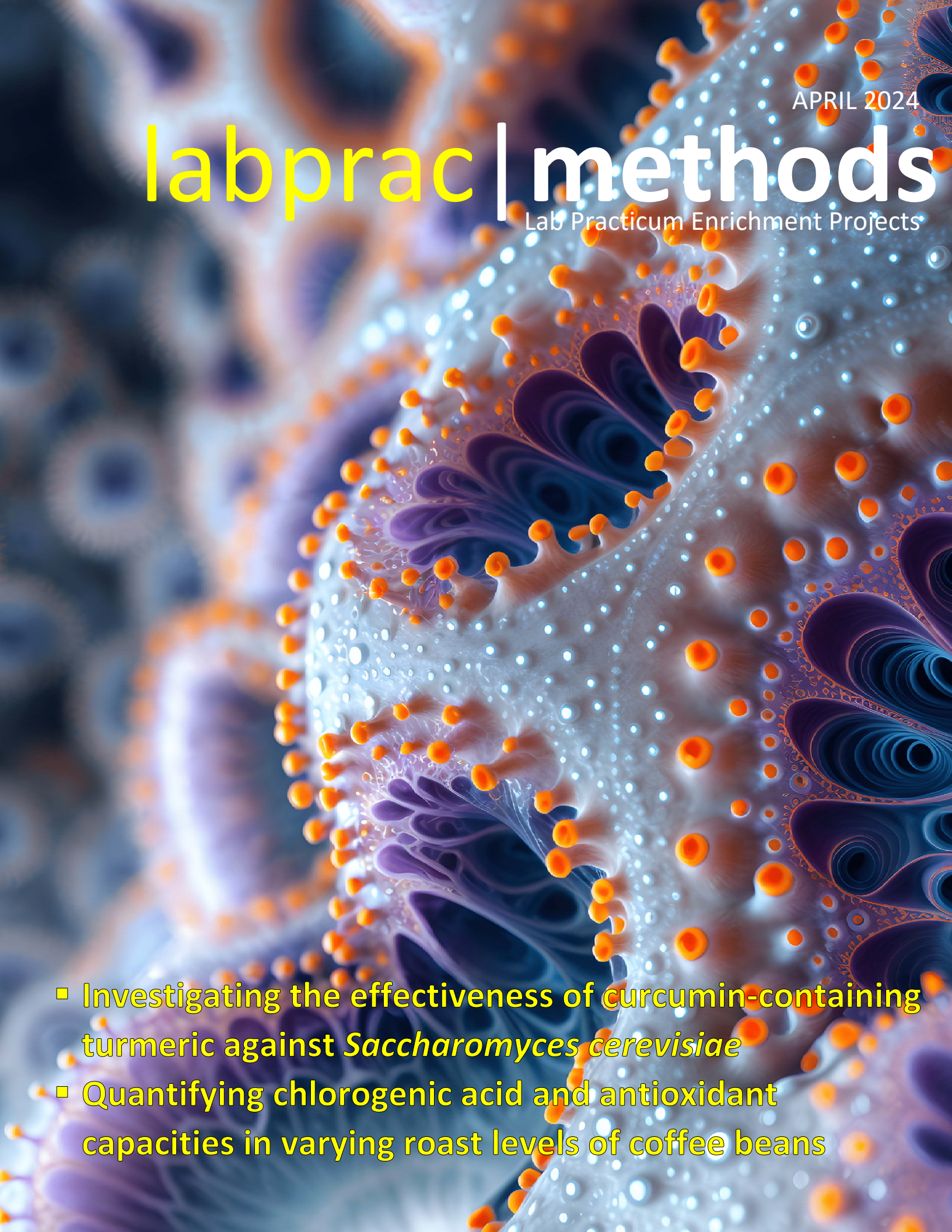

APRIL 2024

labprac | methods

Lab Practicum Enrichment Projects

- Investigating the effectiveness of curcumin-containing turmeric against *Saccharomyces cerevisiae*
- Quantifying chlorogenic acid and antioxidant capacities in varying roast levels of coffee beans

labprac|methods BRIEF COMMUNICATION

Investigating the Effectiveness of Curcumin Against *Saccharomyces cerevisiae* Growth

Bronwen Elkins and Cassidy West

The yeast *Candida albicans* is the cause of the common fungal infection, candidiasis. *C. albicans* is a highly adaptable microorganism that can develop antimicrobial resistance, leading to the investigation of alternative treatment methods. In this study, the efficacy of pure curcumin and curcumin-containing turmeric as antimicrobial agents were assessed against the *C. albicans* analogue, *Saccharomyces cerevisiae*. A study system was created, and data was collected through kinetic assays and the growth on nutrient agar plates. The collected data was analyzed through qualitative and quantitative methods. The pure form of curcumin demonstrated growth inhibition against both non-mature and mature yeast, while the turmeric showed limited growth inhibition.

Antimicrobial resistance (AMR) has been declared one of the top 10 global public health threats by the World Health Organization as it prevents the successful treatment of diseases.[1] AMR arises when microorganisms such as bacteria, viruses, parasites, and fungi grow and adapt after the administration of once-effective antimicrobial agents. Spontaneous evolution, horizontal gene transfer of resistant genes, and bacterial mutations are some of the ways in which AMR may arise.[2] To combat this issue, antimicrobial proteins, probiotics, bacteriophages, and plant-based substances are being studied to determine their efficacy as antimicrobial agents.[3]

As AMR is becoming an increasingly prevalent issue, there is an increased need for alternative treatment methods for common microbial infections, prompting the investigation into natural products. Curcumin, a yellow pigment, is a polyphenol found in the flowering plant turmeric which is believed to have antifungal properties and has demonstrated effectiveness against the causative agent of candidiasis, *Candida albicans*.[4] This study investigated the effectiveness of curcumin in a pure form and in turmeric against *Saccharomyces cerevisiae*, an analogue of *C. albicans*.[4]

Preparation of Yeast Solutions

Two separate concentrations of yeast solutions were prepared and tested. Fleischmann's Quick-Rise Instant Yeast (0.5 g) was added to two 15 mL Falcon tubes, along with either the 6 g/mL or 4 g/mL broth (4 mL). The yeast was dissolved into the broth by mixing with a glass stir rod for 3 minutes with intervals of 30 seconds of stirring followed by 30 seconds of rest. The solution was diluted by filling the tube with the corresponding broth to the 10 mL line. The test tube was placed into the tube rotator to ensure that yeast stayed dissolved in solution until added to a 48-well plate. After mixing, two ten-fold serial dilutions were conducted. All tubes were mixed in the tube rotator until they were plated. A second set of yeast concentrations was created by adding 2.5 g of instant baker's yeast to a 15 mL Falcon tube, followed by the same dilution and storage process.

Nutrient Broth Preparation

A 16 g/L broth was prepared by adding 0.8 g of Difco nutrient broth to a 125 mL Erlenmeyer flask and 50 mL of sterile water. The 4 g/L broth was prepared using an identical method, except only 0.2 g of nutrient broth was placed into the Erlenmeyer flask.

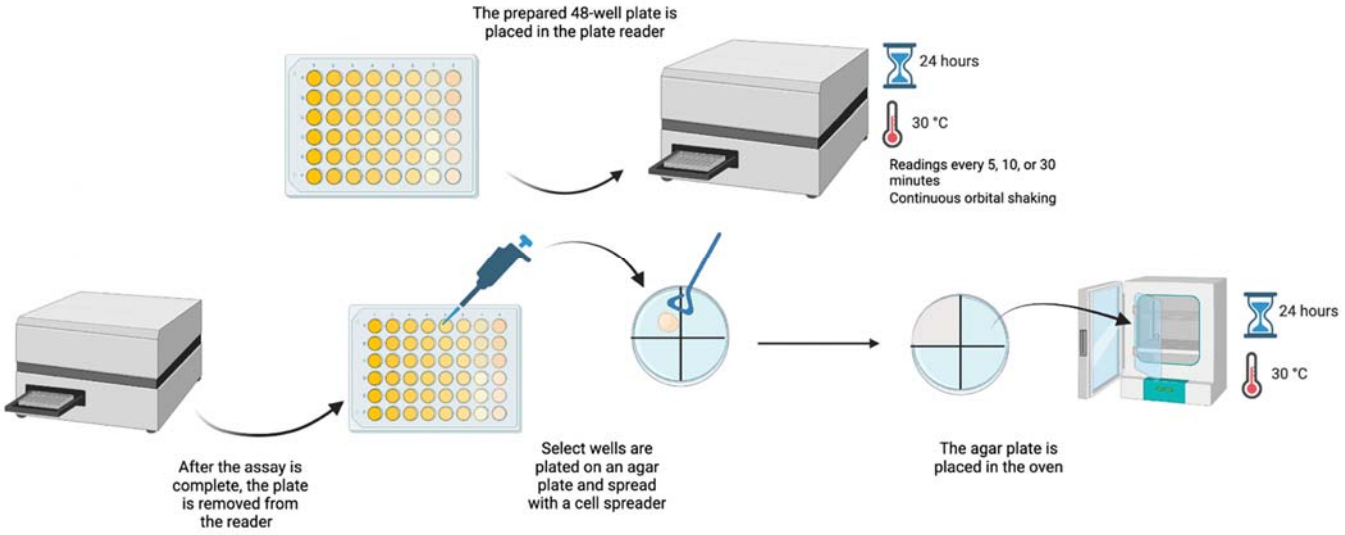

Fig. 1 | A schematic overview of plate reading procedure and the preparation of the agar plates. The 48-well plate is prepared and then placed into the Synergy HTX multi-mode plate reader for 24 hours at 30˚C with continuous orbital shaking and readings being taken automatically every 10 minutes. Once the kinetic assay is complete, the plate is removed from the reader and select wells are placed on a quadrant on separate agar plates and spread using a cell spreader. The agar plates also contain the appropriate controls. These agar plates are then placed in the oven for 24 hours at 30˚C.

Two layers of tinfoil and one piece of tape were placed on the top of the flasks, then the flasks were autoclaved for sterility at 125°C for 90 minutes.

Determination of Optimal Yeast and Broth Solutions

Kinetic assays in 48-well plates were used to determine the ideal growth conditions of yeast. An array of concentrations of yeast and nutrient broth were investigated to determine the optimum growth conditions. The concentrations of broth tested were 4, 8, and 16 g/L, and the concentrations of yeast tested were 0.0005, 0.005, 0.025, 0.05, and 0.25 g/mL. Controls for each broth (1000 µL; 4, 8, 16 g/mL) and yeast (1000 µL; 0.0005, 0.005, 0.025, 0.05, 0.25 g/mL) were included in the study to determine a baseline reading for each component of the assay. The kinetic assay was conducted following the procedure outlined in Figure 1, with readings taken every 30 minutes. This study determined that the 0.0005 g/mL yeast in the 16 g/L broth was optimal and produced the most ideal growth curve as the yeast was able to survive throughout the kinetic trial and produced the smoothest data.

Preparation of Turmeric Solutions

A turmeric solution was created by adding 0.5 g turmeric to a 14 mL Falcon tube and adjusting the final volume to 10 mL with sterile distilled water. The solution was vortexed until fully dissolved. Six 1.5 mL Eppendorf tubes were labelled and 750 µL of sterile water was added to each. A two-fold serial dilution was carried out to obtain solutions from 0.05 g/mL to 0.00078125 g/mL. The pure form of curcumin, procured from Sigma-Aldrich, was prepared in an identical manner with the exceptions of the solvent being DMSO and the starting mass being 0.25 g to create concentrations of 0.025 g/mL, 0.0125 g/mL, 0.003125 g/mL, 0.001563 g/mL, 0.00078125 g/mL, 0.000390625 g/mL.

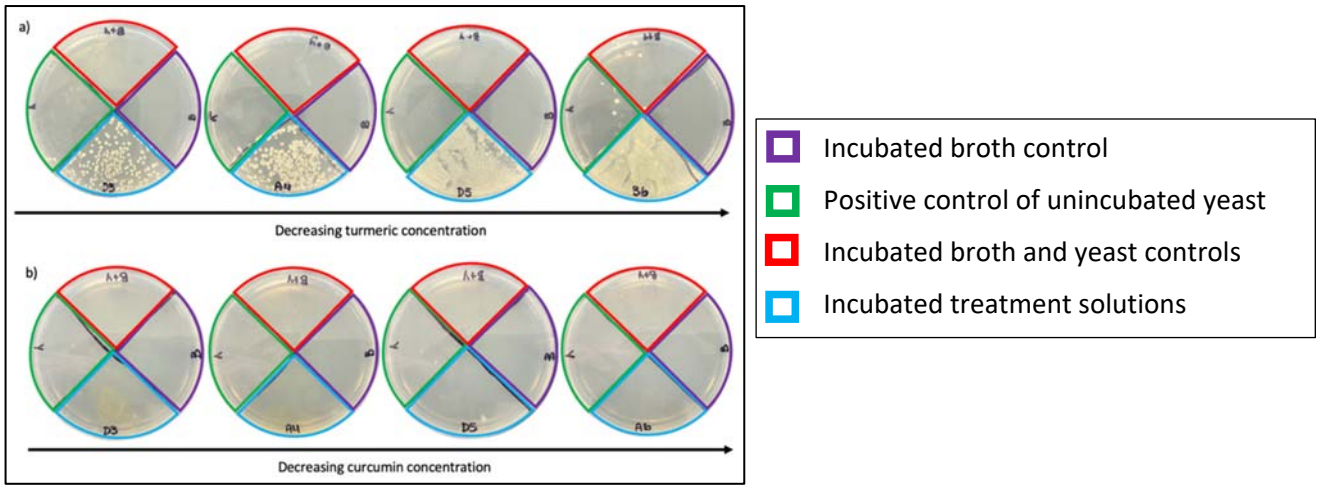

Fig. 2 | Agar plate comparison of (a) turmeric-treated yeast and b. curcumin-treated yeast. (a) Wells of turmeric-treated yeast, with varying concentrations of turmeric, were plated to observe the effects of the turmeric on the growth of yeast after 24 hours of incubation. Concentrations of turmeric are as follows: D3: = 0.00625 g/mL, A4 = 0.003125 g/mL, D5 = 0.0015625 g/mL, and B6 = 0.00078125 g/mL. Though there was an overall increase in growth on the lawns, less growth was observed at the higher concentrations of turmeric. (b) Wells of curcumin-treated yeast, with varying concentrations of curcumin, were plated to observe the effects of the pure curcumin on the growth of yeast after 24 hours of incubation. Concentrations of curcumin were as follows: D3 = 0.00625 g/mL, A4 = 0.003125 g/mL, D5 = 0.0015625 g/mL, and A6 = 0.00078125 g/mL No growth was exhibited on the agar plates, which may be indicative that the curcumin was able to kill the yeast. The lack of growth in the yeast control may be indicative that the yeast did not survive the incubation or kinetic readings.

Plate Reader Protocol

For each plate, the following protocol was used: constant temperature of 30˚C (the ideal incubation temperature for yeast), continuous orbital shaking, and readings taken every 5, 10, or 30 minutes, depending on the trial (Figure 1).[5] All assays were performed with the lid on to avoid any precipitation from heating or any splashing due to shaking, and all readings were taken with a wavelength of 600 nm.

Preparation of 48-Well Treatment Plates

Each well was prepared by adding 225 µL of 16 g/L nutrient broth, 225 µL of 0.0005 g/mL yeast, and 50 µL of treatment, which were various concentrations of turmeric or curcumin solution; each well had a total volume of 500 µL. Control wells included: broth control (500 µL of broth), yeast control (500 µL of yeast), yeast plus broth control (250 µL yeast and 250 µL broth), turmeric control (500 µL of various turmeric concentrations), DMSO control (500 µL DMSO), curcumin in DMSO control (450 µL DMSO and 50 µL of various curcumin concentrations), and broth plus curcumin controls (450 µL broth and 50 µL of various curcumin concentrations). Various controls of turmeric and curcumin were included due to the colour of the test drugs to mitigate this impact on the results.

Preparation of Turmeric and Curcumin Agar Plates

A marker was used to divide and label the underside of each agar plate (Figure 2). The controls were untreated, unincubated yeast (the absolute positive control), incubated broth, and incubated broth and yeast. Treatments were selected based on the growth curves observed from the plate reader. 100 µL of the solutions were removed from the 48-well plate, added to their respective quadrants, and spread onto the agarose gel using a cell spreader. All plates were incubated at 30˚C for 24 hours to qualitatively determine yeast growth and treatment effectiveness.

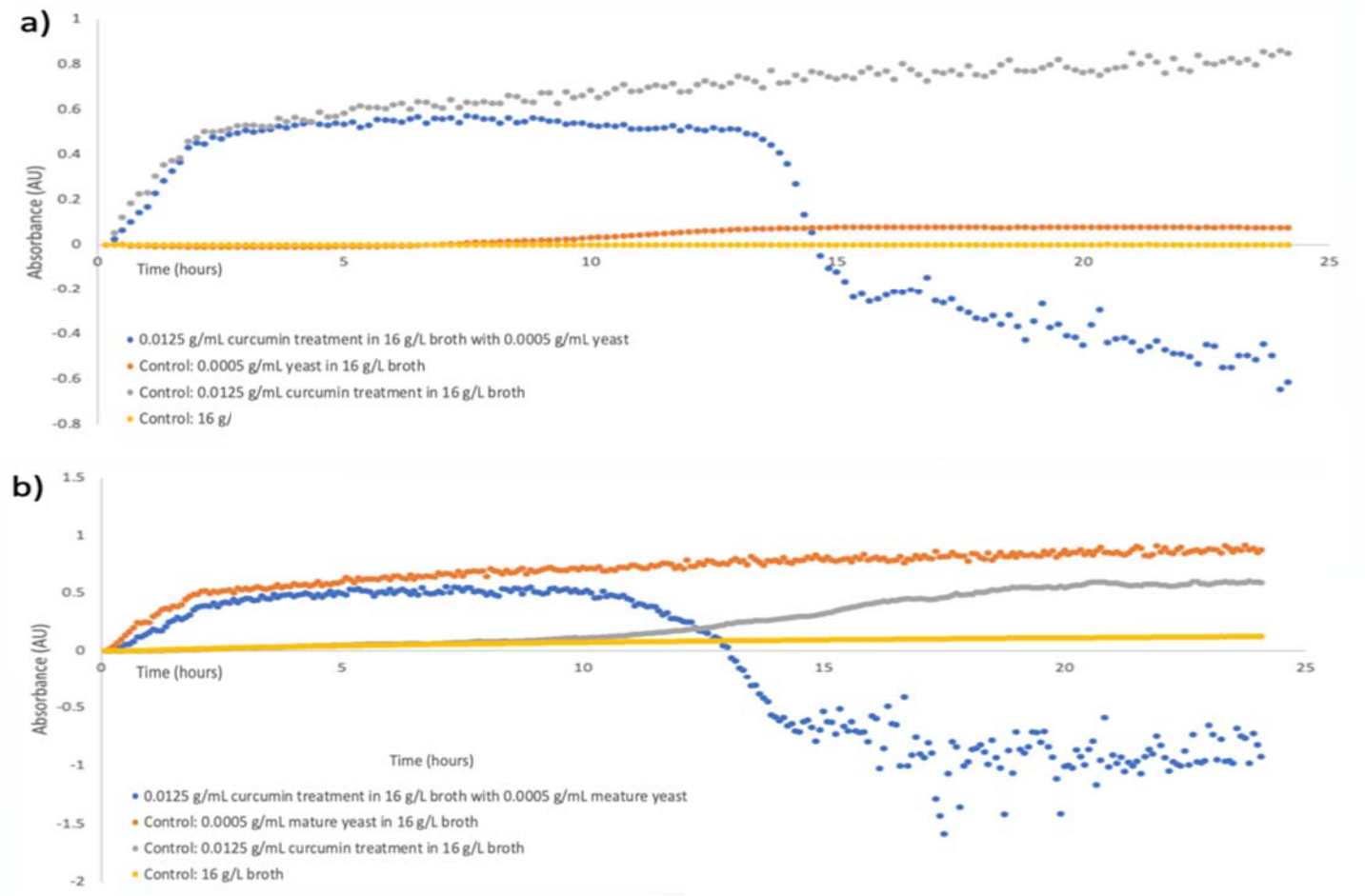

Fig. 3 | Growth curves of non-mature yeast (a) and mature yeast (b) with corresponding controls. The top graph (a) displays the growth curve of 225 µL of non-mature yeast in 225 µL of 16 g/L broth with 50 µL of 0.0125 g/mL treatment of curcumin along with its appropriate controls. The bottom graph (b) displays the same data except the yeast is mature after being incubated for 18 hours prior to plating. To correct for different starting values in absorbance, the first absorbance value of each well from the data set was taken as the baseline and subtracted from every value to produce the graphs.

Treatment of Mature Yeast

The standard yeast solution was prepared in a 125 mL Erlenmeyer flask that was placed in a continuously shaking oven for 18 hours at 30˚C. On the control growth curve, the 18-hour mark coincides with the peak growth, as the absorbance begins to plateau after this time. After 18 hours of growth, the mature yeast was used to create a 48-well treatment plate that underwent the previously defined kinetic protocol in the plate reader for 24 hours, with readings taken every 5 minutes. The reading intervals were reduced to 5 minutes as a sharp drop was observed in initial kinetic readings and additional time points were required to learn more about this event.

Results

After the addition of turmeric, the growth curve of the yeast demonstrated that the yeast grew for approximately 4 hours but led to a plateau. The mature yeast exhibited a growth curve similar to that of the non-mature yeast, and this curve was successfully interrupted by the antimicrobial agent,

curcumin (Figure 3). The incubated solutions were then plated on agar to determine if the yeast was still alive after incubation (Figure 2). The agar plates revealed that turmeric enhanced the growth of yeast compared to the control, but at higher concentrations of turmeric, less growth was observed between treatments. These results suggest that something in turmeric is enhancing yeast growth, and as a result, the pure product curcumin was investigated. In these trials, the growth curve illustrated an initial growth of yeast, but after this period of growth, a steep drop was observed, which was hypothesized to be a result of the curcumin causing the yeast to die (Figure 3). This was confirmed by the samples plated on agar, as there was no observed yeast growth after incubation (Figure 2). Since this differs from the turmeric plates, a possible explanation is that the antimicrobial agent curcumin can inhibit the growth of yeast, but that another agent is present in the turmeric, interacting with the yeast. Although the turmeric did not prevent growth, it was noted that higher concentrations produced less growth than lower concentrations, possibly indicating some correlation between the amount of curcumin present in turmeric and yeast growth. Curcumin also displayed effectiveness against mature yeast as the addition of this agent after 18 hours of incubation produced a similar drop in absorbance to that of non-mature yeast. While the concentrations of curcumin were known, it is important to note that the concentration of curcumin in the turmeric trials was unknown, as well as whether the pure levels that were tested were similar.

Conclusion

Using this preliminary research as a basis, additional studies may be conducted to further the application of curcumin-containing turmeric as an antimicrobial agent. The efficacy of turmeric as the primary source of curcumin should be studied, as the active ingredient demonstrates effectiveness against yeast, with absorbance changes of up to 2.0 Au. Future studies should be conducted to investigate a possible broth-curcumin chemical interaction, as well as increase the biological relevance of the system through the implementation of *C. albicans*.

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Acknowledgements

We would like to thank Mr. Russ Ellis, supervisor and lab coordinator of the Integrated Science Program, for his continuous support, guidance, insight, and use of lab space and materials over the course of this project. We would also like to acknowledge the Integrated Science Program and McMaster University for providing us with the opportunity to complete this project as well as the required space and equipment.

labprac|methods BRIEF COMMUNICATION

Quantifying Chlorogenic Acid and Antioxidant Capacities in Varying Roast Levels of Coffee Beans

Maya Mahmood and Priya Rai

Antioxidants neutralize free radicals linked to various health conditions. Antioxidant properties of coffee are derived from polyphenolic compounds, including chlorogenic acid (CGA), which are all altered by coffee roasting. Coffee extracts, using light to dark bean coffee extracts, were prepared through a multistep ultrasonic extraction. HPLC-UV was used to quantify CGA levels, and a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed to evaluate free-radical scavenging potential. It was observed that, for some extracts, increasing roast levels decreased CGA levels and free-radical scavenging capacities.

A balance between free radicals and antioxidants is crucial for maintaining good health.[1] Excessive free radicals in the body lead to oxidative stress, causing chronic health conditions including cardiovascular diseases, cancer, and inflammation. Antioxidants reduce oxidative stress by stabilizing free radicals.[1] Thus, by investigating the antioxidant compounds found within common dietary sources, free radical-induced diseases can be better managed.

The antioxidant properties of coffee are derived from polyphenolic compounds, including chlorogenic acid (CGA), a major polyphenol found in high concentrations.[2,3] CGA levels can be lowered by coffee roasting, which is known to create varying flavour and aroma profiles by altering the chemical composition of coffee.[4,5] This study examined the effect of varying roast levels on CGA content and free-radical scavenging capacities of coffee beans.

Methods. Coffee beans of varying roast levels were locally sourced, with Colombian-supremo (CS) as light roast, decaffeinated (D) as medium roast, and both French roast (FR) and Colombian-dark (CD) as very dark roast. Following established methods from Rostagno, M. A. *et al.*, these four coffee bean samples were processed through a multistep ultrasonic extraction utilizing increasing methanol concentrations.[6]

Of each bean type, 1.0g was ground to an average grain size of 1.0mm using a mortar and pestle. For the three extractions, 1.0g of each sample was combined with 15mL of 50% methanol, followed by sonication for 20 minutes at 60°C and centrifugation for 10 minutes at 10°C and 4000 rpm. Sequential extractions were similarly done with 75% and 100% methanol. All samples were then filtered through a 0.2µm nylon syringe filter.

CGA levels were quantified using high-performance liquid chromatography (HPLC) coupled with UV detection. The HPLC setup followed the procedure in Król *et al.*, with modifications.[2] The gradient mobile phase contained 10% (phase A) and 55% (phase B) acetonitrile and HPLC-grade water. Phases were pre-mixed, and their pH was adjusted to obtain a stable value of 3. The flow rate was set to 1.0mL min^{-1} with a time program of 1.00–10.00 min, phase A 95% and 5% phase B, and a detection wavelength of 370nm.

A CGA Standard of 1016µM was prepared volumetrically with HPLC-grade water, then diluted to create standard solutions between 847µM and 169µM. 20µL of each standard was injected into the

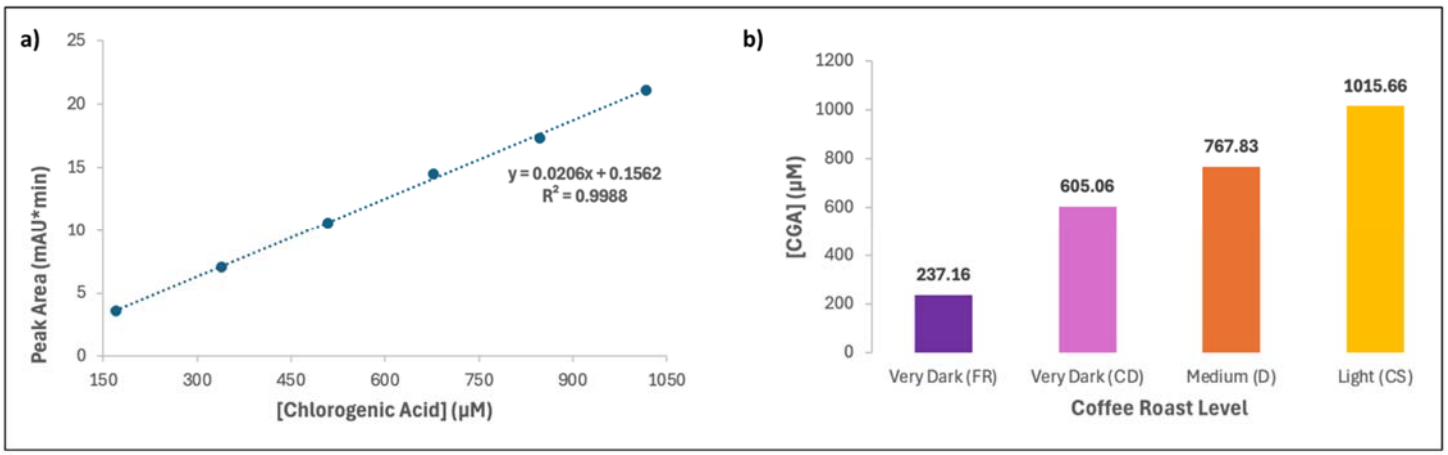

Fig. 1 | Calibration and results from chlorogenic acid quantification. a. External calibration curve of HPLC peak area against concentration of chlorogenic acid standards. The R^2 value calculated was 0.9988 and the coefficients of the slope and y-intercept were 0.0206 and 0.1562, respectively. **b.** Comparison of chlorogenic acid concentrations (µM) in four coffee samples with decreasing roast levels.

sample loop to obtain peak area values, and a calibration curve was created by plotting these values against the CGA standard concentrations (Figure 1a). For each coffee sample, a 20µL injection was analyzed, with unknown CGA concentrations determined from peak area values.

A DPPH assay was conducted in 96-well plates for each coffee type and extract level (Figure 2a). Based on methods by Alnsour *et al*., the methanolic DPPH solution was diluted from 2mM stock to a working concentration 200µM. [4]

A calibration curve of ascorbic acid standards ranging from 0.08mM to 0.2mM were prepared in increments of 0.02mM. This was used to convert absorbance values to ascorbic acid equivalent (AAE) units which are universally translatable as they can be converted and expressed in terms of other standards, such as Trolox.[7]

For the DPPH assay, sample dilutions of 1/25, 1/50, and 1/100 were created in methanol to reduce background noise arising from the natural brown coffee colour. To test the antioxidant capacities, each sample well contained 50µL of coffee extract followed by the addition of 150µL of DPPH. Triplicate preparations of each coffee type and extract at three dilution levels were performed (Figure 2a).

The background absorbance of coffee was measured by replacing DPPH content with an equivalent amount of methanol. A typical plate setup is shown in Figure 2a, with sample, standard, and background measurements, as well as DPPH and methanol controls.

Using a Synergy HTX multi-mode plate reader, DPPH plates were shaken and incubated for 16:00 minutes at 37°C, followed by a single absorbance measurement at 517nm.

Results. CGA standards from HPLC exhibited a consistent peak at an average retention time of 4.95 minutes. a linear calibration curve, allowed for accurate quantification of CGA levels in coffee samples (Figure 1a).

For each coffee type, the CGA concentrations of individual extracts were added together to determine the overall concentrations, and ranged from 237µM to 1016µM, with an increasing trend for decreasing roast levels (Figure 1b). It was found that the light roast coffee (CS) had a CGA concentration 328% greater than the very dark roast coffee (FR), 68% greater than the second very dark roast coffee

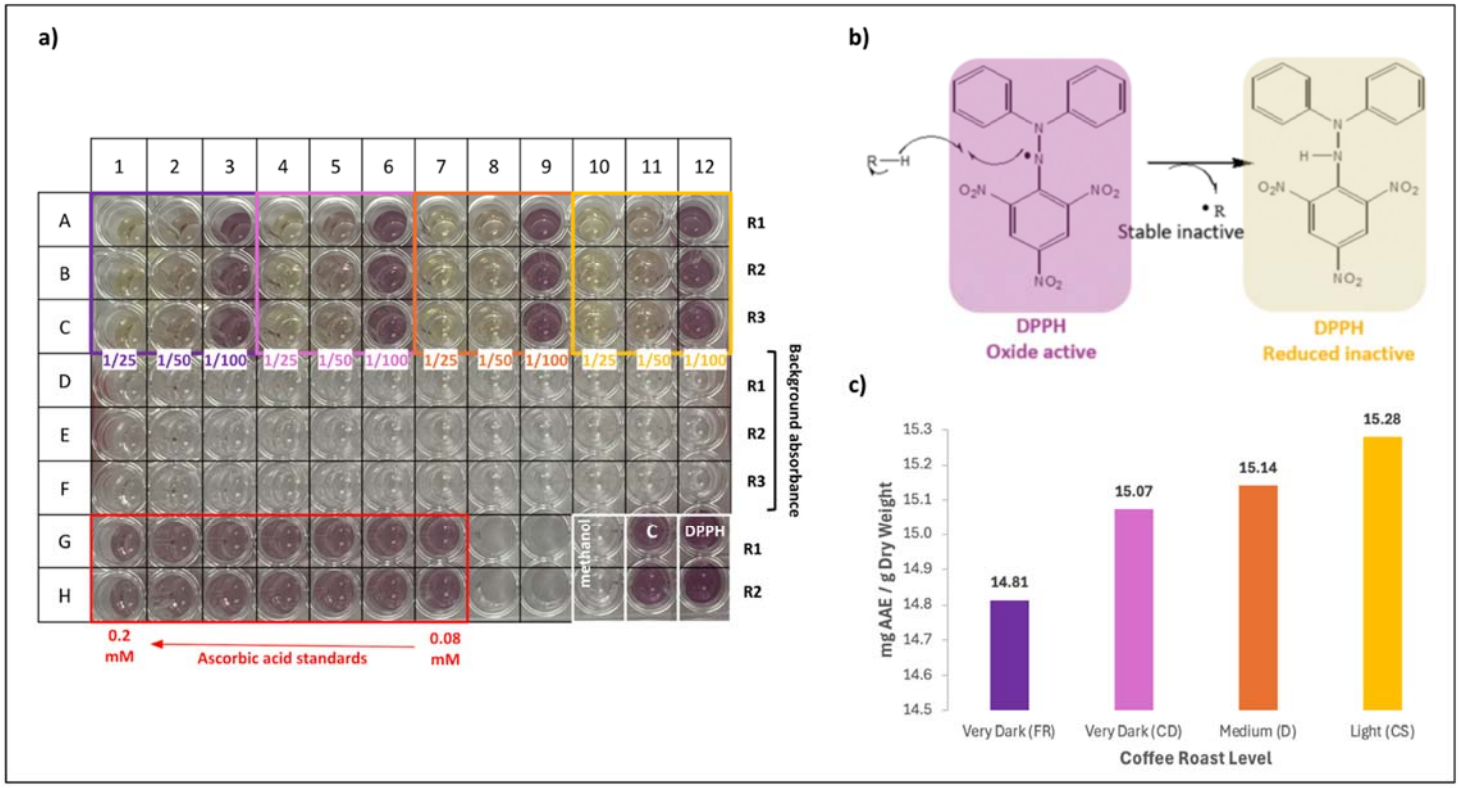

Fig. 2 | DPPH assay schematics and results. a. Annotated photograph of a DPPH plate after absorbance measurement. Visual results from each coffee sample are shown in purple (FR), pink (CD), orange (D), and yellow (CS). Each sample was tested at three dilutions (1/25, 1/50, 1/100) in triplicate (R1-R3) and compared against a background absorbance without DPPH. These wells contained 50µL of sample and 150µL of either DPPH or methanol. Ascorbic acid standards for result calibration are highlighted in red. Control with 50µL methanol and 150µL DPPH (C) along with methanol and DPPH separately are shown in white. **b.** Diagram of DPPH reaction. Dark purple indicates an unreacted DPPH radical which gradually progresses to a light-yellow colour indicating a reduced DPPH radical.[8] **c.** Comparison of antioxidant capacities (mg AAE/g dry weight) in four coffee samples with decreasing roast levels.

(CD), and 32% greater than the medium roast (D). Although there was variation within the very dark roasting category, ultimately, lighter roasts had a higher CGA concentration than darker roasts.

The DPPH assay displayed scavenging potential of samples through a dark-purple to light-yellow colour change as the reaction progressed (Figure 2a, b). Based on the absorbance of ascorbic acid standards at 517nm, a linear calibration curve (R^2=0.9986) of the form y=mx+b was constructed, where y is absorbance and x is the concentration of ascorbic acid (mg/L).

Absorbance measurements from each coffee sample were converted to units of mg AAE/g dry weight of coffee beans. Dry weight was calculated based on the mass of coffee in each 200µL well from an initial weight of 1.0g dissolved in 30mL of methanol from the first two extractions. The first two extracts were combined due to similar absorbance results, while the third extraction was not considered as values fell below detection limits. Additionally, results were taken from the 1/25 dilution of each coffee type as it had minimal background absorbance. At a 1/10 dilution, background absorbance was too large creating noise, while dilutions of 1/50, 1/100, and

1/500 were too diluted causing lost signal. For the four coffee types, antioxidant capacity ranged between 14.81mg AAE/g and 15.28mg AAE/g with lighter roasts being higher than darker roasts (Figure 2c).

Ultimately, a comparison of antioxidant capacity and CGA concentrations of each coffee bean type revealed that, within the parameters of this study, lower roasting levels result in higher CGA concentrations which may be further correlated with an increase in antioxidant capacity. Understanding that lighter coffee roasts may have greater antioxidant potential can then guide consumers to favour antioxidant-rich coffees – in turn, optimizing health outcomes.

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Acknowledgements

We would like to thank our Integrated Science Laboratory Coordinator, Russ Ellis, for supervising our project and providing us with the Integrated Science lab space and materials. We also thank Trisha Martin, Undergraduate Lab Technician, for allowing us to use the HPLC machine and guiding us through the process. Our learning and success can be attributed to their kind encouragement and assistance throughout the project. As well, we are thankful for the Integrated Science Coordinator's fund for covering the costs of our materials.

Editor: Russ Ellis

Created: January 2025

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