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Emily Edwards April 12, 2022

People, plants, and prescriptions: Effects of herbal supplements on pharmaceutical drug metabolism

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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By Emily Edwards

Many people rely on both herbal dietary supplements, which are a form of traditional medicine, and pharmaceutical drugs, which are a form of allopathic medicine, when treating illnesses. In the United States, 18-30% of individuals consume herbal dietary supplements (Clarke et al., 2015). However, there is a need for more research on the interactions between herbal dietary supplements and pharmaceutical drugs. The objective of this study is to identify an herbal dietary supplement that could interfere with drug metabolism and to determine how the use of this herb in traditional medicine could impact the efficacy of pharmaceutical drugs. This project will highlight the importance of considering cultural practices and the uses of traditional medicine when treating patients with allopathic medicine.

The project included a high-throughput screening for cytochrome P450 (P450) inhibition by 20 species commonly used as herbal supplements. Following the initial screening, two species of cinnamon were identified as having a strong inhibitory effect on at least one drug metabolizing P450. A concentration-response curve was completed for these *Cinnamomum* spp. against P450 isoenzyme CYP2C9. Half-maximal inhibitory concentrations (IC_{50}) values of CYP2C9 were calculated for both *Cinnamomum verum* and *Cinnamomum burmannii. C. burmannii* bark extracted in 80% ethanol showed the greatest inhibition of CYP2C9.

This study aims to reveal more about how dietary supplements interact with pharmaceutical drugs. This knowledge will benefit both traditional medicine users and physicians who prescribe pharmaceutical drugs. As a result, this project will improve the safety and efficacy of pharmaceuticals by increasing our understanding of herb-drug interactions.

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Chapter 1: Introduction

Plants as medicine: Complementary medicine use in the United States

The relationship between humans and plants has been critical to human survival. Throughout history, humans have relied on plants to provide sources of food, clothing, shelter, and more. Humans have learned how to utilize both the physical and chemical properties of plants to meet our daily needs.

Ethnobotany is a subfield of anthropology focused on the interactions between humans and plants (Quave & Pieroni, 2015). One important relationship between humans and plants is the use of plants as medicine. Humans have relied on plants as medicine for thousands of years, with historical texts such as the Ebers Papyrus documenting medicinal plant use as early as 1500_{BCE} (Quave, 2020). Today, over four billion people in developing countries rely on traditional, plant-based medicine as their primary form of healthcare (Ekor, 2014). Additionally, allopathic medicine, also known as Western medicine, relies on plants for the development of many pharmaceutical drugs (Houghton, 2001). Plants remain a critical part of healthcare around the world (WHO, 2019).

Herbal supplements are a common form of plants used as medicine. The National Center for Complementary and Integrative Health (NCCIH, 2020) defines herbal supplements as "a type of dietary supplement containing one or more herbs." Herbal supplements are taken both to promote health and to treat illnesses (Thakkar et al., 2020). They come in many forms, including liquid extracts, tablets, capsules, and powders, and they contain plant parts ranging from roots and seeds to berries, leaves, and flowers (Thakkar et al., 2020). In the United States, it is estimated that over a third of adults take herbal supplements (Council for Responsible Nutrition, 2019). Herbal supplement usage in the United States has been steadily increasing since 2003

(Tyler Smith, Majid, Eckl, & Morton Reynolds, 2021). In the wake of the COVID-19 pandemic, there was a 17.3% increase in herbal supplement sales in the United States (Tyler Smith et al., 2021). Many people use herbal supplements because they feel like they are a safer or more natural form of medicine (T Smith, May, Eckl, & Reynolds, 2020).

In 2020, cinnamon was a popular herbal dietary supplement used in the United States (Tyler Smith et al., 2021). Cinnamon is a spice derived from the bark of small evergreen trees in the genus *Cinnamomum* of the Lauraceae family. Two species of cinnamon, *Cinnamomum burmannii* and *Cinnamomum verum*, known as Indonesian cinnamon and true cinnamon, respectively, are commonly used as both a seasoning in food and as herbal dietary supplements. Cinnamon is used as a supplement to promote blood sugar metabolism, lower cholesterol, and improve heart health (Maierean et al., 2017). In 2020, over $12 million was spent on cinnamon supplements in the United States (Tyler Smith et al., 2021).

Many people also combine supplement use with pharmaceutical drugs. This is a form of complementary medicine, which is when traditional medicine is combined with allopathic medicine (NCCIH, 2022). Since herbal supplements are not perceived as the same as allopathic medicine, many people fail to report their supplement usage to their doctor or pharmacist (Mehta, Gardiner, Phillips, & McCarthy, 2008). This could lead to potentially harmful herb-drug interactions.

Herb-drug interactions: Drug metabolism

One harmful herb-drug interaction occurs when herbal supplements interfere with drug metabolism. Cytochrome P450 enzymes (P450s) are a class of liver enzymes responsible for the majority of pharmaceutical drug metabolism. When developing drugs, the FDA recommends

screening P450s for drug interactions. Identifying drug interactions with P450s is an important part of pharmaceutical drug development but is not required for herbal supplements (Food and Drug Administration, 2020)(Cohen, 2003). Since there are few regulations on testing herbal supplements for efficacy before they are sold, there is little information available on how herbal supplements interfere with metabolic pathways. It is known, however, that some supplements can have an inhibitory effect on P450s. For example, grapefruit juice is known to inhibit the effects of P450 isoenzyme 3A4, which is responsible for over a third of drug metabolism (Lown et al., 1997). This inhibition can be dangerous, since drugs can build up to a potentially toxic concentration in the bloodstream if they are not properly metabolized. P450 inhibition is important to understand to improve the safety of all medications, both traditional and allopathic.

Project overview

Twenty of the top 40 herbal supplements sold in the United States were analyzed for inhibition against P450s. Following an initial screening of each herbal supplement at a concentration of 8 μg/mL, further concentration-response screenings were completed with two species of cinnamon, *Cinnamomum burmannii* and *C. verum*. With the widespread usage of herbal supplements in combination with pharmaceutical drugs in the United States, it is critical to identify herbal supplements with inhibitory properties for P450s. By deepening our understanding of the ways that people use plants, we can increase the safety and efficacy of both traditional and allopathic medicine. Additionally, an increased understanding of how plants interact with human physiology can pave the way for future drug development and discovery.

Chapter 2: Literature Review and Background Information

Herbal supplement use in the United States

Herbal supplement use in the United States has been increasing since 2003, with over a third of US adults using at least one herbal supplement in the last year (Council for Responsible Nutrition, 2019). In 2020, herbal supplement usage increased by 17.3% (Tyler Smith et al., 2021). This is likely in response to the COVID-19 pandemic. Supplements taken to promote immune health and reduce stress had particularly large increases in sales from 2019 to 2020 (Tyler Smith et al., 2021). For example, elderberry—a supplement taken to improve immune function—increased in sales by 150% from 2019 (Tyler Smith et al., 2021).

Sales increased of both single-herb supplements and combination formulas. A combination formula is defined as a formula that "contain[s] multiple herbs that typically work together (additively or synergistically), often to support a general health function or body system" (Tyler Smith et al., 2021). Although there were more overall sales of single-herb supplements, there has been a greater increase in sales of combination formula herbs over the last several years. Most Americans report taking supplements for "overall health and wellness benefits," rather than for one specific desired outcome (Council for Responsible Nutrition, 2019). This suggests a shift from a more traditional "magic bullet" approach to healthcare, where a single drug is taken in order to target a specific health problem, to a more holistic approach to healthcare (Fiandaca et al., 2017; Strebhardt & Ullrich, 2008). This shift is consistent with the increase in complementary and alternative medicine use throughout the United States.

An investigation into *Cinnamomum* spp.

Cinnamon is one of the top 40 most commonly used herbal supplements in the United States (Tyler Smith et al., 2021). According to the United Nations Comtrade database, 31,814,027 kg of cinnamon was imported to the United States in 2021, increasing from 30,629,360 kg imported the year before. Cinnamon is a spice used throughout the world as a single-herb supplement, a combination supplement, and a functional food.

Cinnamon is the common name for several species from the genus *Cinnamomum*, which are a part of the Lauraceae family (USDA, NRCS 2022). Two *Cinnamomum* species of interest were reviewed for this study: *Cinnamomum burmannii* (Nees & Th. Nees) Nees ex Blume, commonly known as "Indonesian cinnamon," and *Cinnamomum verum* J. Presl, commonly known as "true cinnamon" or "Sri Lankan cinnamon" (USDA, NRCS 2022) (Chen, Sun, & Ford, 2014). *C. burmannii* and *C. verum* are both evergreen trees with oval leaves and small yellow flowers that produce a small, dark purple drupe fruit (Pathak, 2021). Both species grow to approximately 6-8 meters high. Cinnamon trees grow best in wet, tropical climates. *C. burmannii* is native to Southeast Asia, China, and the Philippians (Chen, Sun, & Ford, 2014). *C. verum* is native to Sri Lanka and Southern India (Pathak, 2021). Compared to *C. burmannii*, *C. verum* has a more mild taste and is considered a higher quality spice. *C. verum* is used more frequently as a supplement, while *C. burmannii* is often used commercially, as it is a cheaper spice to produce (Muhammad & Dewettinck, 2017).

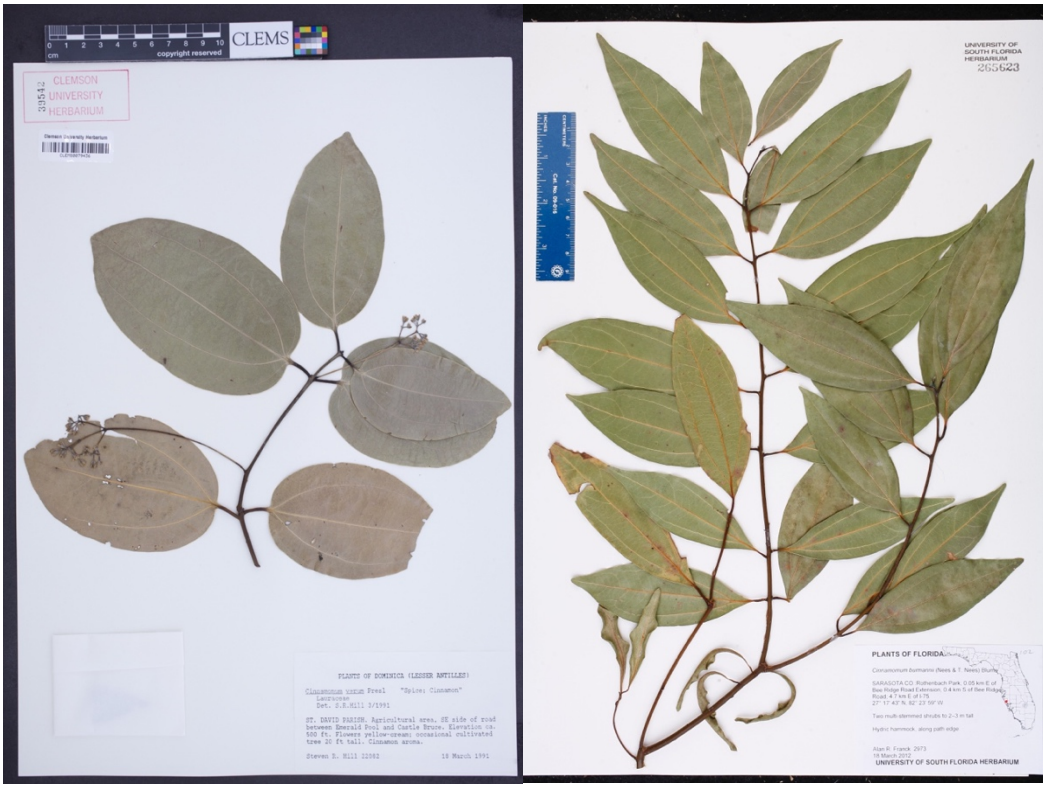


Figure 2.1 Herbarium voucher specimens of *Cinnamomum verum* (left) and *Cinnamomum burmannii* (right). Sources: Clemson University Herbarium and University of South Florida Herbarium.

Cinnamon has been used traditionally by many cultures in a variety of ways ranging from food preparation to religious rituals. During the 16th and 17th century, international cinnamon trade began when Portuguese traders brought cinnamon to Europe. Cinnamon was an important export by the East India Company in the 17th century, and continues to be a top export from China, Sri Lanka, and Indonesia (Figure 2.2). In ancient Egypt, cinnamon was used for embalming practices (Pathak, 2021). It has been used in traditional Chinese medicine to treat fever and diarrhea ((Heinrich, Williamson, Gibbons, Barnes, & Prieto-Garcia, 2017). Single-herb cinnamon supplements are marketed as promoting blood sugar metabolism, lowering cholesterol, and improving heart health (Maierean et al., 2017). Cinnamon supplements are also commonly

prepared as teas in combination supplements marketed to promote weight loss (Yazdanpanah, Azadi-Yazdi, Hooshmandi, Ramezani-Jolfaie, & Salehi-Abargouei, 2020).

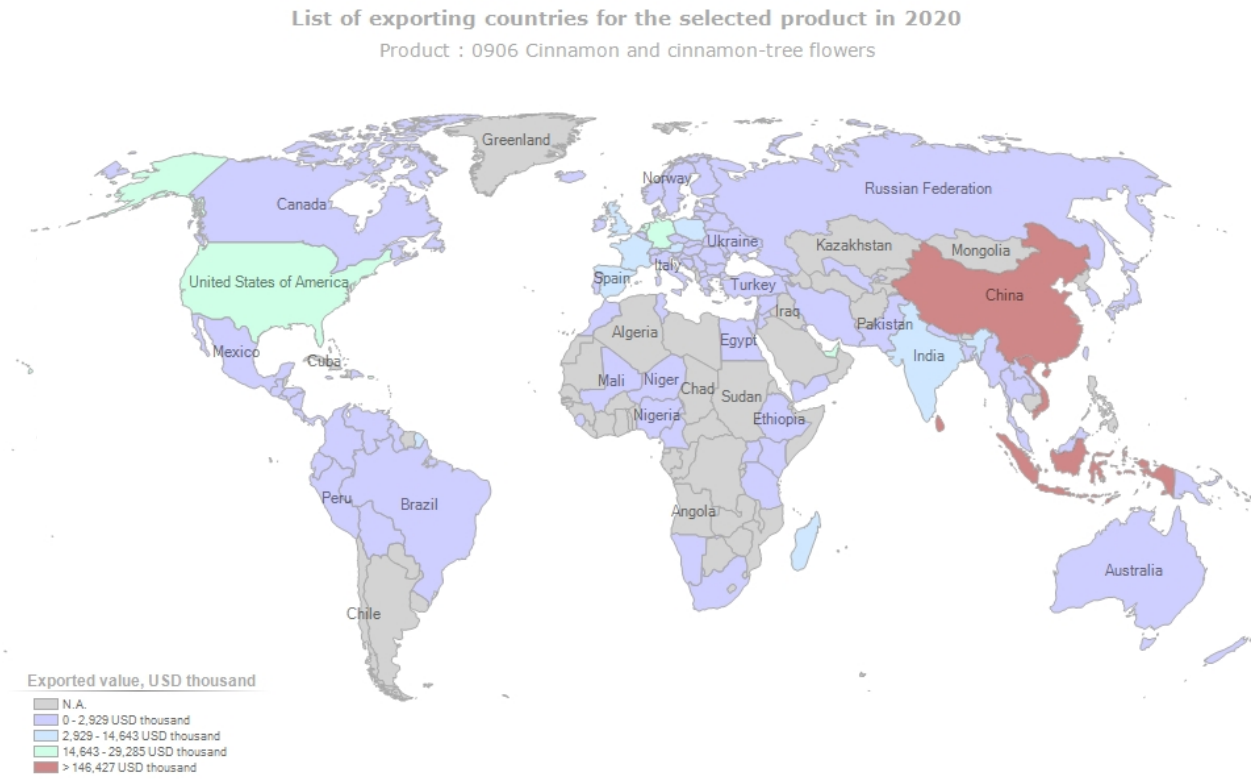


Figure 2.2 Map of global exports of cinnamon species. Source: Trade Map, International Trade Centre, www.trademap.org

As both a spice and a supplement, cinnamon is most commonly prepared as extractions from the inner bark of *Cinnamomum* trees. Cinnamaldehyde is the most common compound found in the bark of *Cinnamomum* spp., making up approximately 65-80% of its chemical constituents(Rao & Gan, 2014). Cinnamaldehyde is responsible for the fragrance and taste of cinnamon. Cinnamaldehyde is reported to have antioxidant activity, and derivatives of cinnamaldehyde have been found to reduce tumor growth (Rao & Gan, 2014). Eugenol is the second most common chemical constituent of *Cinnamomum* bark, making up approximately 5-10% of its chemical constituents (Rao & Gan, 2014). Eugenol also has reports of antioxidant

activity (Rao & Gan, 2014). *C. cassia* bark oils contain 80.40–88.50% cinnamaldehyde, and 0.03–1.08% eugenol compounds (Zachariah, 2006). *C. burmannii* bark contains approximately 77.06-84.71% cinnamaldehyde (Fajar, 2019). *C. verum* bark contains approximately 77.1 % cinnamaldehyde and 3.0% eugenol compounds. Beyond species differences, individual members of each species have varying concentrations of compounds in their barks (Jayaprakasha, 2011). Factors such as age of the tree and location can impact chemical composition (Fajar, 2019).

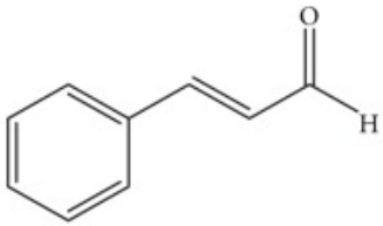
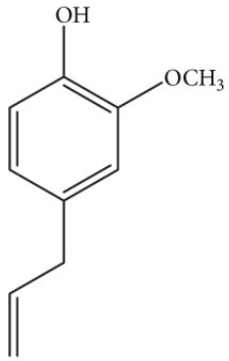
Chemical Structure	Compound name
	Cinnamaldehyde
	Eugenol

Figure 2.3 Common chemical structures found in *Cinnamomum* spp. bark. Source: Rao & Gan, 2014.

Figure 2.4 Herbarium voucher specimens of *Cinnamomum verum* bark. Source: New York Botanical Garden.

Function of Cytochrome P450 enzymes

P450s are a major class of enzymes. They increase the rate of reaction of many catabolic pathways, in which large molecules are broken down into smaller molecules to be utilized by the body. These enzymes increase the rate of reaction by oxidizing the substrate and forming more polar, and therefore more reactive, intermediates. First, the P450 forms a binding complex with

the substrate. Next, NADPH donates electrons to NADPH-cytochrome P450 reductase, and this enzyme transfers electrons to the P450 complex, which is reduced. Then, molecular oxygen binds to the P450, oxidizing it. Another NADPH by donates electrons to the NADPH-cytochrome P450 reductase, which then reduces the P450 complex again. This results in an oxygen becoming highly reactive. This oxygen oxidizes the substrate, and finally the oxidized substrate is released from the P450 (Murray & Reidy, 1990).

Beyond understanding the way that P450s function, it is also important to understand mechanisms that prevent the proper functioning of P450s. There are several ways that P450 function is inhibited. Competitive inhibition, also known as reversible inhibition, occurs when more than one substrate is competing for the binding site of the P450 (Murray & Reidy, 1990). These interactions are temporary, because once one substrate is released from the P450, the binding site is free to interact with the other substrates.

Other methods of inhibition cause more permanent changes to P450 function. One is known as “metabolite intermediate complexation.” This occurs when a substrate is converted by the P450 into an intermediate metabolite that is strongly attracted to the P450. The intermediate metabolite forms a stable complex with the P450. As a result, that P450 is no longer able to bind to other substrates (Murray & Reidy, 1990). Another long-lasting mechanism of inhibition occurs when a metabolite inactivates a P450 by denaturing the cytochrome (Murray & Reidy, 1990). This destroys the active site of the P450 and prevents substrate from binding.

Inhibition could also be caused through interference with cofactors necessary for P450 function. NADPH is an important reducing agent that donates electrons to the P450 reaction cycle. If a substrate interacts with the NADPH, it could decrease the availability of the NADPH necessary for the catalytic reaction to occur (Murray & Reidy, 1990).

There are 57 types of P450s in the human body, most of which are found in the liver. P450 expression in humans is not static and can vary within individuals and populations. Factors that affect P450 expression include, but are not limited to, sex, age, and illness.

P450s are responsible for a majority of pharmaceutical drug metabolism. Eight P450s metabolize >95% of drugs: CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5.

CYP2C9 is responsible for the metabolism of about 20% of pharmaceutical drugs. (Van Booven et al., 2010). It is primarily found in the liver, and has the second highest expression of all P450s, following 3A4. Drugs metabolized by 2C9 include sulfonylureas, which are drugs used to treat diabetes, non-steroidal anti-inflammatory drugs (NSAIDs), which are used to reduce inflammation and treat pain, and anticoagulants. Sulfaphenazole, an antibacterial drug, is a known 2C9 inhibitor and is known to cause interference with metabolism of other pharmaceutical drugs (Van Booven et al., 2010).

Herb-drug safety

Overall, public perception of herbal supplements is that they are safe because they are natural (Heinrich et al., 2017). Many people fail to report their herbal supplement use to their physicians (Kennedy, 2005). Herbal supplements are often used by more vulnerable populations, such as children and the elderly, because of their perceived safety (Heinrich et al., 2017).

The FDA requires that all herbal supplements are labeled as supplements but does not require proof of efficacy related to the claimed health benefits. Herbal supplements cannot be marketed to treat or cure a disease. Herbal supplements are not required to be proven safe before they are marketed, but the FDA regulates safety of products once they are on the market.

Since P450s metabolize so many pharmaceutical drugs, the inhibition of P450s can prevent proper drug metabolism. This can result in the build-up of drug in the bloodstream to a toxic level. P450 inhibition is a known danger, and pharmaceutical drugs must be tested for inhibition against seven P450s during preliminary testing (FDA 2020). However, under current FDA regulations, herbal supplements are not required to undergo this same safety screening.

There is known P450 inhibition by cinnamon extracts. One common form of cinnamon, *Cinnamomum cassia*, commonly known as Chinese cinnamon, is a known inhibitor of 3A4 and 2C9 enzymes (Kimura, Ito, & Hatano, 2010). *C. burmannii* has reported inhibition of 3A4 and 2D6 enzymes (Usia, Iwata, Kadota, & Tezuka, 2006).

Chapter 3: Materials and Methods

Experimental overview

Experiments were conducted utilizing the three specialty areas of the Quave research group: the Emory University Herbarium, the phytochemistry lab, and the microbiology lab. First, herbal supplements and plant parts were collected and authenticated via the Emory University Herbarium. Next, test compounds of herbal supplements were prepared via organic and aqueous extraction in the phytochemistry lab. Then, a high-throughput screening assay was used to test herbal supplements for inhibition of three common P450s: CYP2C8, CYP2C9, CYP2C19, CYP3A4, and CYP3A5. Following the initial screening, assays were conducted to determine the half-maximal inhibitory concentration (IC_{50}) values of *Cinnamomum burmannii* and *Cinnamomum verum* extracts for CYP2C9.

Materials

Herbal supplement materials

Plant materials of the herbal supplements of interest were obtained from the Quave Natural Product Library (QNPL) for testing. All source plants were authenticated and deposited with herbaria stored in the Emory University Herbarium. Retention vouchers were also created of all materials before and after processing by grinding in a Wiley mill. Plants purchased from Mountain Rose were chemically authenticated via High Performance Thin Layer Chromatography (HPTLC).

Test extract preparation

The test extracts were prepared via decoction, maceration, and sonication. Decoctions, which are extractions prepared in boiling solvent, were performed by soaking dried plant material in boiling water for 20 minutes (Salam et al., 2019). Maceration, which are extractions prepared by soaking plant material in liquid, were performed in a closed container at room temperature using 80% ethanol solvent for 72 hours and repeated 3 times (Salam et al., 2019). Sonication, also known as ultrasound-assisted extraction, uses ultrasonic waves to speed up extraction by creating currents that increase contact between solvent and plant materials (Herrero, Plaza, Cifuentes, & Ibáñez, 2012; Salam et al., 2019). Sonication was completed with 80% ethanol solvent for 30 minutes and repeated three times.

Following extraction, all extract samples were dried through rotary evaporation and lyophilization and stored at -20 °C before being prepared at a stock concentration of 10 mg/mL in either deionized water (diH_2O) or dimethyl sulfoxide (DMSO). Stock concentrations for extracts prepared via decoction were dissolved in diH_2O, and extracts prepared via maceration or ultrasonic extraction were dissolved in DMSO.

The 10 mg/mL stock concentration of extracts were then diluted in phosphate buffered saline (PBS) and buffer in order to decrease the inhibitory effects of the extraction solvent on the P450s. First, the extracts were diluted in PBS to a concentration of 0.625 mg/mL. Then, they were further diluted in Vivid® CYP450 Reaction Buffer to a concentration of 20 µg/mL. These buffer dilutions resulted in a >1% v/v of the extraction solvent, which minimizes its inhibitory effects.

Control preparation

Vehicle controls were prepared using extraction solvent, PBS, and Vivid® CYP450 Reaction Buffer using the same concentrations at which test extracts were prepared. Known P450 inhibitors were used as positive controls. The positive controls were prepared according to their manufacturer, then diluted to the concentration recommended by the Vivid® CYP450 High-Throughput Screening Assay Protocol in Vivid® CYP450 Reaction Buffer. A list of positive controls can be found in Table 3.1.

P450	Positive Control
2B6	30 µM miconazole
2C8	10 µM montelukast
2C9	30 µM sulfaphenazole
2D6	10 µM quinidine
3A4	10 µM ketoconazole
3A5	10 µM ketoconazole

Table 3.1 Positive controls for P450 assays of known P450 inhibitors. Source: Vivid® CYPP450 High-Throughput Screening Assay Protocol

P450 inhibition screening materials

Vivid® CYP450 Screening Kits are an *in vitro* model used to identify P450 inhibition according to current FDA guidelines. Kits were purchased from LifeTechnologies. Each kit contains the following:

- P450 BACULOSOMES® Plus Reagent: The P450 BACULOSOMES® each express a specific P450. They are insect microsomes, or fragments of a nuclear membrane involved in protein synthesis. These insect microsomes have been modified to express a human

P450 enzyme. Each baculosome expresses only one P450, and they metabolize specific substrates.

- Vivid® Substrate: The substrate used for each kit a is a "blocked dye" that is metabolized by the P450 to produce fluorescence. If there is no P450 inhibition, the substrate will produce a highly fluorescent "free-dye." Figure 3.1 shows how substrates are metabolized from a blocked dye to a free dye by their respective P450s. The enzyme-specific substrates are listed in Table 3.2.

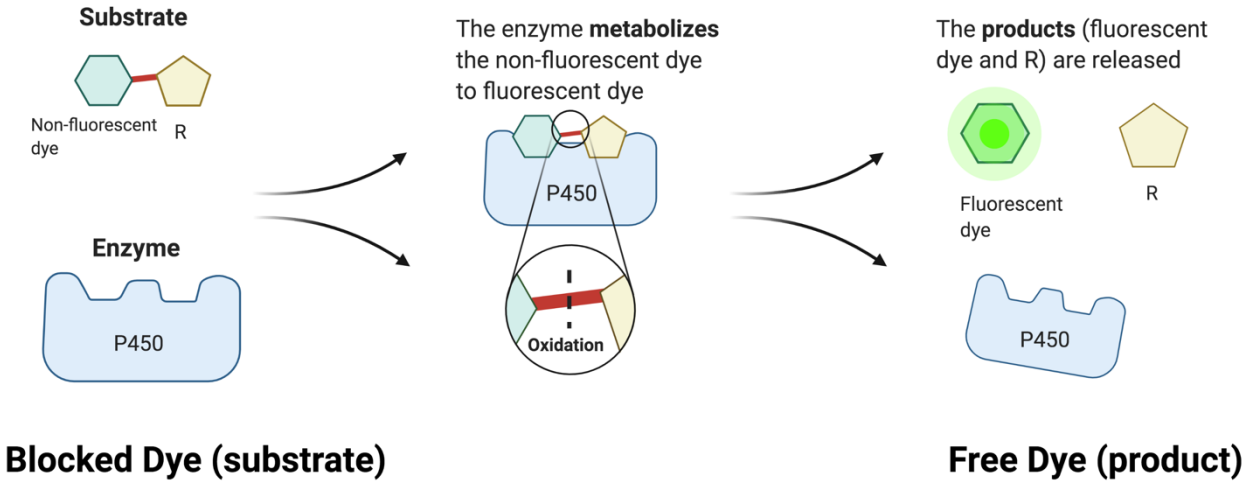


Figure 3.1 Mechanism of blocked dye metabolized into fluorescent free dye by P450 isoenzyme. Figure illustrates the process by which the blocked dye (substrate) is metabolized by the P450 isoenzyme to become a free dye and produce. Fluorescence is produced as a result of this process. Source: Vivid® CYP450 High-Throughput Screening Assay Protocol. Created with BioRender.com

- Vivid® Regeneration System, 100X: The regeneration system reduces $NADP^+$ to NADPH by donating a hydrogen atom to $NADP^+$. It is made of 333mM Glucose-6-phosphate and 30 U/mL Glucose-6-phosphate dehydrogenase prepared in potassium phosphate buffer.

- Vivid® $NADP^+$: The $NADP^+$ is reduced to NADPH by the regeneration system. The NADPH acts as a proton donor for the enzymatic reaction. The stock $NADP^+$ is prepared at a concentration of 10 mM NADP+ in 100 mM potassium phosphate, pH 8.0
- Vivid® CYP450 Reaction Buffer I and II: Reaction buffers 1 and 2 are 200 mM and 100 mM of potassium phosphate, respectively. Both buffers have a pH of 8.0. This buffer is used to dilute test compounds, controls, and reagents, and to help the assay maintain a balanced pH throughout the reaction
- Vivid® Fluorescent Standard: The fluorescent standard is a "free dye" prepared in DMSO at a known nanomolar concentration. It is measured for fluorescence and used to create a standard curve of nanomolar concentration versus fluorescence. The standard curve is used to determine the concentration of product formed during the assay based on the amount of fluorescence produced.

Kit specific materials and their part numbers are listed in Table 3.2. Screening kits were prepared as aliquots in order to minimize the effects of repeated freeze-thawing cycles, and final concentrations of reagents are included in Table 3.2.

Vivid® CYP450 Screening Kit	BACULOSOMES® Plus Reagent screening concentration (nM)	Vivid® Substrate	Vivid Substrate screening concentration (µM)	$NADP^+$ screening concentration (µM)
2B6 (Cat. no. P3019)	5	BOMCC	3	30
2C8 (Cat. no. PV6141)	5	DBOMF	1	100
2C9 (Cat. no. P2860)	10	BOMF	1	100
2D6 (Cat. no. P2972)	10	EOMCC	10	30
3A4 (Cat. no. P2857)	5	DBOMF	2	100
3A5 (Cat. no. P2969)	5	DBOMF	2	100

Table 3.2. Screening concentrations for Vivid® CYP450 Screening Kit reagents. Source: Vivid® CYP450 High-Throughput Screening Assay Protocol

Methods

Initial screening of P450 inhibition

An initial screening was conducted testing 20 of the top selling herbal supplements in the US for P450 inhibition at a concentration of 8 µg/mL. P450 screening assays were run according to the Vivid® CYP450 High-Throughput Screening Assay Protocol, with minor modifications. The assay was scaled down to 20µL final volume and completed in a black flat bottom 384-well plate (Corning, 77776-798). The reaction was completed at room temperature in low light conditions. Reagents were dispensed using the BRAND Robotic Liquid Handling System, with final concentrations of reagents listed in Table 3.2.

First, QNPL test extracts were diluted to a concentration of 0.02 mg/mL in 1X Vivid CYP450 Reaction Buffer. Next, the master premix was prepared using P450 BACULOSOMES® Plus Reagent and Vivid® Regeneration System diluted in 1X Vivid®

CYP450 Reaction Buffer. The master premix was prepared for each enzyme tested using the concentrations described in Table 3.2.

While the test extract dilutions were being prepared by the BRAND liquid handling station, a master premix containing P450 BACULOSOMES® Plus Reagent and Vivid® Regeneration System was prepared by hand in 1X Vivid® CYP450 Reaction Buffer.

Then, the BRAND liquid handling system dispensed extracts into unique wells of a black 384-well plate in triplicate. Master premix was added to the extracts and the plate was incubated for 10 minutes. During this time, a standard curve was prepared by hand at low light in the black 384-well plate. 500nM of fluorescent standard was 2-fold serially diluted in buffer for the standard curve.

After the incubation period, a pre-read of the black 384-well plate was completed using a Cytation3 Imaging Reader. During the pre-read, a mixture of Vivid Substrate and Vivid $NADP^+$ was prepared at low light according to Table 3.2.

Following the pre-read, the mixture of substrate and $NADP^+$ was added to each well containing extract and master premix. The final reaction volume was 20 µL, and the final test concentration for the QNPL extracts was 0.08 µg/mL.

Finally, the black 384-well plate was returned to the Cytation3 Imaging Reader and fluorescence was measured once every ~3 minutes for twenty-five minutes.

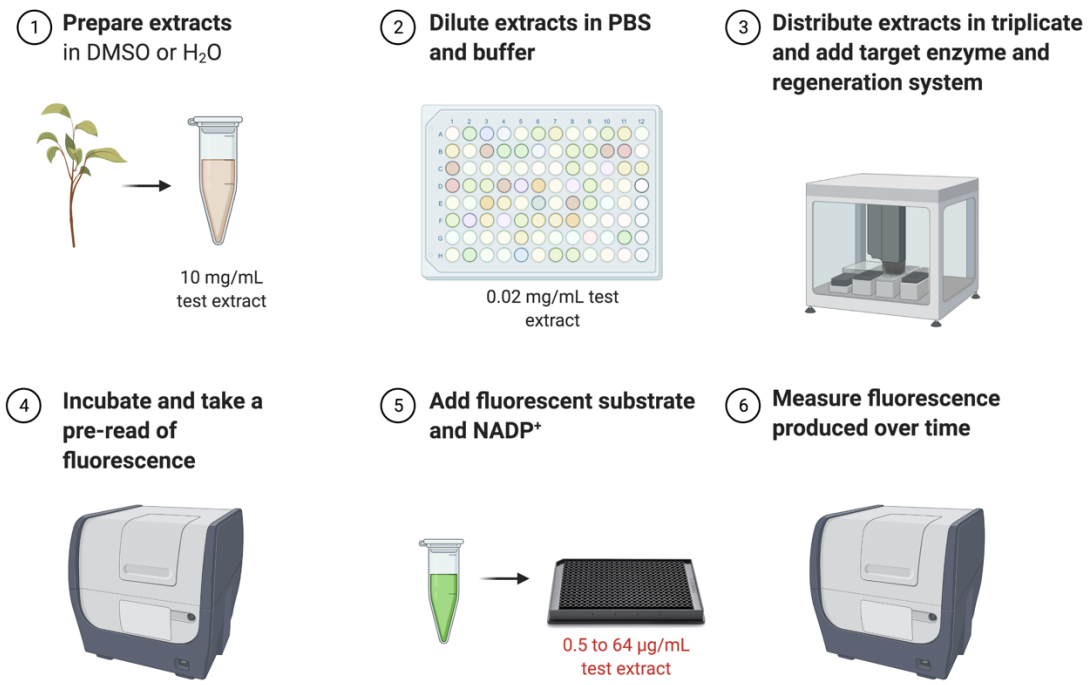


Figure 3.2 Overview of high-throughput screening assay protocol. Created with BioRender.com

Half-maximal inhibitory concentration assay for cinnamon extracts

Half-maximal inhibitory concentration (IC_{50}) assays were performed to identify the concentration of test extract that inhibits 50% of CYP2C9 enzyme activity (Sebaugh, 2011). IC_{50} assays were conducted for four extracts: one extraction of *C. burmannii* bark in 80% EtOH, one extraction of *C. burmannii* bark in H_2O one extraction of *C. verum* bark in 80% EtOH, and one extraction of *C. verum* bark in H_2O. Extracts were diluted in PBS and 1X Vivid® CYP450 Reaction Buffer for final test concentrations of 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, and 0.5 µg/mL. Vehicle controls of DMSO and diH_2O were prepared at the same final concentrations for each screening concentration. Additionally, assays were completed for sulfaphenazole, a known inhibitior of CYP2C9, at the same final concentrations.

The IC_{50} assays were run according to the Vivid® CYP450 High-Throughput Screening Assay Protocol in 96-well plates. The final reaction volume was 100 µL, with 40 µL of test extract, 50 µL of master pre-mix containing P450 BACULOSOMES®, Vivid® Regeneration System, and 1X Vivid® CYP450 Reaction Buffer, and 10 µL of Vivid® Substrate plus $NADP^+$. Final test concentrations of reagents are described in Table 3.2. Fluorescence was measured by the Cytation3 Imaging Reader once every ~1 minute for twenty-five minutes.

Each extract concentration was tested in triplicate, and two biological replicates were completed for a total of six assays for each test extract concentration.

Data analysis

Initial screening

Data analysis for the initial screening of P450 inhibition was performed using Microsoft Excel and Prism 9. First, fluorescence readings were exported from the Cytation3 Imaging Reader as excel files. Then the pre-read of the plate without fluorescent substrate was subtracted from the kinetic reading. This ensures that background fluorescence from the test compound is not included in the calculations of fluorescence produced by the metabolized substrate.

Prism 9 was used to calculate a simple linear regression analysis of the fluorescence vs nanomolar concentration of the fluorescent standard. The equation produced by the simple linear regression was used to calculate the concentration of product formed in the presence of each test compound.

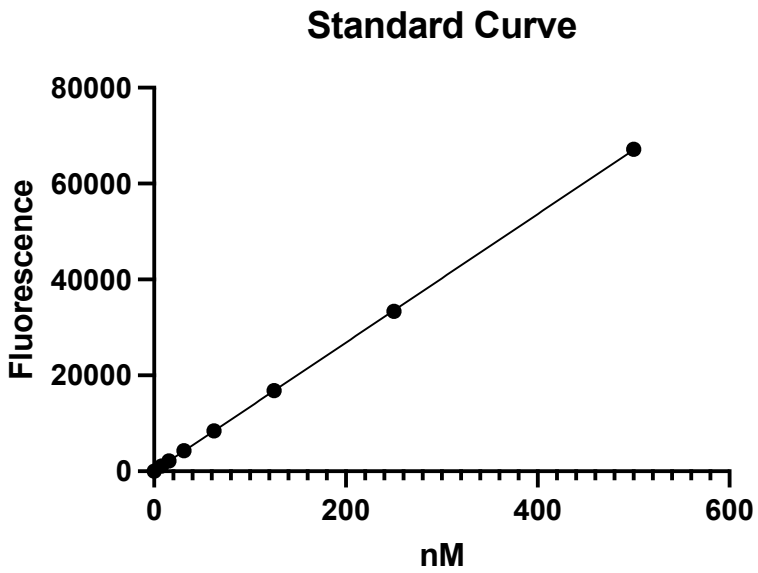


Figure 3.3 Example of standard curve used to calculate concentration (nM) of product formed from fluorescence reading. Each point represents the average fluorescence produced for a given concentration. The line represents a simple linear regression analysis of the relationship between concentration of dye and fluorescence produced. N = 3 for each concentration. Error bars represent standard deviation (some error bars are too small to be seen).

Once the concentration of product formed was calculated for each timepoint, Prism 9 was used to calculate a simple linear regression analysis of product formed vs time. The slope of the line was used to determine the rate of product formation in the presence of each test compound and control.

Percent inhibition was calculated using the following formula:

$$\%\ inhibition = \left(1 - \frac{X - P}{V - P}\right) \times\ 100$$

X = rate of product formation in the presence of test compound

P = rate of product formation in the presence of known inhibitor (positive inhibition control)

V = rate of product formation in the absence of inhibitor (vehicle control)

Use of a vehicle control takes into account inhibition caused by the solvent that the test compound is prepared in. The test compound is considered to have 0% inhibition. The positive control is considered 100% inhibition because it is known to inhibit P450 activity at the recommended concentrations. It is used in calculations to subtract background fluorescence from the assay.

Half-maximal inhibitory concentration data analysis

The initial screening data analysis was completed for each concentration screened. The percent inhibition was reported for each test concentration. A nonlinear regression was calculated to determine the line of best fit for the model of percent inhibition vs concentration. Using the line of best fit, the IC_{50} value was determined at a 95% confidence level.

Outliers were identified and removed using the robust regression and outlier removal method on Prism 9 with a Q value of 1%.

Chapter 4: Results

Initial Screening: Inhibitory activity of herbal supplements on Cytochrome P450 enzymes

An initial screening was conducted in order to identify herbal supplements that inhibit P450 metabolism, and to identify a supplement of interest for further testing. All extracts were tested at a concentration of 8 µg/mL. This concentration was chosen in order to identify extracts with inhibitory properties at a low concentration.

Results range from 0% inhibition, meaning that the test compound did not prevent P450 metabolism, to over 100% inhibition. Results of greater than 100% inhibition suggest that the test compound screened at 8 µg/mL has a greater inhibitory effect on the P450 than the known P450 inhibitor used as the positive control.

The initial screening included fifty-two extractions from twenty top-selling herbal supplement species. The fifty-two extractions were tested primarily against P450s 3A4, 2C8, and 2C9. Four supplement species showed evidence of ≥ 50% inhibition of at least one P450 tested, which demonstrates strong inhibition of that P450. An extract of St. John's wort (*H. perforatum*) flowering tops extracted in 95% ethanol showed strong inhibition of of P450 3A5, 2C8, and 2C9 (average percent inhibitions of 69.4%, 75.6%, and 51.1%, respectively). These results indicate that St. John's wort extraction has potential for interactions with multiple drug metabolizing pathways. Green tea (*Camellia sinensis*) leaf extracts prepared in both H_2O and 80% ethanol showed strong inhibition (≥ 50%) of CYP2C8, and elderberry (*Sambucus candadensis*) roots extracted in methanol showed strong inhibition of CYP3A4, while elderberry leaves extracted in methanol showed strong inhibition of CYP2C8. These results indicate that several species of herbal supplements interfere with normal P450 metabolism by when tested *in vitro*, which indicates a potential for herb-drug interactions.

A detailed report on the results from the initial screening can be found in Appendix A. Further testing should be conducted on species identified as inhibitory, since there have not been biological replicates performed from the initial screening.

Two species of cinnamon, *C. burmannii* and *C. verum*, were identified as having strong inhibition of at least one P450 in the initial screening. Assays were repeated at concentration of 8 µg/mL for additional P450s (3A5 and 2B6) at a volume of 100 µL

Table 4.1 summarizes P450 inhibition by the two species of cinnamon.

	% inhibition			
Extract	CYP2B6	CYP2C9	CYP3A4	CYP3A5
C. burmanniiI (2017, Bark, 80% EtOH)	8.25 (8.8)	67.73 (2.2)	35.50 (8.2)	91.62 (0.40)
C. burmanniiI (2018, Bark, H2O)	6.99 (6.8)	26.05 (0.41)	16.8 (7.7)	30.82 (6.3)
C. verum (2019, Bark, 80% EtOH)	17.84 (3.7)	48.98 (2.3)	17.67 (18.9)	51.2 (14.9)
C. verum (2020, Bark, H2O)	3.36 (1.42)	25.9 (4.6)	4.57 (15.46)	19.76 (9.3)

Table 4.1 Summary of average P450 inhibition by extracts of *Cinnamomum* spp. at 8 µg/mL. Data is reported as Mean (Standard Deviation). N = 3 for CYP3A4, CYP 2B6, CYP 2C9, and CYP3A5. N = 6 for CYP2C9.

Figures 4.1 and 4.2 shows a graphical representation of the amount of product formed over time in the presence of cinnamon extracts. The blue line indicates the product formed in the presence of a known P450 inhibitor, which is considered 100% inhibition for the calculations. The pink line represents the product formed in the presence of the vehicle control, which is considered 0% inhibition for the calculations. The black line represents the product formed in the

presence of the test compound. A steeper slope indicates less inhibition, because more product formed.

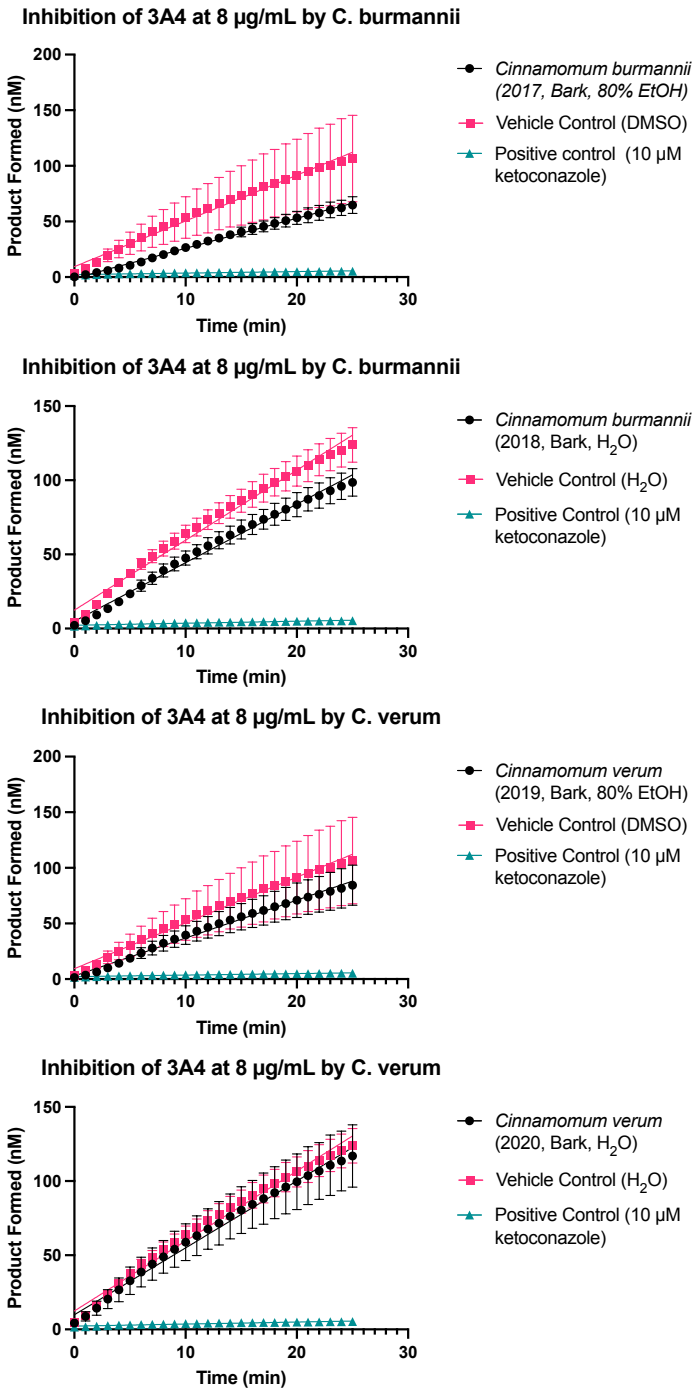


Figure 4.1 Inhibition of CYP3A4 by extracts of *Cinnamomum* spp. at 8 µg/mL. Graphs show fluorescense produced over time by metabolized CYP3A4 substrate. The blue line represents a simple linear regression of the fluorescense produced over time in the presense of a known CYP3A4 inhibitor (Pos. Control). The pink line represents a simple linear regression of the fluorescense produced over time in the presense of the extraction solvent (vehicle control). The black line represents a simple linear regression of the fluorescense produced over time in the presense of the test extract. Error bars represent standard deviation.

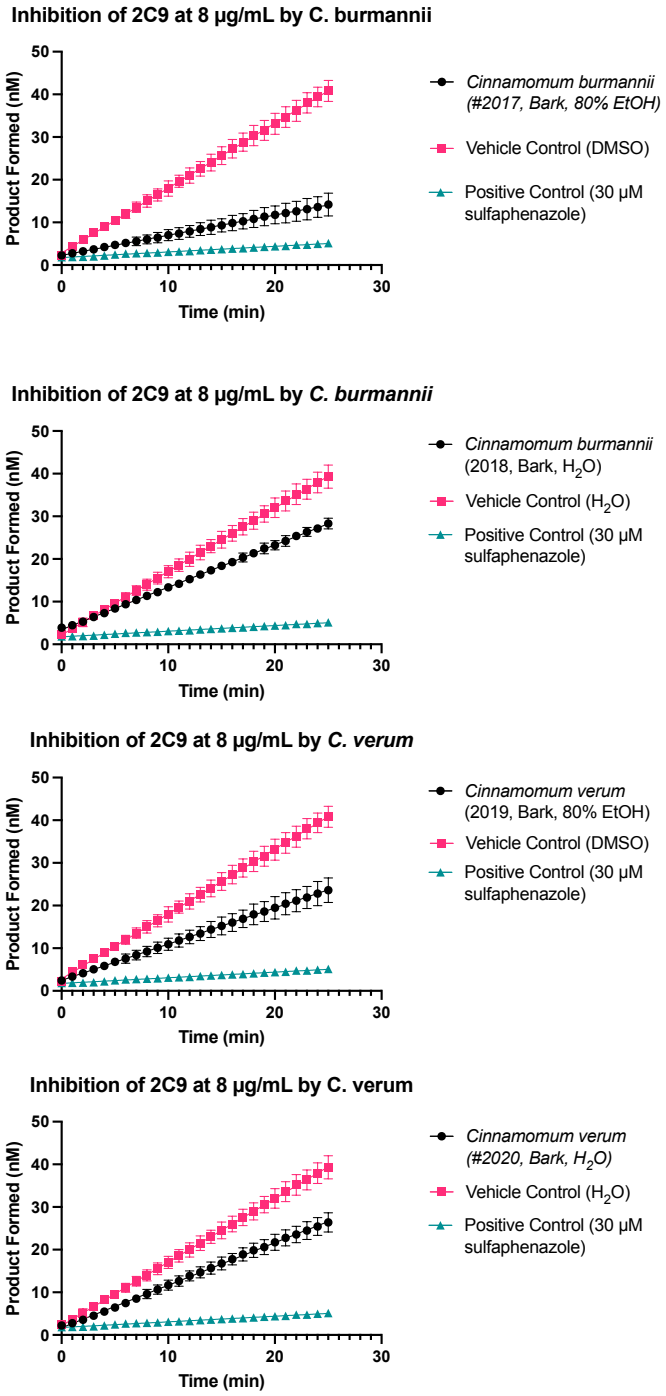


Figure 4.2 Inhibition of CYP2C9 by extracts of *Cinnamomum* spp. at 8 µg/mL. Graphs show fluorescense produced over time by metabolized CYP2C9 substrate. The blue line represents a simple linear regression of the fluorescense produced over time in the presense of a known CYP2C9 inhibitor (Pos. Control). The pink line represents a simple linear regression of the fluorescense produced over time in the presense of the extraction solvent (Vehicle Control). The black line represents a simple linear regression of the fluorescense produced over time in the presense of the test extract. Error bars represent standard deviation.

Half-maximal inhibitory concentration screening of *Cinnamomum spp*. on CYP2C9

C. burmannii and *C. verum* were further tested for half-maximal inhibitory concentration (IC_{50}) of CYP2C9. CYP2C9 was selected since it is responsible for ~20% of drug metabolism, and inhibition by *Cinnamomum burmannii* has not been previously reported.

The IC_{50} reported is the concentration of test extract that inhibits 50% of enzyme activity. In order to calculate accurate IC_{50} values, it is recommended to include at least two points with greater than 50% inhibition and two points with less than 50% inhibition, so percent inhibition of P450s was tested at eight different concentrations (Sebaugh, 2011). This ensured enough data was available to make a more accurate estimation of the IC_{50} values. 64 µg/mL was selected as the maximum screening volume to ensure that the % v/v vehicle control was >1% for all screenings. This reduced the interference of P450 inhibition by solvents. Lower IC_{50} values mean that a compound has greater inhibitory properties, because they inhibit 50% of enzymatic activity at a lower concentration.

All four extractions screened showed 100% inhibition of CYP2C9 at concentrations of at least 32 µg/mL. *C. burmannii* (2017, Bark, 80% EtOH) inhibited CYP2C9 with the greatest potency, with an IC_{50} value of 7.122 µg/mL (95% CI: 6.725 to 7.518 µg/mL). Table 4.3 summarizes the IC_{50} calculations for *Cinnamomum* spp. IC_{50} values against CYP2C9. *C. burmanniiI* (2017, Bark, 80% EtOH) had the greatest inhibition of 2C9, with an IC_{50} value of 7.122 µg/mL (95% CI: 6.725 to 7.518 µg/mL).

Extract	IC_{50} 95% CI (µg/mL)	R Squared Value
C. burmannii (2017, Bark, 80% EtOH)	6.725 to 7.518	0.9863
C. burmannii (2018, Bark, H2O)	10.62 to 12.10	0.9904
C. verum (2019, Bark, 80% EtOH)	8.460 to 9.458	0.9879
C. verum (2020, Bark, H2O)	10.60 to 12.85	0.977

Table 4.2 Summary of 2C9 IC_{50} values calculated by Prism 9 nonlinear regression analysis.

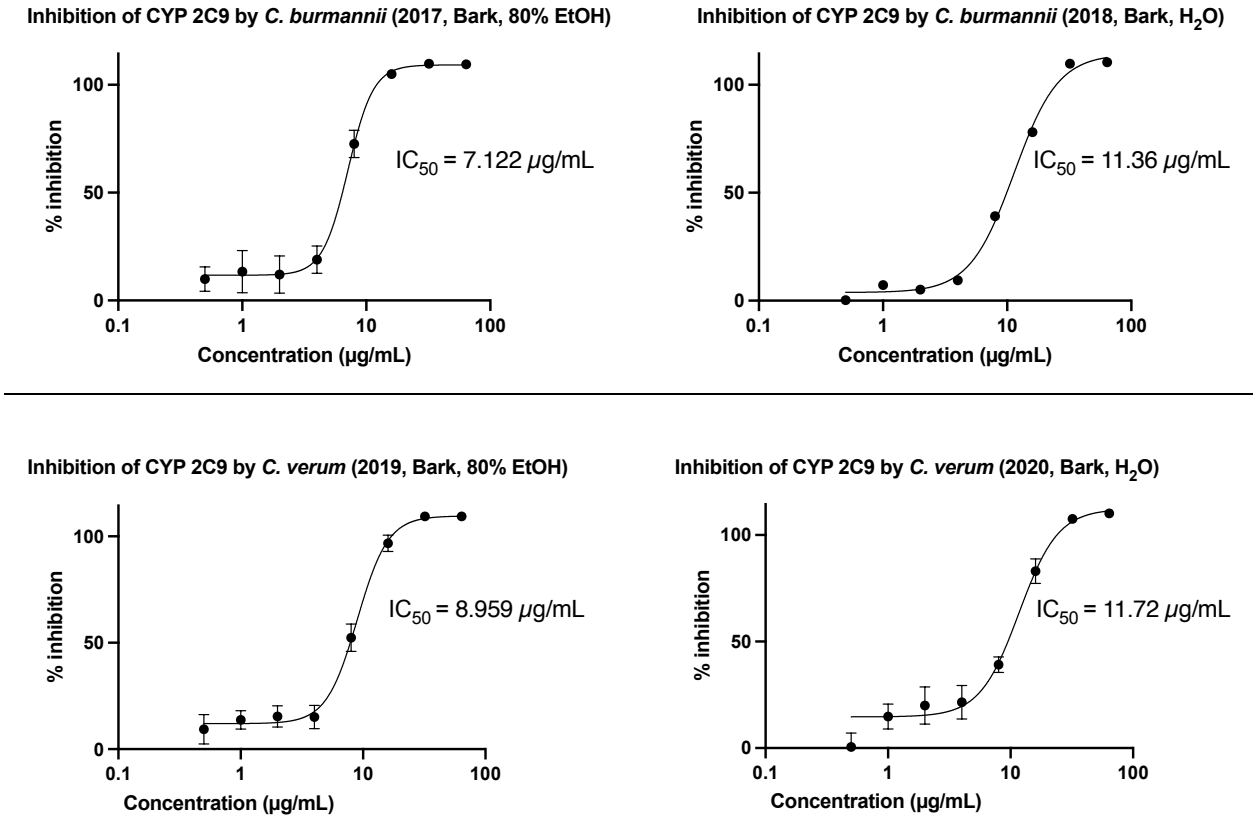


Figure 4.3 Inhibition of CYP2C9 by extracts of *Cinnamomum* spp. from 0.5 - 64 µg/mL with IC_{50} values. Concentration of test extract is reported on the x-axis on a log 10 scale, and percent inhibition is reported on the y-axis. Each point represents the average percent inhibition for a given concentration (n=6). The line represents a nonlinear fit of the relationship between concentration of test extract and percent inhibition of CYP2C9 calculated using Prism9. Error bars represent standard deviation (some error bars are too small to be seen).

Chapter 5: Discussion

This study found that several common herbal dietary supplements inhibit P450 enzymes in *in vitro* studies. Notably, this study identified previously unreported P450 inhibitions by commonly consumed species of cinnamon.

Although there is no standardized process for preparation of herbal supplements sold in the United States, they are commonly prepared as extracts. Common extraction methods for herbal supplements include decoctions, teas, and tinctures (NCCIH, 2020). Decoctions and teas are both prepared by soaking plant material in hot water, but decoctions are usually performed for approximately 15 to 20 minutes and are used to extract tougher plant materials, such as bark and roots, whereas teas are usually steeped for a shorter period of time using more delicate plant material, such as leaves and flowers (Salam, Lyles, & Quave, 2019). Tinctures are prepared by soaking the plant parts in alcohol. The herbal supplement materials in the QNPL were prepared using aqueous and organic extraction methods in order to best mimic common preparation methods of herbal supplements.

By testing at a low concentration, the initial screening identified herbal supplements with the greatest potential for physiological effects. The results from the initial screening at 8 μg/mL are consistent with earlier in vitro reports of P450 inhibition by species of St. John's wort and green tea (Misaka et al., 2013; Obach, 2000). However, these results highlight the need for further testing. Although St. John's wort had been identified as a P450 inhibitor in early in vitro screenings, later in vivo screenings identified it as a P450 inducer instead (Markowitz et al., 2003). These results demonstrate the need for further in vivo testing in order to identify potential differences between herb-drug activity in vitro and in vivo.

Our data strengthens the previous studies demonstrating inhibition of CYP2C9 by *C. verum*. Although a previous study found evidence of inhibition by "Sri Lanka cinnamon" (a common name for *C. verum*), this is the first study that has been completed with chemically and botanically authenticated source plant materials (Kimura et al., 2010).

The results of the 2C9 IC_{50} assays show stronger inhibition by *C. burmannii* of 2C9 than previously reported inhibition of 3A4 (Subehan, Kadota, & Tezuka, 2008). This suggests that low concentrations of *C. burmannii* could have a greater effect on drugs metabolized by 2C9 enzymes. Since the IC_{50} estimation of *C. burmannii* for 2C9 was found to be 7.122 µg/mL (95% CI: 6.725 to 7.518 µg/mL), it is possible that a standard 6 mg dose of cinnamon supplements could interfere with drug metabolism (Muhammad & Dewettinck, 2017).

One study proposed a mechanism-based inactivation of 3A4 by *C. burmannii*. This means that metabolites of *C. burmannii* generated during metabolism by 3A4 were then inhibitory of 3A4. Previous research suggests that compound 5′-hydroxy-5-hydroxymethyl-4″,5″-methylenedioxy-1,2,3,4-dibenzo-1,3,5-cycloheptatriene, derived from *C. burmannii* bark extraction in methanol, is responsible for 3A4 inhibition (Subehan, 2008). This is a compound of interest for further studies on 2C9 inhibition, which may also be inhibited by mechanism-based inactivation.

Although the P450 inhibition screenings used in this study are consistent with FDA recommendations for initial screenings of P450 inhibition, these *in vitro* studies cannot be used to draw conclusions about how herbs will interact with P450 metabolism *in vivo*. More information is needed on the bioavailability of supplements screened. Additionally, further screenings should be conducted in order to identify the inhibition of P450s by compounds derived from herbal supplements. For example, fractionations of *C. burmannii* and *C. verum*

should be screened against 2C9 in order to identify the specific compound responsible for P450 inhibition of this enzyme.

In conclusion, the results of this study identified P450 inhibition by commonly used herbal supplements, as well as supported previous findings of P450 inhibition. The results show *in vitro* inhibition of 2C9 by *C. burmannii*, which could cause harmful herb-drug interactions. Since so many people around the world rely on complementary medicine, it is important to have more information available on herb-drug interactions. Further screenings should be conducted identify if these mechanisms occur *in vivo*. Additionally, major compounds of cinnamon species should be tested for P450 inhibition in order to identify the most likely mechanism of inhibition. The results of this study can be used to increase knowledge for complementary medicine users and increases the knowledge of plant and human interactions.

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Appendix A. Percent inhibition of P450 enzymes by herbal supplement extracts.

						% Inhibition		
Common name	**Latin Binomial**	**Extract #**	**Plant Part**	**Extraction Solvent**	**Extraction Method**	**CYP3A4** Mean (SD)	**CYP2C8** Mean (SD)	**CYP2C9** Mean (SD)
Acai	*Euterpe oleracea*	2038	Fruit	H_2O	20 minute decoction	–	19.0 (5.3)	30.8 (9.6)
	Euterpe oleracea	2037	Fruit	80% EtOH	2x 72h maceration	6.9 (7.8)	13.7 (4.3)	–
Ashwagandha	*Withania somnifera*	2030	Roots	H_2O	20 minute decoction	–	0.18 (5.3)	-2.9 (2.5)
	Withania somnifera	2029	Roots	80% EtOH	2x 72h maceration	7.0 (3.0)	0.40 (11.0)	–
Black cohosh	*Actaea racemosa*	2234	Roots	H_2O	20 minute decoction	–	-0.62 (9.7)	–
Chamomile	*Matricaria chamomilla syn M. recutita*	27	Leaves, Flowers, Stems, Roots	H_2O	20 minute decoction	–	11.1 (8.2)	–
	Matricaria chamomilla syn *M. recutita*	1925	Flowers	80% EtOH	2x 72h maceration	–	10.6 (4.4)	–
Cinnamon	*Cinnamomum burmannii*	2018	Chips	H_2O	20 minute decoction	–	81.5 (4.5)	59.9 (6.8)
	Cinnamomum burmannii	2017	Chips	80% EtOH	2x 72h maceration	51.0 (3.1)	92.6 (2.2)	–

	Cinnamomum camphora	787	Leaves	H_2O	20 minute decoction	–	11.8 (8.9)	11.7 (9.9)
	Cinnamomum camphora	789	Leaves	H_2O	20 minute decoction	–	7.3 (12.6)	3.5 (28)
	Cinnamomum camphora	909	Stems	MeOH	2x 72h maceration	89.0 (1.8)	78.2 (4.1)	18.2 (13.9)
	Cinnamomum camphora	1024	Bark	MeOH	2x 72h maceration	41.7 (11.6)	–	28.6 (70.9)
	Cinnamomum verum	2020	Chips	H_2O	20 minute decoction	–	64.9 (11.8)	31.4 (25.3)
	Cinnamomum verum	2019	Chips	80% EtOH	2x 72h maceration	28.1 (7.8)	79.2 (12.5)	–
Echinacea	*Echinacea purpea*	2006	Roots	H_2O	20 minute decoction	–	1.2 (10.6)	19.2 (5.0)
	Echinacea purpea	2008	Leaves	H_2O	20 minute decoction	–	-1.7 (22)	16.3 (8.9)
	Echinacea purpea	2005	Roots	80% EtOH	2x 72h maceration	-8.0 (6.2)	5.1 (6.6)	–
	Echinacea purpea	2007	Leaves	80% EtOH	2x 72h maceration	-19.0 (12.7)	3.6 (3.8)	–
Elder Berry	*Sambucus canadensis*	838	Flowers	MeOH	2x 72h maceration	81.1 (6.1)	49.0 (11.4)	21.1 (7.3)
	Sambucus canadensis	935	Leaves	MeOH	2x 72h maceration	53.6 (10.0)	22.5 (24.1)	(18.9 (4.9)
	Sambucus canadensis	940	Roots	MeOH	2x 72h maceration	-8.83 (12.0)	59.2 (3.5)	15.1 (8.2)

	Sambucus nigra	89	Flowers	H_2O	20 minute decoction	–	-5.1 (8.9)	-13.6 (6.6)
	Sambucus nigra	90	Leaves	H_2O	20 minute decoction	–	1.9 (12.2)	-4.8 (14.5)
	Sambucus nigra	2082	Berries	80% EtOH	2x 72h maceration	–	-2.2 (14.6)	-12.6 (1.9)
Fennel	*Foeniculum vulgare*	1448	Aerial Parts	H_2O	20 minute decoction	-16.9 (5.3)	–	–
Fenugreek	*Trigonella foenum-graecum*	2090	Seeds	80% EtOH	2x 72h maceration	–	3.7 (10.8)	-2.2 (2.4)
Garcinia	*Garcinia gummi-gutta*	2093	Fruits	H_2O	20 minute decoction	–	-7.4 (10.1)	–
	Garcinia gummi-gutta	2092	Fruits	80% EtOH	2x 72h maceration	–	5.5 (3.6)	0.65 (4.3)
Ginseng	*Panax ginseng*	2164	Roots	H_2O	20 minute decoction	–	7.4 (8.9)	–
	Panax ginseng	2163	Roots	80% EtOH	2x 72h maceration	–	-1.1 (17.0)	-4.8 (21.3)
Goji berry	*Lycium barbarum*	2054	Fruits	H_2O	20 minute decoction	–	4.6 (15.7)	13.63 (24.6)
	Lycium barbarum	2053	Fruits	80% EtOH	2x 72h maceration	-11.2 (6.0)	5.8 (7.9)	–
Green tea	*Camellia sinensis*	446	Leaves	H_2O	20 minute decoction	–	54.2 (3.0)	48.0 (6.0)
	Camellia sinensis	2057	Leaves	H2O	20 minute decoction	–	24.5 (5.7)	31.1 (0.53)

	Camellia sinensis	2152		H_2O	20 minute decoction	–	42.9 (49.9)	-
	Camellia sinensis	2056	Leaves	80% EtOH	2x 72h maceration	0.04 (14.8)	17.4 (31.0)	-
	Camellia sinensis	2151	Leaves	80% EtOH	2x 72h maceration	–	66.3 (7.9)	6.9 (6.7)
	Camellia sinensis	471	Leaves			–	65.6 (2.2)	14.8 (25.8)
Horehound	*Marrubium vulgare*	42	Infloresecence, Leaves, Stems	H_2O	20 minute decoction	–	9.1 (7.4)	-4.1 (3.85)
	Marrubium vulgare	2115	Aerial parts	H_2O	20 minute decoction	–	1.8 (6.0)	–
	Marrubium vulgare	2114	Aerial parts	80% EtOH	2x 72h maceration	–	20.7 (3.5)	28.5 (4.2)
Horny goat weed	*Epimedium grandiflorum*	2004	Leaf	H_2O	20 minute decoction	–	6.9 (30.9)	17.1 (18.7)
	Epimedium grandiflorum	2003	Leaf	80% EtOH	2x 72h maceration	-9.9 (10.6)	14.9 (1.2)	–
Maca	*Lepidium meyenii*	2040	Roots	H_2O	20 minute decoction	–	3.0 (15.9)	20.5 (2.6)
	Lepidium meyenii	2039	Roots	80% EtOH	2x 72h maceration	-0.2 (5.5)	0.2 (11.6)	–
Milk thistle	*Silybum marianum*	1381	Inflorescence	H_2O	20 minute decoction	4.6 (11.4)	–	–

	Silybum marianum	1406	Inflorescence	H_2O	20 minute decoction	-5.8 (12.3)	–	–
Saw palmetto	*Serenoa repens*	2238	Berries	H_2O	20 minute decoction	–	-3.8 (16.1)	–
St John's wort	*Hypericum perforatum*	1231	Flowering tops	95% EtOH	2x 72h maceration	69.4 (11.7)	75.6 (2.6)	51.1 (11.6)
	Hypericum perforatum	1232	Leaves, Stems	95% EtOH	2x 72h maceration	29.0 (10.4)	55.9 (10.9)	35.4 (12.0)
Valerian	*Valeriana officinalis*	2186	Roots	H_2O	20 minute decoction	–	-15.1 (21.5)	–