *C. arabica* plant were obtained from AmazonFresh, Colombia (AmazonFresh, Cat# B071WWCVJV). Dry, medium-roasted beans of the *C. liberica* plant were obtained from Teofilo Coffee Company, Philippines (Teofilo, Barako Type Beans). Dry, medium-roasted Robusta coffee beans of the *C. canephora* plant were obtained from the Dalat Highlands, Vietnam (Heirloom Coffee LLC, Cat# B008A0ACNC). In order to create extraction solutions of each coffee bean species, a 1:1 water-ethanol solvent was prepared using 150 mL of deionized water and 150 mL ethanol (>95% purity, Innovating Science, Cat# IS14018).

Thereafter, a water-bath extraction using the solvent was performed for each coffee bean species in triplicate, using the method detailed by Silva et al. (16). Following homogenization of the whole beans using a coffee bean grinder (Hamilton Beach, Cat# 80335R), 10 grams of each species of ground coffee bean was added to a beaker and combined with 1:1 water-ethanol solvent to create 100 mL of extract solution. Each solution was vigorously mixed for 3 minutes before placing in a water-bath (JOANLAB, Cat# BHS-1) at 60 degrees Celsius for approximately 1 hour. Following the water-bath extraction, each solution was centrifuged (QOR Labs, Cat# QLC-1606-B7T3) for 5 minutes at 5,000 G, and supernatant was filtered using quantitative filter paper (Labasics, Cat# B08PNXLFJK). The filtered extract solutions were stored at 4 degrees Celsius until usage in the assays, for approximately one week.

Total Flavonoid Content Assay

The total flavonoid content colorimetric assay for each species of coffee beans' extract solutions was performed in triplicate using the method detailed by Fattahi *et al.* (17). Initially, 0.1 mL of each extract solution was added to 4 mL of deionized water for dilution. Thereafter, 0.3 mL of 5% w/v sodium nitrite solution (prepared using anhydrous >99% sodium nitrite, Sinodream Pharmaceuticals, Cat# 7632-00-0) was mixed with the diluted solution and left to react for 5 minutes. Then, 0.3 mL of 10% w/v Aluminum Chloride solution (prepared using anhydrous >99% aluminum chloride, Lab Alley, Cat# C1361) was then added and left to react for 6 minutes. Finally, 2 mL of 1M NaOH solution (prepared using >99% anhydrous NaOH, Belle Chemicals, Cat# LYEJAR-32) was added alongside 3 mL of deionized water and mixed thoroughly. For the blank solution, the above processes were performed with the addition of 0.1 mL ethanol instead of extract solution. Absorbance readings for each of the solutions was recorded at 510 nm using UV-Visible Spectrophotometry (Ocean Insight, Cat# SR4), with a path length of 1 centimeter. Values were compared against a calibration curve prepared using (+)-Catechin (Santa Cruz Biotechnology, Cat# SC-204673A), allowing experimental values to be measured in milligrams of catechin per mL of extract (mg CE / mL). Calibration curve values were plotted using linear regression to form a line $y = ax + b$, where

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y indicated an experimental absorbance value, *x* indicated concentration of (+)-Catechin (in milligrams) per milliliter of extract, and *a* and *b* were constants. This model was thus used to determine experimental flavonoid concentrations relative to the calibration curve.

DPPH Antioxidant Capacity Assay

The DPPH• free radical assay was conducted in triplicate using the method described in Xiao et al. 2020 (18). Firstly, DPPH solution was prepared by mixing 7.89 mg of DPPH radicals (Santa Cruz Biotechnology, Cat# SC-202591) with 100 mL ethanol and setting the solution in the dark for 2 hours at room temperature. Following the solution preparation, 1 mL of DPPH solution was mixed with 0.8 mL of Tris-HCl buffer, pH 7.5 (ThermoFisher, Cat# 15567027) in each of 4 test tubes. Following this, 0.2 mL of each coffee bean extract was added to its own test tube and mixed vigorously, then set aside. Following the addition of each sample to DPPH-containing solution, a change in color from deep-purple to pale-yellow was expected to be noticed, in accordance with general literature regarding the color of the diphenylpicrylhydrazine compound (11). For the fourth tube, 0.2 mL of ethanol was added – this was labeled the control. The test tubes were left in the dark at room temperature for 30 minutes, following which absorbance values were measured at 516 nm using UV-Vis spectrophotometry. Measured values were expressed in inhibition percentages, by subtracting the absorbance value of each experimental solution from the absorbance value of the control solution and dividing the result by the control absorbance value.

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