Cytochrome P450-3A4

Inhibition of Human Cytochrome P450-3A4 isoform by Crude Extracts from Acai Berry

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Abstract

The oxidoreductase enzymes that comprise the human liver cytochrome P450 (CYP450) system are vital in metabolism, lipid synthesis, and detoxification of the human body. It is known that enzymes in this pathway process the majority of pharmaceuticals, thus the study of these enzymes proves to be valuable in determining potentially harmful drug interactions. The amazon acaí berry has gained popularity in recent years due to its reported health benefits and has been deemed by many health organizations as a superfood. The purpose of this research was to determine the effects of isolated acaí berry extracts on the activity of the CYP 450 enzyme 3A4 and 2D6 to determine if there is a possible drug interaction. The results obtained suggest that compounds in certain acaí fractionations, specifically 94-C (Diosmetin) inhibit the enzymes' activity by approximately 68%. While further research into the metabolism of acaí berries must be conducted, these results imply that eating acaí berries may be harmful while taking certain medications due to the inability of CYP 3A4 and 2D6 enzymes to adequately metabolize the drug.

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The Liver is one of the most important organs in vertebrates. It is located in the upper right quadrant of the abdomen, below the diaphragm. The liver has a wide variety of functions such as detoxification of various metabolites, protein synthesis, and biochemical secretion. In addition, the liver's unique role is that it is responsible for eliminating foreign compounds (xenobiotics) like drugs and toxins from the body by specific types of metabolism that involve using cytochrome P450 enzymes, which are a family of isozymes responsible for the biotransformation of several drugs (1). The synthetic role of the liver will be disregarded and the detoxification role of the liver will be focused on due to the importance of this research on the human body and the danger that a natural product can produce.

It is well known that the inhibition or activation of human liver cytochrome P450 enzymes by compounds of natural product origin can result in adverse drug interactions(19). By inhibiting one or all of the P450 isoforms, the Brazilian Acai berry has gained considerable popularity due to its health benefits. It can suppress inflammation in individuals with cancer and arthritis. It improves digestion along with sexual performance and general health (12). Acai products range from the raw freeze-dried powder, supplement tablets, beverages such as juice, wine, smoothies or energy drinks, and even food products such as jelly or ice cream (19).

Human liver cytochrome P450 consists of six different isozymes that play

important roles in drug metabolism. CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 (17, 21). The idea that these isoforms could be affected by Acai berry came from a classic example of Grapefruit Juice and the plant St.John's Wart (4). Grapefruit Juice was found to have the furanocoumarins compound that is responsible for inhibiting the CYP3A4 in two ways, if furanocoumarins inhibits the hepatic CYP3A4, it will affect the medication metabolism (10, 13). On the other hand, if it inhibits the enterocyte CYP3A4, the medication absorption in the intestine would be affected (5). Likewise, Black cohosh extracts was found to have a herbal drug interactions that inhibit the 3A4 and 2D6 human isoforms (22). Human P4501A2, P4503A4, and P4502C9 isoforms metabolize anticoagulant drugs such as warfarin and clopidogrel that are susceptible to pharmacokinetic-related drug interactions with natural products (8). Clopidogrel – if used in wrong dosages- would affect the formation of platelets and cause internal bleeding (2). Whereas warfarin interacts with nonsteroidal anti-inflammatory drugs and causes a serious bleeding complications (7).

The amazon acaí berry has gained popularity in recent years due to its reported health benefits and has been deemed by many health organizations as a superfood. In this research, different extracts from the Brazilian acai berry were examined for their potential to inhibit human liver cytochrome P450 enzymes to determine the effects of isolated acaí berry extracts on the activity of the CYP 450 enzymes 3A4 to determine if there was the potential for adverse drug interaction. The isoform 3A4 was explored with the goal of identifying potential drug-extract interactions or identifying specific P450 interactions with potential health benefits

(1, 24). To establish this, the activity of the enzyme was measured using a commercial assay kit in the presence and absence various acai fractions. The results obtained suggest that compounds in certain acaí fractionations inhibit the activity of the human 3A4 up to 70%. Furthermore, by using an activity-guided fractionation approach it was determined that one of the primary compounds in acai berries that is responsible for this inhibition is diosmetin, which is a known flavonoid inhibitor of CYP 450 enzymes. The 3A4 isoform in particular is significance with respect to drug interactions involving anticoagulant medications. P4503A4 was affected critically by St. John's wort (A herbaceous plant) (16). This plant is a source of hyperforin, an active ingredient that has a strong affinity for the pregnane xenobiotic receptor (PXR), as a PXR ligand, hyperforin promotes expression of CYP3A4 enzymes in the small intestine and liver. This in turn causes induction of CYP3A4 and can reduce the oral bioavailability of many drugs making them less effective (14, 23). Furthermore, goldenseal was found to be inhibitory to 3A4.

7-Benzyloxy quinoline, 7-benzyloxy-trifluoromethylcoumarin, testosterone are the main substrates that is responsible of inhibiting P4503A4, These substrate can bind to different domains at the active site of CYP3A4 and that any pair can interact with each other in a manner that is distinct from simple competitive inhibition. Researches have shown that BQ and BFC show a larger inhibition when interacting with a natural product like the ones discussed earlier. This is emphasized by highlighting the investigation and prediction of CYP3A4-ligand interactions. This research would also give knowledge about enzyme

substrate binding properties as it may play a major key to the understanding of cytochrome P450 inhibition patterns from herbs and drugs (3, 15).

Materials and Methods

Acai berry freeze dried extract was purchased from optimally organic Inc. The identity of the dried powder was identified by submitting it for genetic analysis, which ultimately showed that it is closely related to the authentic acai berry sample.

Fractionation of the Acai Powder

455g of freeze dried acai powder was extracted in 4L MeOH to generate methanol extract. The volume was reduced to 2L by filtering the methanol fraction. The solution consisted of 9:1:10 ratio of MeOH:H2O:Hexane in a total volume of 4L. Since the aqueous MeOH is immiscible with hexane, 19.3g of hexane fraction would generate by defatting the original MeOH. The aqueous MeOH fraction was dried and partitioned with 4:1:5 chloroform:MeOH:H2O in a total of 4L. Finally, the chloroform layer produced 7.2g chloroform and the remaining aqueous solution resulted 6.5g butanol and 16.8g aqueous fractions.

$P450_{3A4}$

10 mM NADP+ stock solution: 382 mg NADP+ in 50 ml Milli-Q water. Store at 4 °C. 100 mM glucose-6-phosphate: 3.4 g in 100 ml Milli-Q water. Store at 4 °C. 103 IU/ml yeast glucose-6-phosphate dehydrogenase: 1.0 mg in 1 ml 10 mM Tris-acetate buffer (pH 7.4), containing 20% glycerol (v/v) and 1.0 mM EDTA. Store at 4°C. 4 mM BFC stock solution: 2.56 mg in 2 ml methanol. Stored in Teflon-sealed amber glass vial at 4 °C. 1 mM ketoconazole stock solution: 5.31 mg in 10 ml methanol. Stored in Teflon-sealed glass vial at 4°C. NADPH-generating system: combine 50 parts 10 mM NADP+, 100 parts 100 mM glucose-

6-phosphate and 1 part 1 mg/ml yeast glucose 6-phosphate dehydrogenase. Prepare fresh daily and store on ice when not in use. Stop buffer: 80% acetonitrile and 20% 0.5 M Tris-base (v/v), stored at ambient temperature. P450-BFC 2× mix: mix P450 3A4 bicistronic membranes and 4 mM BFC stock solution in 100 mM potassium phosphate buffer (pH 7.4) so that the final concentration of P450 is 20 nM and BFC is 40 μM. Prepare the mix fresh daily and keep on ice. Dispense 60 μl 100 mM potassium phosphate buffer into the wells (column 2–12) in a 96-well microplate using a multichannel pipette. Dispense 118 μl 100 mM potassium phosphate and 2 μl of 1 mM ketoconazole into the wells (column 1). Serially dilute 60 μl inhibitor solution from the wells in column 1 to the other wells (2-10). Discard the extra 60 μl solution in the wells in column 10. Dispense 100 μl P450-BFC 2× mix in all the wells. Add 75 μl stop buffer to the wells in column11.Preincubate the plate at 37 °C for 5 minutes.Add 40 μl NADPH generating system into each well to initiate the reaction. Incubate the plate at 37 °C for 20 minutes. Stop the reaction by adding 75 μl stop buffer to each well (except wells in column 11) (6, 18).

Methodological summary of the fractionation approach that was used in this research can be followed by the following: A luminescent commercial enzyme assay (Promega P450-Glo Assay) was performed in the presence of various acaí berry extracts. The reaction was started using 0.2 mM NADPH and stopped using lucifierin detection reagent. The generated luminescent product was quantified using a Tecan M200 Pro Infinite plate reader. The assay conditions and preparation of samples were in accordance with the

recommended Promega protocol. The percent activity of each experimental sample was determined and was used to determine the relative amount of inhibition caused by each fraction. An identical assay was preformed using various concentrations of Diosmetin (94-C) to obtain a dose response curve. The results were obtained and analyzed through Microsoft excel.

Results and Discussion

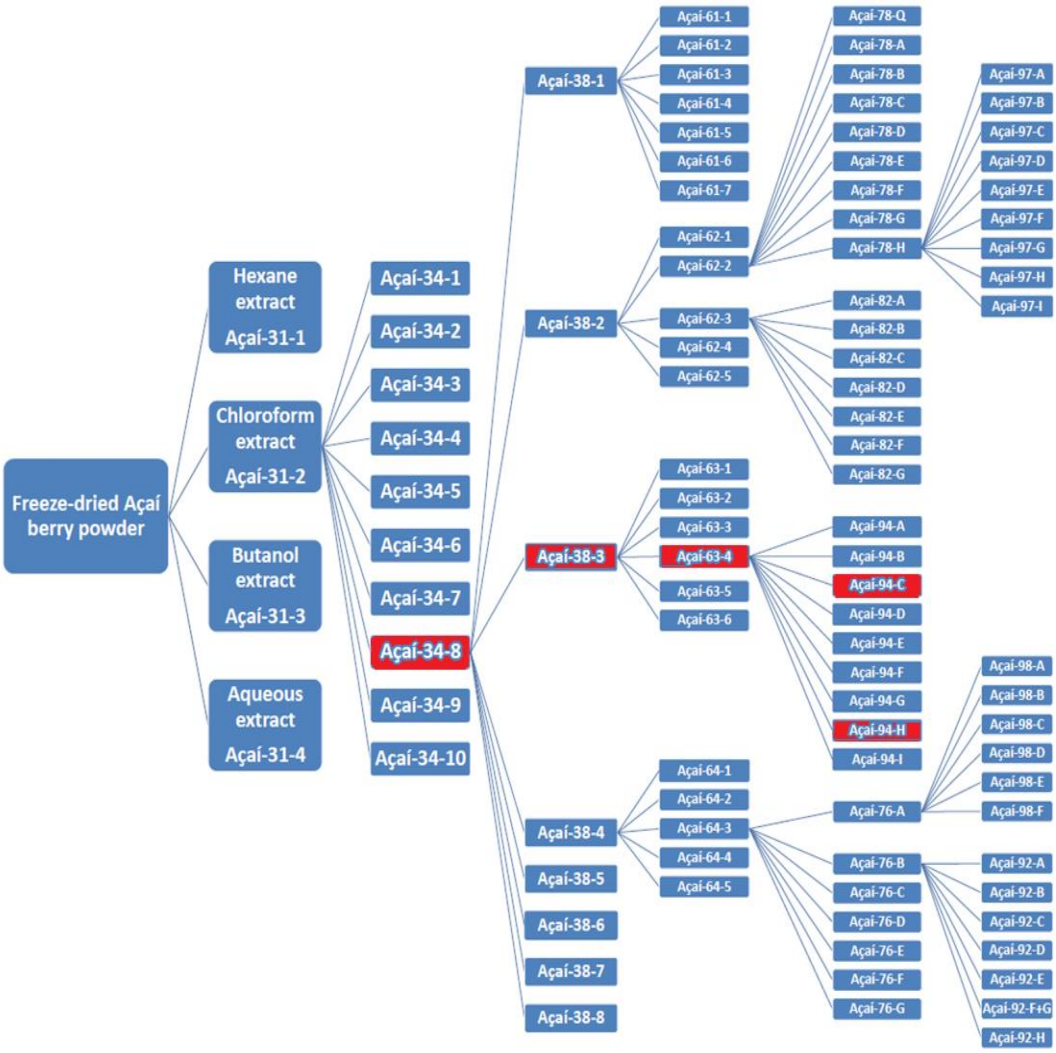


***Figure 1*. Freeze Dried Acaí Berry fractionation Scheme.** Amazon dried Acai berry was fractionated into multiple

Table 1. *% Activity 3A4-34 Series fractions*

Fraction	Luminescence	Percent of +
Positive	**7746.5**	**100**
34-7	1086.667	14
34-8	768	10
34-9	1973.5	25
Positive	**3378.25**	**100**
34-1	618	18
34-2	1083	32
34-6	984.125	29
34-10	558.125	17
Positive	**5729.5**	**100**
34-3	1339	23
34-4	2532.25	44
34-5	1545.25	27

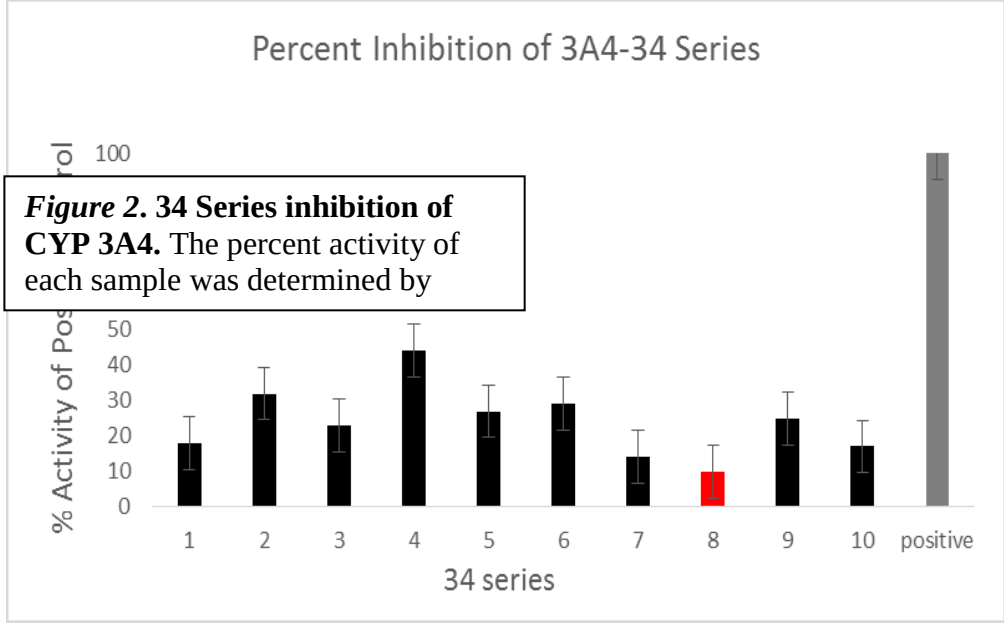


***Figure 2*. 34 Series inhibition of CYP 3A4.** The percent activity of each sample was determined by

Table 2. *% Activity 3A4-38 Series fractions*

Fraction	Percent of +
Positive	**100**
38-1	37
38-2	27
38-3	30
38-4	26
38-5	28

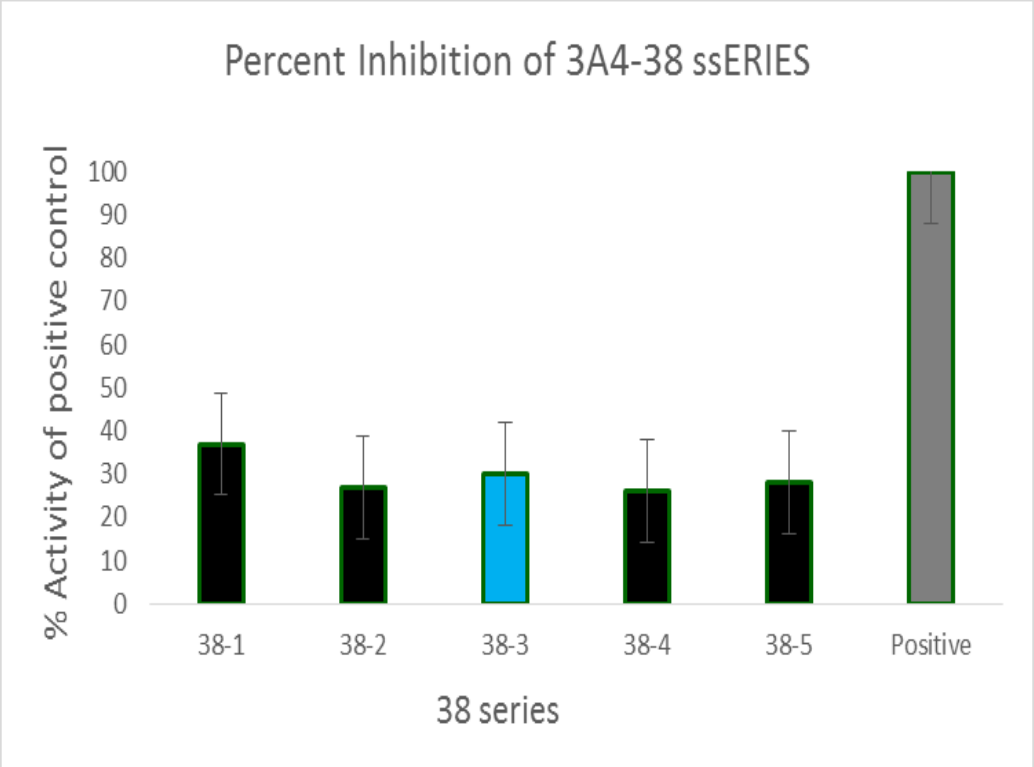


***Figure 3*. 38 Series inhibition of CYP3A4.** The final concentration of fraction in each sample was 25 mg/mL. While all fractions evidenced a similar level of inhibition, all with a percent activity 30-40%, it was determined that subsequent fractionations from 38-3 would be tested due to their relative availability.

Table 3. *% Activity of 3A4-62 Series.*

Fraction	Luminescence	Percent of +
Positive	**5346**	**100**
62-1	3972.75	74
62-2	2029	38
Positive	**7309**	**100**
62-3	2501	34
62-5	2447.5	33

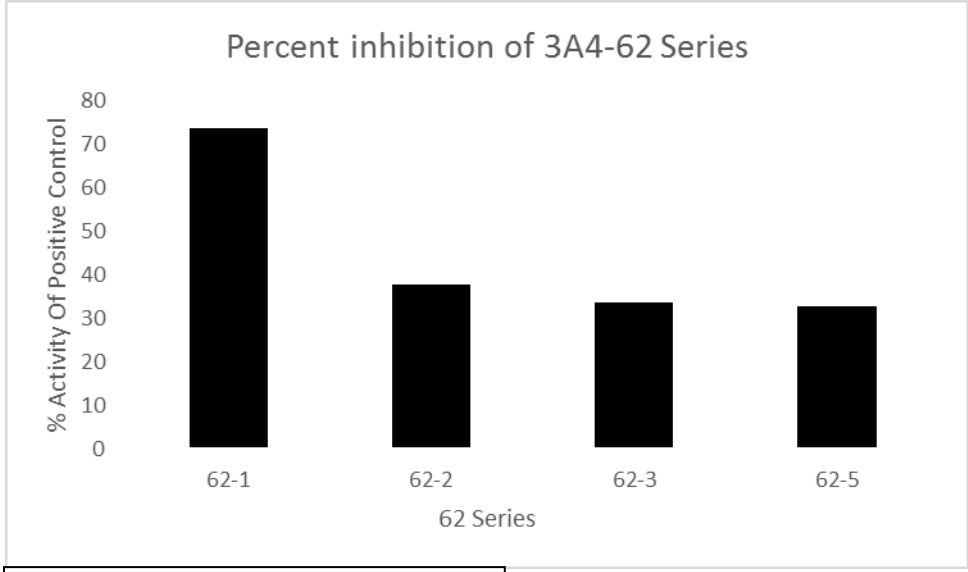


***Figure 4*. Percent inhibition of 3A4-62 series.** The activity of the fractions of the 62 series was

Table 4. *% Activity of 3A4-63 series*

Fraction	Luminesence	Percent of +
Positive	21704	100
4	6966	32
5	8479.5	39
6	6186	29

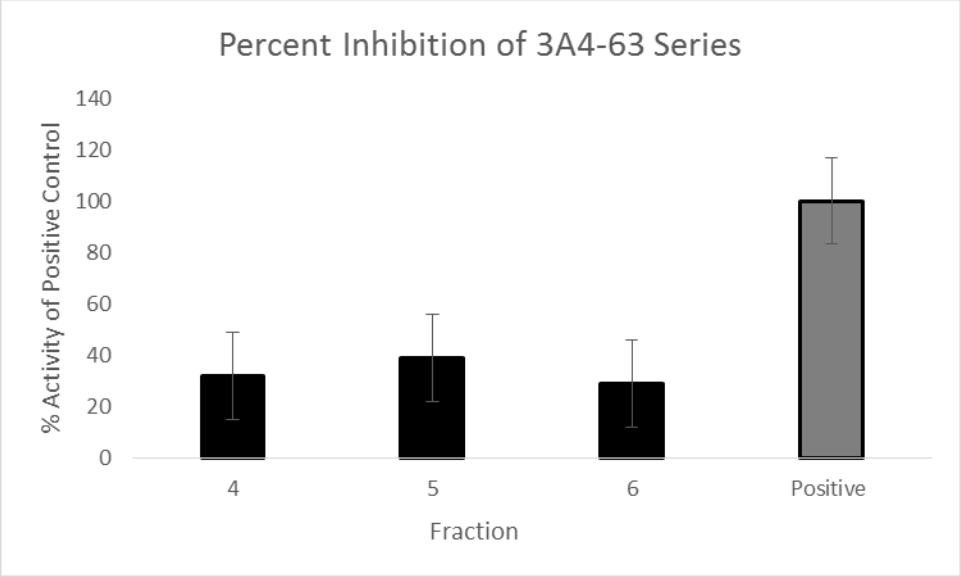


***Figure 5*. 63 Series inhibition of CYP3A4.** The luminescence assays preformed with the 63 series had a

Table 5. *% Activity of 3A4-98 Series.*

Fraction	Luminescence	percent of +
Positive	**821**	**100**
A	659	80
B	524	64
Positive	**3705.5**	**100**
C	1429	39
D	1253	34
E	1308	35
Positive	**1759**	**100**
H	448	27
I	776	44

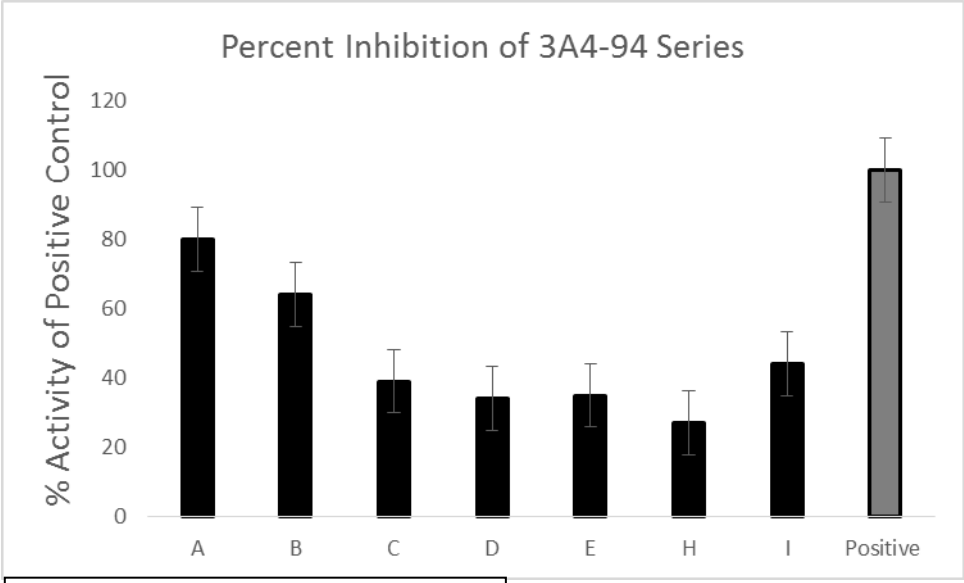
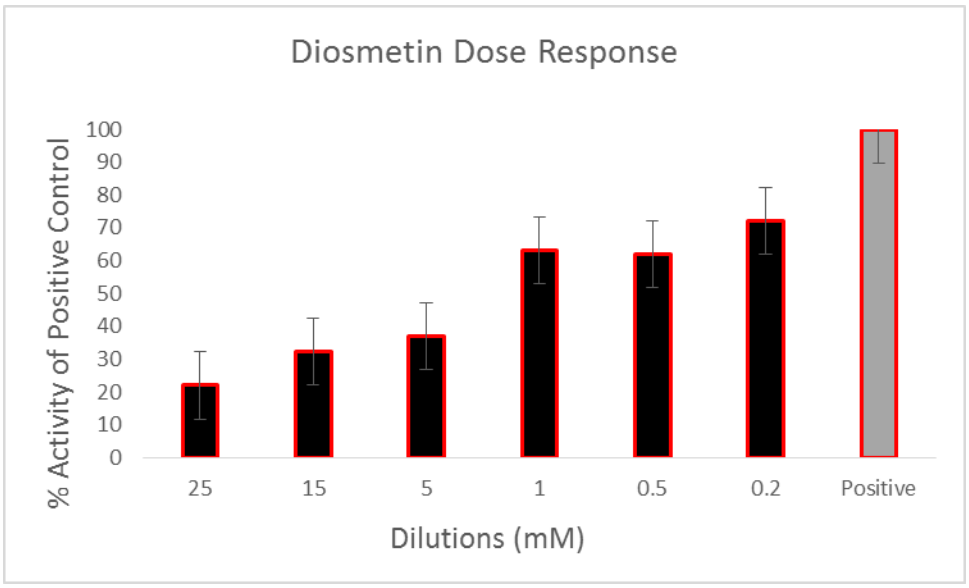


Figure 6. 94 Series inhibition of CYP3A4. The assays carried out

Table 6. *% activity of four different dilutions of diosmetin.*

Dilutions	**precent of +**
Positive	**100**
25	23.75596616
1	63.71390766
0.5	62.26312556
0.2	71.72156325



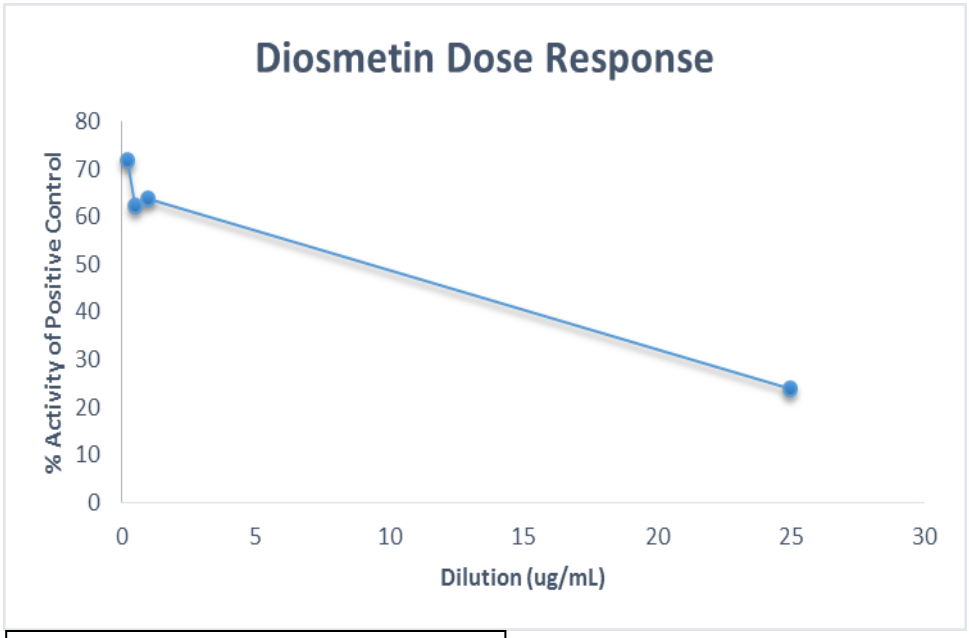


***Figure 7*. Percent inhibition of four different dilutions of diosmetin. A)** The luminescent

The results of this experiment strongly suggest that compounds in acaí berries inhibit CYP 3A4 *in vitro*. The results from the most crude chloroform extracts tested, 34 series, suggest that the majority of these fractions significantly inhibit CYP3A4 (Figure 1). These results were obtained by comparing the luminescence activity of each fraction to the value of the positive control. Reactions were carried in a 25µg/ml molar concentration. Concentrations were determined based on the purity of the sample factions which differ depending on the fractionation method and the place where they have originated from. Most notably, 34-8 proved to be the strongest inhibitor of CYP3A4, which is evidenced by 3A4's low percent activity of 10% in the presence of this fraction. A low percent activity means that there is large percent inhibition of the enzyme in the fraction.

Upon continuation of the activity guided fractionation approach, it was discovered that fractions in the 38 series all inhibit 3A4 to a similar level (30-40% activity); however, fractions from 38-3 were further pursued due to its relative availability (Figure 2). Table 2 shows the values of the luminescence activity of the 38 series fractions. Percentages are relatively close (26%-37%).

The activity guided fractionation approach was further performed on the 63 series. The assay results from the 63 series indicated that both 63-6 and 63-4 were inhibitors of CYP 3A4, both yielding a percent activity around 40% (Figure 3). Reactions were carried on a 5µg/ml plate reader wells. This reduction in concentration is due to the purity of the fractions. Due to its significant inhibition, fractionations from 63-4, 94 series, were tested. Potency of diosmeting on

CYP3A4 was determined using the same fractionation approach, it was discovered that 94-C and 94-H were the most potent inhibitors with a percent activity of 39% and 27%, respectively.

The identity of 94-C was previously determined to be the flavonoid compound Diosmetin, also known as 5,7,3'-trihydroxy-4'-methoxyflavone, which is a known inhibitor of CYP enzymes (2). Diosmetin is an O-mythelated flavone that was found to waken tyrosine kinase enzyme. In addition, it was found to play a role pharmacologically by being anti cansourous, antioxidant, anti-inflammatory, and also, antimicrobial. In order to determine the effective inhibitory dose of Diosmetin on 3A4, a dose response curve was produced (Figure 6) (11). It was determined that the effective inhibitory dose of Diosmetin is between 1-5mM, which is evidenced by the large decrease in enzyme activity between these two concentrations (Figure 6b).

The quantity of acaí berries that must be consumed to reach this inhibitory dose of Diosmetin is unknown, as the concentration of this compound in crude acaí berry has not yet been determined. It was also found that fractions 34-1 and 94-H strongly inhibit 3A4. The identity of these fractions is currently being determined by an outside source.

The cytochrome p450 family has a liege group of enzyme that is found predominantly in the liver. This group of enzymes is responsible for various functions, the most primary functions being metabolism of both nutrients and exogenic components such as pharmaceuticals. It is known that cytochrome p450-3A4 is larger responsible for metabolizing pharmaceuticals. It has been

approximated that this enzyme is responsible for metabolizing 30% of pharmaceuticals that are currently on the market. Further studies have shown that this enzyme is inhibited by certain components of grapefruit. Thus, inhibition of this enzyme while taking certain drugs, such as statins, has been shown to be harmful due to the inability of drug clearance; as a result, toxic levels of drugs build up in the system resulting in prolonged defects of these drugs and magnified effects of these drugs, both of which lead to undesirable outcome.

Similar studies are currently being explored and pursued as acai berry is being tested for potential inhibition effects on this enzyme looking for potential DDI. While diosmetin was found to be largely responsible for inhibition of CYP3A4 in the presence of acai berry fractions, further research must be done to further explore how acai berry is actually metabolized in *vivo* and weather diosmetin is conjugated with other proteins or flavonoids in *vivo*.

Results from these further studies can help illustrate how relevant acai berry in to inhibiting CYP 3A4 in a physiological settings. Another component of further research that must be determined is the amount of diosmetin per gram or volume of acai berry as it will prove to be relevant in dosage information regarding how much acai has to be consumed to revive an effective dose of diosmetin to inhibit CYP 4A4. The goal of this research was to determine if there was an adverse drug interaction caused by acai berry on CYP 3A4. These preliminary studies suggest that there is such a negative correlation between diosmetin and CYP 3A4 potentially leading to verifying that acai berry fractions and components of acai berry, specifically diosmetin, may inhibit the enzyme

3A4, thus leading to a toxic build-up of certain substrates of 3A4 mainly which are pharmaceuticals (9), thus leading to potential toxicity of these drugs while consuming acai berry. The relevance of this research might be avoiding consumption of acai berry while taking certain drugs such as statins.

Future Research

Future research is to be conducted in order to reinforce the finding that diosmetin is the primary inhibitor of CYP3A4 found in acaí berries. The dose response that this flavonoid can cause in the bod due to a specific amount of acai berries intake is also to be determined. Pharmaceutical products consumption along with the acai berries is dangerous and can cause drug-drug interactions. Furthermore, 94-H fraction is marked as a potential inhibitor of CYP3A4 isoform (20).

References

1. **Aitken AE, and Morgan ET**. Gene-specific effects of inflammatory cytokines on cytochrome P450 2C, 2B6 and 3A4 mRNA levels in human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* 35: 1687-1693, 2007.

2. **Androutsopoulos VP, Mahale S, Arroo RR, and Potter G**. Anticancer effects of the flavonoid diosmetin on cell cycle progression and proliferation of MDA-MB 468 breast cancer cells due to CYP1 activation. *Oncology reports* 21: 1525-1528, 2009.

3. **Anwar MA, Al Disi SS, and Eid AH**. Anti-Hypertensive Herbs and Their Mechanisms of Action: Part II. *Frontiers in pharmacology* 7: 50, 2016.

4. **Bailey DG, and Dresser GK**. Interactions between grapefruit juice and cardiovascular drugs. *Am J Cardiovasc Drugs* 4: 281-297, 2004.

5. **Bailey DG, Malcolm J, Arnold O, and Spence JD**. Grapefruit juice-drug interactions. *British journal of clinical pharmacology* 46: 101-110, 1998.

6. **Brantley SJ, Graf TN, Oberlies NH, and Paine MF**. A systematic approach to evaluate herb-drug interaction mechanisms: investigation of milk thistle extracts and eight isolated constituents as CYP3A inhibitors. *Drug metabolism and disposition: the biological fate of chemicals* 41: 1662-1670, 2013.

7. **Choi KH, Kim AJ, Son IJ, Kim KH, Kim KB, Ahn H, and Lee EB**. Risk Factors of Drug Interaction between Warfarin and Nonsteroidal Anti-Inflammatory Drugs in Practical Setting. *Journal of Korean medical science* 25: 337-341, 2010.

8. **D'Agostino J, Zhang H, Kenaan C, and Hollenberg PF**. Mechanism-Based Inactivation of Human Cytochrome P450 2B6 by Chlorpyrifos. *Chemical research in toxicology* 28: 1484-1495, 2015.

9. **Fowler S, Guerini E, Qiu N, Cleary Y, Parrott N, Greig G, and Mallalieu NL**. Low Potential of Basimglurant to Be Involved in Drug-Drug Interactions: Influence of Non-Michaelis-Menten P450 Kinetics on Fraction Metabolized. *The Journal of pharmacology and experimental therapeutics* 360: 164-173, 2017.

10. **Hansen TS, and Nilsen OG**. In vitro CYP3A4 metabolism: inhibition by Echinacea purpurea and choice of substrate for the evaluation of herbal inhibition. *Basic Clin Pharmacol Toxicol* 103: 445-449, 2008.

11. **Holderness J, Schepetkin IA, Freedman B, Kirpotina LN, Quinn MT, Hedges JF, and Jutila MA**. Polysaccharides isolated from Acai fruit induce innate immune responses. *PloS one* 6: e17301, 2011.

12. **Marcason W**. What is the acai berry and are there health benefits? *Journal of the American Dietetic Association* 109: 1968, 2009.

13. **Monroe KR, Murphy SP, Kolonel LN, and Pike MC**. Prospective study of grapefruit intake and risk of breast cancer in postmenopausal women: the Multiethnic Cohort Study. *British journal of cancer* 97: 440-445, 2007.

14. **Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, and Nebert DW**. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6: 1-42, 1996.

15. **Ogu CC, and Maxa JL**. Drug interactions due to cytochrome P450. *Proc (Bayl Univ Med Cent)* 13: 421-423, 2000.

16. **Palovaara S, Pelkonen O, Uusitalo J, Lundgren S, and Laine K**. Inhibition of cytochrome P450 2B6 activity by hormone replacement therapy and oral contraceptive as measured by bupropion hydroxylation. *Clinical pharmacology and therapeutics* 74: 326-333, 2003.

17. **Preissner SC, Hoffmann MF, Preissner R, Dunkel M, Gewiess A, and Preissner S**. Polymorphic cytochrome P450 enzymes (CYPs) and their role in personalized therapy. *PloS one* 8: e82562, 2013.

18. **Raner GM, Cornelious S, Moulick K, Wang Y, Mortenson A, and Cech NB**. Effects of herbal products and their constituents on human cytochrome P450(2E1) activity. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 45: 2359-2365, 2007.

19. **Sadowska-Krepa E, Klapcinska B, Podgorski T, Szade B, Tyl K, and Hadzik A**. Effects of supplementation with acai (Euterpe oleracea Mart.) berry-based juice blend on the blood antioxidant defence capacity and lipid profile in junior hurdlers. A pilot study. *Biology of sport* 32: 161-168, 2015.

20. **Shirasaka Y, Sager JE, Lutz JD, Davis C, and Isoherranen N**. Inhibition of CYP2C19 and CYP3A4 by omeprazole metabolites and their contribution to drug-drug interactions. *Drug metabolism and disposition: the biological fate of chemicals* 41: 1414-1424, 2013.

21. **Van Booven D, Marsh S, McLeod H, Carrillo MW, Sangkuhl K, Klein TE, and Altman RB**. Cytochrome P450 2C9-CYP2C9. *Pharmacogenetics and genomics* 20: 277-281, 2010.

22. **Wanwimolruk S, Phopin K, and Prachayasittikul V**. Cytochrome P450 enzyme mediated herbal drug interactions (Part 2). *EXCLI journal* 13: 869-896, 2014.

23. **Wanwimolruk S, and Prachayasittikul V**. Cytochrome P450 enzyme mediated herbal drug interactions (Part 1). *EXCLI journal* 13: 347-391, 2014.

24. **Zhou SF**. Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4. *Current drug metabolism* 9: 310-322, 2008.