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The Efficacy Of Curcumin And Oil Palm Phenolics, As Potential Antidepressants, On The Serotonin Pathway In A Neuronal Rat Cell Line

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THE EFFICACY OF CURCUMIN AND OIL PALM PHENOLICS, AS POTENTIAL ANTIDEPRESSANTS, ON THE SEROTONIN PATHWAY IN A NEURONAL RAT CELL LINE

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DEDICATION

I would like to dedicate this to my family for their never-ending love and support. They taught me not to be afraid of taking a unique path in life.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Introduction

1.1 Depression

The onset of major depressive disorder (MDD), or depression, can happen at all ages and overall is considered to be the largest contributor to disabilities. Worldwide, over 300 million people experience some form of depression.[1] Various forms of depression can develop.[2] These include persistent depressive disorder, which is having a depressed mood for two or more years; postpartum depression, an onset of depressed mood among women who are pregnant or post-delivery; psychotic depression, suffering from severe depression along with a form of psychosis; and seasonal affective disorder, onset of depression during the wintertime.[2] MDD is one of the most prevalent psychiatric disorders; an episode is diagnosed based on having the presence of five or more of specific symptoms that need to be persistent for at least two weeks while affecting one's mood, cognition, and behavior. All of these symptoms happen on a nearly daily basis and include depressed mood, fatigue, weight or appetite fluctuations, feeling worthless or unreasonably guilty, inability to think or concentrate, lack of interest or decreased ability to find pleasure in normal activities, recurrent thoughts of death/suicidal ideation, sleep disturbances, and psychomotor retardation or agitation.[2; 3; 4] Having just one episode of major depressive disorder is a risk factor for relapse – up to 85% of those who suffer from an episode may experience a relapse.[3; 5] Nationwide, numerous Americans also suffer from depression: a survey done showed that 13.3% of adolescents, 12-17 years old, and 7.1% of adults had at least one instance of an episode of major depression during 2017; in both groups, a major depressive episode was more prevalent in females than in males.[4]

Depression can affect quality of life and may be related to having a higher likelihood of comorbidities such as stroke, metabolic syndrome, and increased risk of mortality from cardiovascular disease.[6] Dementia and depression are also believed to be correlated; however, it is unknown whether depression is a result of dementia, vice versa, or depression and dementia coexist, yet are unrelated.[6; 7] Depression is also a symptom that is prevalent in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.[7; 8; 9]

Multiple hypotheses exist as to the biochemical cause of depression (Figure 1.1), with the monoamine hypothesis as the prevailing thought; which includes both the catecholamine and serotonin hypotheses.[10] The catecholamine hypothesis states that depression is caused by a catecholamine deficiency, especially norepinephrine, while mania is caused by an excess of catecholamines at critical brain synapses.[11; 12] Initially, it was believed that serotonin was not involved in the incidence of depression; however, this thought process changed with the creation and administration of selective serotonin reuptake inhibitors (SSRIs), observing that the depletion of tryptophan in formerly depressed patients could lead to a relapse, and observing a relationship between severely depressed patients and decreased plasma tryptophan.[13; 14]

Current treatment of depression involves utilization of psychotherapies and pharmaceuticals. There are multiple types of antidepressant medications, including the first generation of antidepressants, monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs); and the second generation, norepinephrine and dopamine reuptake inhibitors (NDRIs), selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), serotonin antagonist and reuptake inhibitors (SARIs).[15] While these classes of antidepressants have side effects specific to their class, such as patients who take SSRIs are more likely to develop osteoporosis, there are many common side effects with nutritional and

gastrointestinal implications, including changes in weight, loss of appetite, nausea, vomiting, diarrhea, and constipation.[15; 16] Antidepressants also can take 2-4 weeks for noticeable improvement, which may be discouraging to patients and result in decreased adherence to treatment plans.[2; 15]

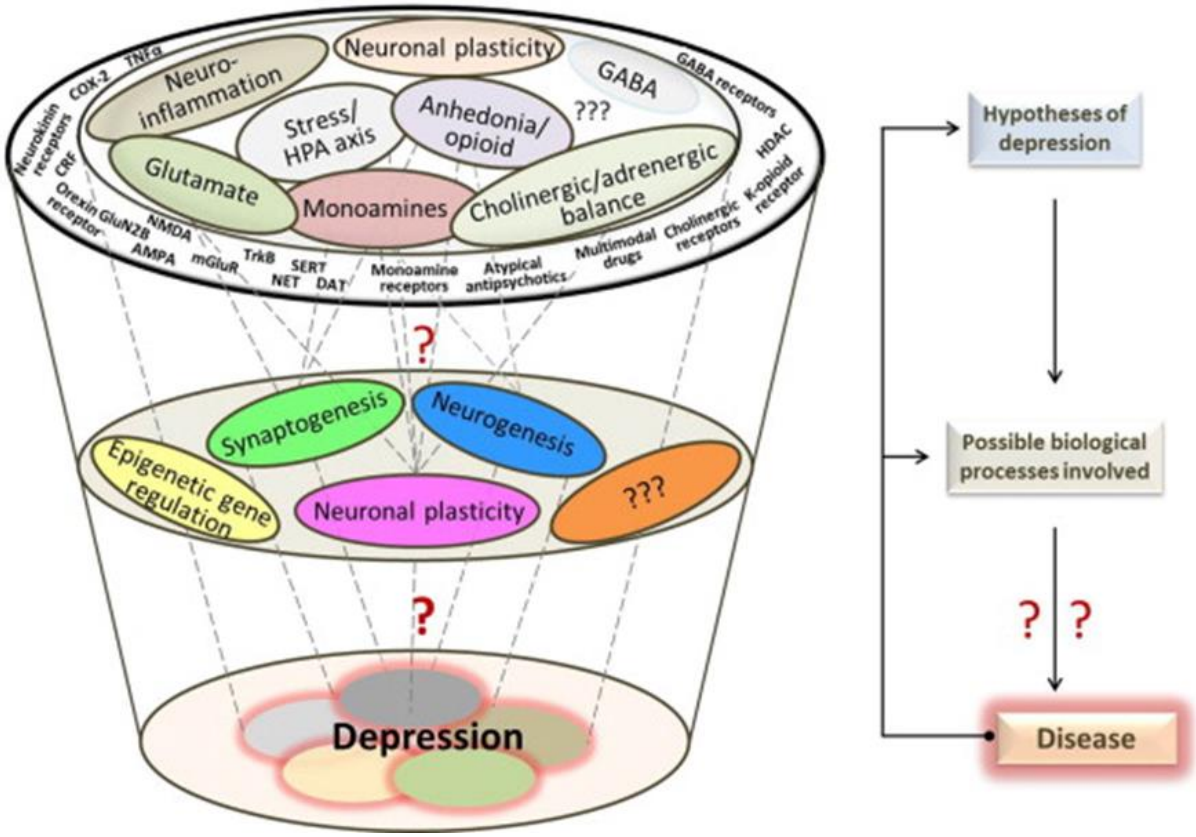


Figure 1.1: Potential Hypotheses of Depression. The top oval shows the possible causes of depression, along with their associated drug targets; the middle oval is representative of biological processes that are involved in the etiology of depression; the bottom oval shows the heterogeneity of the disease, including etiology, diagnosis, and manifestation.[10]

1.2 Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT) was first identified in the 1940s, as a vasoconstrictor.[16] Since then, it has been found to play a role in cognition, mood, appetite, and sleep.[17; 18; 19] Low levels of serotonin have been found to be associated with depression.[14] It is now known that the majority of serotonin is found peripherally: approximately 90-95% of

serotonin is found in the enterochromaffin cells, regulating intestinal mobility. The remaining ~5-10% is found in the brain where it functions as a neurotransmitter.[17]

Neurotransmitters, such as serotonin, allow for neurons in the brain to communicate with each other via chemical signals, which is necessary for proper functioning of the central nervous system. For this to happen, the enzymes and compounds required to synthesize the neurotransmitter must be present in the presynaptic cell. The neurotransmitter is then packaged, and the vesicle attaches to the bottom of the presynaptic cell. Entry of calcium into the presynaptic cell allows for depolarization to occur, which releases the neurotransmitter into the postsynaptic terminal. Here, the correct, specific receptors must be present to allow for the neurotransmitter to be able to bind to the postsynaptic cell.[20]

Tryptophan (TRP), an essential amino acid, can be metabolized through the kynurenine pathway, to quinolinic acid and kynurenic acid, which have neurotoxic and neuroprotective properties, respectively via interaction with the gamma-aminobutyric acid (GABA) receptor. Further, it can also be metabolized into serotonin (Figure 1.2). The hydroxylation of tryptophan into serotonin's precursor, 5-hydroxytryptophan, is carried out via the rate-limiting enzyme, tryptophan hydroxylase.[21] Serotonin is then synthesized by the enzyme, aromatic amino acid decarboxylase; serotonin can be further degraded to 5-hydroxyindoleacetic acid and melatonin by serotonin-N-acetyltransferase and hydroxyindole-O-methyltransferase.[22] Serotonin is unable to cross the blood-brain barrier (BBB); however, the precursors tryptophan and 5-hydroxytryptophan can.[23; 24; 25]

The first-generation antidepressants, MAOIs and TCAs, were designed by chance, and worked through interactions with multiple receptor sites; however numerous side effects resulted. This led to rational drug development in the late 1960s, as evidence started to suggest that serotonin plays

a significant role in the incidence of MDD, leading to a focus on inhibiting the reuptake of serotonin.[26; 27] Research by Carlsson and colleagues suggested that imipramine, a TCA, may alleviate depression by not only targeting noradrenaline, but also through blocking the reuptake of serotonin.[28; 29] This led to the development of SSRIs, which are currently one of the most prescribed pharmaceuticals for treating depression. They function by focusing more specifically on serotonin, and serotonin receptors (SERT); they bind to the serotonin receptor, which allows for the accumulation of serotonin within the synapse once released due to not being transported back into the neuron.[30] The first SSRI, zimeldine (Zelmid), was introduced to the market in Europe through the Swedish pharmaceutical company Astra AB in 1982; however, it was removed from the market after 16 months due to adverse side effects, including causing Guillain-Barre syndrome and hypersensitivity.[27] Fluoxetine (Prozac) was being investigated at the same time due to its potential as an SSRI, with a comparatively weak affinity for the norepinephrine transporter; it was approved in December of 1987 by the FDA, and introduced to the market in January of 1988, and is still prescribed today as an antidepressant, as well as to treat bulimia nervosa, obsessive compulsive disorder, and panic disorder.[27; 31]

However, SSRIs are only able to treat the symptoms of depression, can take weeks for patients to see any effects of treatment, can result in a relapse of depression, and may lead to adverse side effects such as abnormally low blood sodium levels, sedation, or gastric irritation.[10; 32] Also, taking SSRIs in combination with other classes of antidepressants or excess intake of SSRIs due to uninformed or unaware patients may lead to serotonin syndrome/toxicity, which may even be fatal.[33; 34] To determine if a patient is suffering from serotonin toxicity, diagnostic criteria has been developed due to the lack of availability of laboratory tests; some of the criteria involve taking or increasing the dosage of a serotonergic agent; symptoms including elevated mood, coma, altered

consciousness, hyper/hypotension, restlessness, and diarrhea; and having symptoms that are not from a preexisting psychiatric disorder.[33; 34] Discovering a treatment that treats the disease as opposed to just reducing symptoms, is more effective, and has fewer adverse effects is important for improving quality of life and decreasing the global burden of illness.

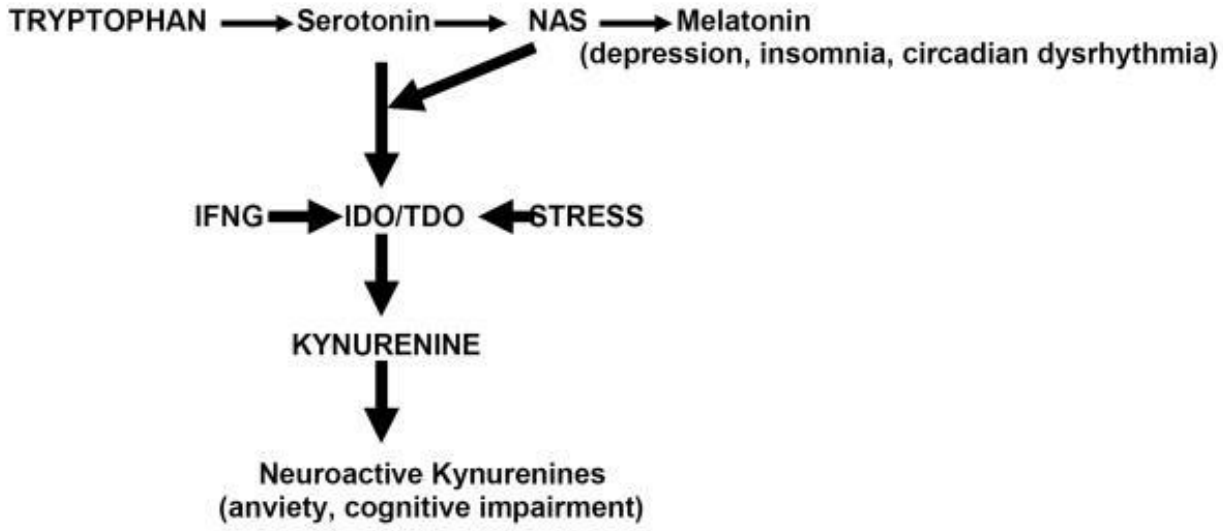


Figure 1.2: Metabolism of tryptophan. NAS – N-acetylserotonin; IFNG – interferon-gamma; IDO – indoleamine 2,3-dioxygenase; TDO – 2,3-dioxygenase tryptophan[35]

1.3 Nutraceuticals

The saying "let thy food be thy medicine and medicine be thy food",[36] commonly attributed to Hippocrates, is a thought process that still prevails in certain traditional medicinal practices, such as Ayurveda and Traditional Chinese Medicine, where nutrition is believed to play a role in disease prevention. In the westernized world, the initial thought of nutrition was not related to disease prevention; instead, due to the population expansion, food that was safe while providing enough energy and nutrients was the most important concern.[36; 37] More recently, awareness of the importance of a balance between nutrition and a healthy lifestyle has become more prominent, eliciting an increase in multidisciplinary research in an attempt to increase the overall quality of life.[36; 37]

The term nutraceutical was first used by Dr. Stephen DeFelice in 1989, combining the words nutrition and pharmaceuticals. He defined it is "a food or part of a food, such as a dietary supplement, that has a medical or health benefit, including the prevention and treatment of disease".[38; 39; 40] Foods and beverages rich in phenolic content, such as fruits, chocolate, tea, and coffee fit within this category, and exhibit many promising properties, including antioxidant, anti-inflammatory, anticarcinogenic, antiviral, anticancer, and neuroprotective.[41]

The Canadian Network for Mood and Anxiety Treatments (CANMAT) has recommended certain dietary agents that have been examined for their potential to reduce the depressive side effects seen in those with MDD, including omega-3 fatty acids, saffron, S-Adenosyl-L-Methionine (SAM), folate, lavender, acetyl-L-carnitine (ALC), dehydroepiandrosterone (DHEA), and St. John's Wort; however, more research is being done on other agents such as curcumin, citicoline, vitamin D, vitamin C, inositol, and tryptophan, with results that are currently inconclusive.[42; 43]

1.4 Curcumin

The bioactive constituent of Curcuma longa, also known as turmeric, is the polyphenol, curcumin.[44] It is fat-soluble, used as a spice, and as a coloring agent, giving foods a yellow color. Although medicinally, curcumin has been consumed for thousands of years, it wasn't officially discovered until a couple of centuries ago, in the early 1800s, by Vogel and Pelletier.[45; 46] The rest of the 19th century was spent working on identification of the structure; in 1910 the structure was finally identified, and then work began towards synthesis of the compound (Figure 1.3).[46; 47] During the mid-1940s the first paper on biological characteristics of curcumin was published; Schraufstätter and Brent reported curcumin exhibited anti-bacterial properties; inhibiting the growth of *Staphylococcus aureus*, *Salmonella paratyphi*, *Trichophyton gypseum*, and *Mycobacterium tuberculosis*.[48] Following that publication, other research has shown that

curcumin exhibits other properties as well, including neuroprotective, anti-inflammatory, anti-oxidative, and anti-cancer, anti-diabetic, and an ability to lower cholesterol.[44; 49; 50; 51; 52] An individual's ability to absorb curcumin is low due to the compound's poor bioavailability, although, with the addition of other compounds such as piperine the bioavailability does increase.[44] Due to curcumin having a low bioavailability, dosage for treatment becomes an important focus. It is imperative to ensure that an individual gets a large enough dosage of curcumin for there to be a noticeable and positive effect. However, too high of a dose may be toxic, but reported side effects of curcumin toxicity are mild, including nausea, diarrhea, and dizziness in humans, with unknown long-term side effects; although in some cell lines adverse effects, including inhibition of cytochrome P450 activity and nuclear and mitochondrial DNA damage were observed.[44; 49; 53]

Curcumin has demonstrated antidepressant effects; a meta-analysis examined the effects of curcumin on depression in humans, and even though some studies showed no significant effect of treated patients as compared to the control there was still evidence of reduction in anxiety and depressive symptoms.[53] A pilot study has shown that an anti-inflammatory drug, acetylsalicyclic acid (ASA) in addition to antidepressants may improve symptoms of those suffering with depression in a shorter time, leading to increased compliance with treatment regimen, suggesting that other anti-inflammatory compounds, such as curcumin, may exhibit a similar effect.[54]

Curcumin's anti-inflammatory properties indicates that it may also be able to enhance treatment outcomes when given in addition to an antidepressant drug; a study conducted in rats induced with a stressor led to increased protein level of indoleamine-2, 3-dioxygenase (IDO), as well as an increase in the kynurenine (KYN) and KYN/TRP ratio, with a concurrent decrease in serotonin levels; rats that were induced with a stressor but also receiving an oral gavage of

curcumin resulted in a significantly decreased protein level of IDO, significantly decreased levels of KYN and KYN/TRP, and significantly elevated levels of serotonin with respect to the stress induced group, suggesting that the anti-inflammatory effects of curcumin may be part of the mechanism of curcumin's antidepressant properties.[55] Previously in our lab, we investigated the effect of curcumin on a high cholesterol diet induced Alzheimer's model. In addition to alleviating markers of the disease, including amyloid β deposition, using the metabolomics technique we observed an impact on the tryptophan/serotonin pathway. Thus the purpose of the current study was to determine if curcumin also exhibits antidepressant properties within a neuronal rat cell line.

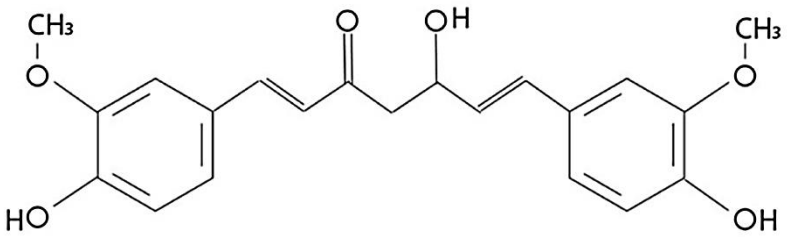


Figure 1.3: Structure of curcumin[56]

1.5 Oil Palm Phenolics

Elaeis guineensis jacq, the African oil palm, is the highest oil producing plant, and is believed to have originated in West Africa, with evidence suggesting the existence of this plant as early as 3000 BC in Egypt. However, the current leading manufacturers are in the southeastern Asian region, specifically Indonesia and Malaysia.[57; 58] The kernel of the fruit is where palm kernel oil is extracted from, commonly used in oleochemical applications. The edible portion, palm oil, is extracted from the mesocarp of the oil palm; oil palm phenolics (OPP) are the phenolic compounds that get dissolved and are removed in the aqueous waste product during the process of oil extraction. As determined by high-pressure liquid chromatography (HPLC) analysis, oil palm phenolics have a high concentration of the three isomers of caffeoylshikimic acid, *p*-

hydroxybenzoic acid, protocatechuic acid, hydroxytyrosol, and gallic acid; also present in substantial amounts is 2,3-dihydroxybenzoic acid, chlorogenic acid, caffeic acid, and ferulic acid.[59; 60]

OPP has been reported to have antioxidant and anticancer properties; it may also offer protection against diabetes.[61; 62; 63] Based on a study done by Sambanthamurthi *et al.*, OPP may also have cardioprotective effects: OPP was found to dose-dependently inhibit the copper mediated oxidation of human LDL *in vitro*; they also observed a relaxation in the aorta and resistant vessels in rat tissue, suggesting that OPP has the potential to lower blood pressure throughout the whole organism.[59]

Resveratrol, a phenolic compound that is found in wine, grapes, berries, and peanuts, has been reported to exhibit beneficial properties, including antioxidative, anticancer, anti-inflammatory, cardioprotective, and neuroprotective. Although the mechanism is not fully understood, a few studies have also suggested that resveratrol may exhibit anti-depressant properties: Xu et al. found elevated serotonin levels in the frontal cortex, hippocampus, and hypothalamus of mice along antidepressant effects, while Ahmed et al. saw restoration of serotonin levels with treatment of resveratrol in rats induced with depression.[64; 65] Since resveratrol and OPP are both phenolic compounds, and share certain properties such as antioxidative and anticancer, they may share similar antidepressant properties as well.

Furthermore, previous work in the lab in a high cholesterol diet induced model of Alzheimer's disease suggested that OPP is able to modulate the tryptophan pathway, observing elevated levels of serotonin through plasma and urinary metabolomics.[66; 67] Because of this we wanted investigate the effects of OPP on the serotonin pathway in a neuronal cell line to see if it would be able to be a potential agent for the management of depression.

1.6 RN46A-B14 Cell line

For this study a neuronal rat cell line, biologically from embryonic (day 13) medullary raphe, was used. This cell line, RN46A-B14, was supplied by the European Collection of Cell Cultures (ECACC; Salisbury, United Kingdom) as catalog number 12061303, and was purchased from CellBank Australia (Westmead, NSW, Australia). The cell line has been described per the originator of the cell line, Dr. Scott R. Whittemore.[68] These cells were isolated after transfecting the RN46A cell line with brain-derived neurotrophic factor (BDNF); because of this the RN46A-B14 cells are able to secrete and synthesize BDNF, while also able to synthesize serotonin.[68] They have been infected "with a retrovirus encoding the temperature-sensitive mutant simian vacuolating virus 40 (SV40) large T antigen".[69] In a proliferative state, these cells have fibroblast-like morphology; however, increasing the temperature from 33°C to 39°C cause the oncogene which drives proliferation to be inactivated, resulting in differentiation of the cells to a neuronal morphology, which is why we wanted to use this cell line to investigate the effects of OPP and curcumin on depression (Figure 1.4).[68]

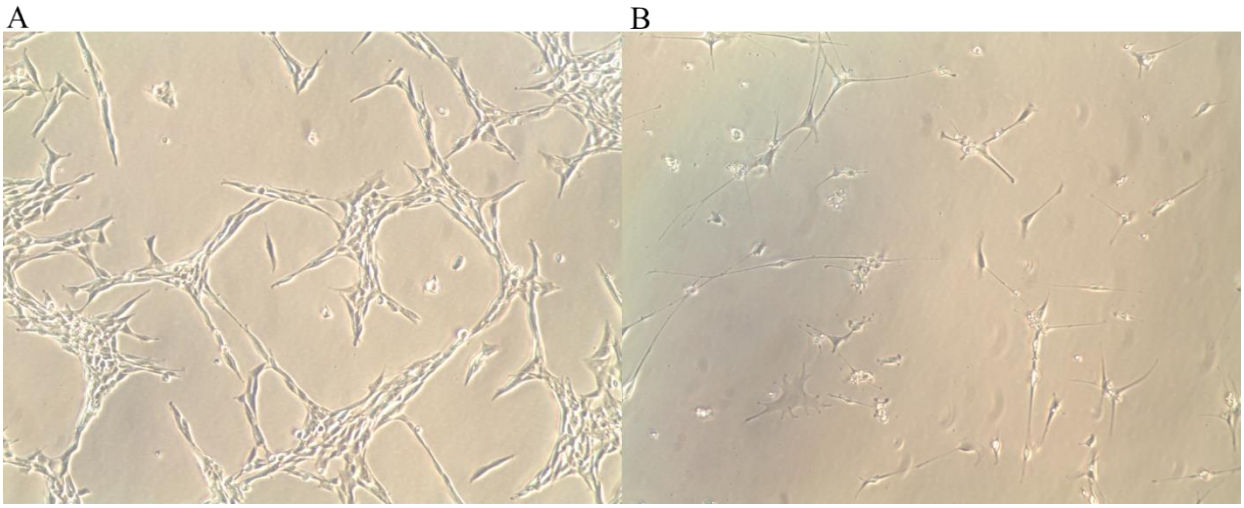


Figure 1.4: Morphology of RN46A-B14 cell-line. A) Morphology of the proliferating cells, two days after passaging, and B) the differentiating cells, eight days after induction of differentiation.

1.7 Metabolomics

It has been commonplace for branches of science to use the reductionist approach, meaning that the problem being researched, observed, or diagnosed has been reduced to the simplest level possible. While this does allow for better understanding of a certain mechanism or area within a pathway, it does not account for the fact that within a living organism, multiple systems are working together, as an interconnected network.[70] Systems biology has been an increasingly popular approach used to study an overall living organism, and involves "omics" sciences – genomics, transcriptomics, proteomics, and metabolomics (Figure 1.5).[71] Systems biology is less focused on homeostasis; instead, the idea of dynamic stability is important, which allows for small alterations to occur, but the summative effect of the changes leads to no change overall.[70]

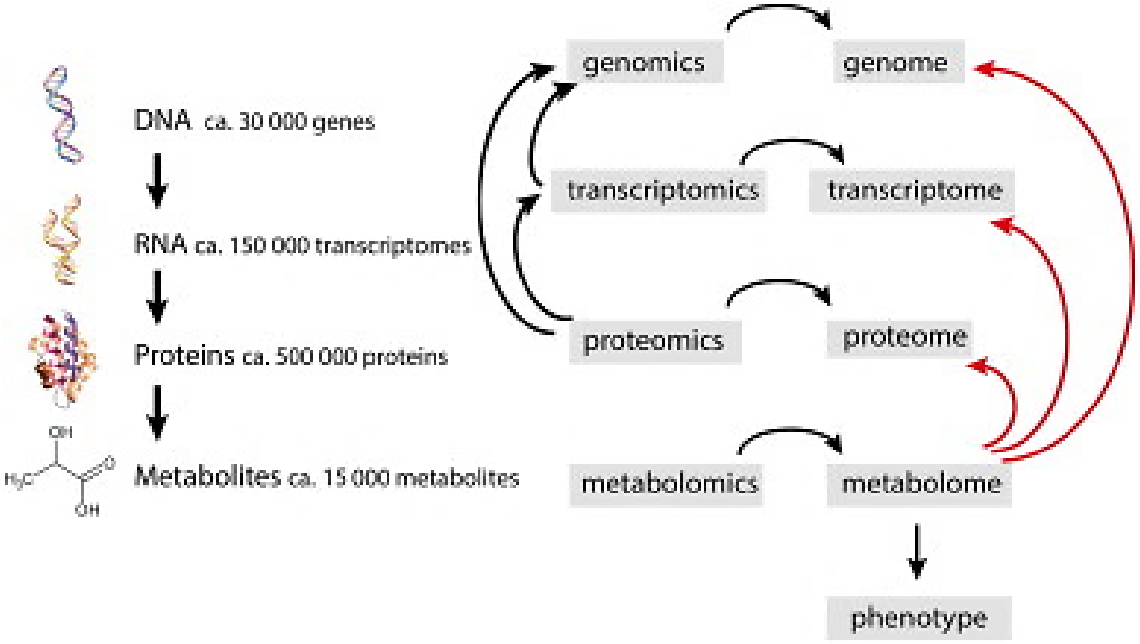


Figure 1.5: Approach of systems biology. The omics approach is seen here, where each system can be looked at individually; however, the relationship between all the omics cannot be downplayed. Changes seen in genomics, transcriptomics, and proteomics may be indicative of behavior changes present within the organism, while alterations in metabolomics are related to changes in the current status.[71]

Metabolomics is the newest branch of the omics approach; it involves the identification and quantification of small molecular metabolites, at a certain time point similar to taking a picture of the current functional status of the cells, which is indicative of whether there have been any positive or negative changes due to adaptations to dietary supplementation or as a response to stress, respectively.[72] The endometabolome and exometabolome can be both be studied, where the endometabolome includes all intracellular metabolites, such as cell extract for cell culture studies and plasma, saliva, and cerebral spinal fluid, for organisms; the exometabolome is composed of external metabolites that have been excreted, such as the growth media for cell culture and urine, feces, and saliva for organisms.[73]

1.8 Hypothesis

Previous data from the lab indicated that supplementation with curcumin and OPP might attenuate some of the effects seen in rats with hypercholesterolemia induced Alzheimer's disease. Examination of urinary metabolomics indicated that curcumin played a role in inhibiting the neurotoxic pathway of tryptophan metabolism to quinolinic acid, while increasing the presence of mood-elevating serotonin.[67] The mechanism for curcumin increasing the metabolism of tryptophan to serotonin is of interest and needs to be better understood. Curcumin and OPP exhibit similar properties, such as antioxidative and anticancer. Due to the antidepressant effect that has been seen with supplementation of curcumin, we wanted to investigate whether OPP would act in a similar way.

Aim 1: To determine the appropriate concentrations or dose of curcumin and OPP treatment in the proliferating and differentiating neuronal cells using a cell viability assay on the proliferating cells.

Aim 2: To examine changes in the gene expression of serotonin pathway-related genes, *Tph1, Tph2, Aanat,* and *Asmt* and investigate if there are protein level changes of TPH1 and TPH2 due to treatment of curcumin and OPP.

Aim 3: To conduct metabolomic analysis on the cell extracts, to evaluate differences between the proliferating and differentiating cells, as well as any differences in each subset between curcumin and OPP treatments; identify and quantify which metabolites are responsible for the differences of metabolic profiles.

CHAPTER 2

METHODS

2.1 Cell culture

Since RN46A-B14 cells exhibit both proliferative and differentiating properties, specific cell culture media was needed to be made for each subset. The base for both was 1:1 Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F-12) (Gibco). For proliferation of the cells, 10% fetal bovine serum (FBS), 2 mM L-Glutamine, and antibiotics: 0.25 mg/ml geneticin (G-418) and 0.1 mg/ml hygromycin (Thermo Fisher Scientific) were added to the DMEM/F12. For differentiation, 0.1% bovine serum albumin (BSA) (Sigma Aldrich), 0.1 mg/ml hygromycin, 1X N-2 and 1X B-27 supplements (Thermo Fisher Scientific) were added to the DMEM/F12.

Curcumin (Sigma Aldrich) and oil palm phenolics (Malaysian Palm Oil Board (MPOB), Malaysia) were used as treatments for the cell culture. In brief, cells were plated into two sets of 150 mm Petri dishes (Thermo Scientific) and fed with proliferation media. One set was for the proliferating cells, where the cells were seeded, and after 48 hours the media was replaced with untreated or treated media; the other set of plates were seeded, and after 4 days the plates were moved to the 39°C incubator with the differentiation media. After an additional 6 days, untreated or treatment media was added. Both the proliferating and differentiating cells were treated with 6 µM of curcumin, 1.5% OPP (stock solution of 1500 parts per million (ppm) gallic acid equivalents (GAE)), or dual-treatment of 6 µM and 1.5% OPP for 48 hours prior to sample collection.

RN46A-B14 cells were maintained in T-75 flasks (Fisher Scientific). The proliferating cells were kept in a humidified incubator (Thermo Fisher Scientific) at 33°C with 5% CO_2. For inducement of differentiation, proliferating cells at 70-80% confluency were moved into a 39°C

humidified incubator, also at 5% CO_2, with the addition of differentiation media. The cells were considered fully differentiated after 8 days.

These cells are adherent in nature, so in order to passage the cells, a protocol needed to be used that allowed for the cells to be detached from the bottom of the flasks. Once 70-80% confluence was reached in the proliferating cells, phosphate buffered saline (PBS) was added to the flask to rinse the cells, ensuring that the FBS added to the media was removed; if FBS were still present in the flask, the trypsin would be inactivated. Trypsin/ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific) was added to cleave the cells from the bottom of the flask. Media was added to collect the cells, and pelleted down via centrifugation, resuspended, and split into new flasks or plates.

For freezing the proliferating cells, the above steps were followed, but during the resuspension the media was either a lab-made solution of 90% proliferation media plus 10% dimethyl sulfoxide (DMSO), or commercial cell culture freezing medium (Thermo Fisher Scientific). The cells were then placed into 1.2 ml cryovials (Thermo Fisher Scientific) and cooled in stages to avoid shocking the cells. First, the cells were cooled overnight at -20°C, moved to the VWR -80°C freezer (Thermo Fisher Scientific) for another 24 hours, and finally placed in liquid nitrogen for long-term storage.

2.2 Cell Viability Assay

To determine the cell viability of proliferating, treated RN46A-B14 cells in relation to the control cells, a 3-(4,5–dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)–2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was carried out by using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). Briefly, proliferating cells were counted and plated at a volume of 200 µl in a 96-well plate at a density of 5,000 cells per well. After 24 hours, the original media

was removed, and varying concentrations of curcumin in media (0-20 µM) or OPP in media (0-10%) was added, with a final volume of 200 µl per well. Then, after 72 hours of treatment, 10 µl of CellTiter was added to each well. The plate was incubated at 33°C for 1 hour, and the absorbance was then read on a microplate reader (Biotek ELx800) at 490 nm. The average absorbance of pure proliferation media was subtracted from the average absorbances of the control and treated cells. Then the relative cell viability was calculated by dividing the average cell viability (absorbance) of the treatment by the average cell viability (absorbance) of the control, and multiplying by 100. Six replicates were used for each condition.

2.3 Real-time quantitative Polymerase Chain Reaction analysis

To determine the expression of genes present in the serotonin pathway, real-time quantitative polymerase chain reaction (qPCR) analysis was done. The RN46A-B14 cells from each treatment type were then from the flasks and counted; 2 million cells from all four treatment conditions of the proliferating and differentiating cells were used for total RNA extraction.

The protocols from QIAshredder and RNeasy Mini Kit (Qiagen) were followed, with an additional RNA cleanup step done to eliminate the presence of genomic DNA (gDNA) contamination. Briefly, the cells were lysed and homogenized using the QIAshredder, ethanol was added to allow the total RNA to bind to the membrane of the column, 3 washes were then done to eliminate possible contaminants, and the total RNA was eluted; as an additional RNA purification step, the procedure was repeated, excluding the lysing and homogenization.

Total RNA was then converted to complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Briefly, the total RNA was quantified using the NanoDrop (Thermo Fisher Scientific); for each sample, 1 µg of total RNA was added to the reverse transcription master mix, containing random primers, deoxyribose nucleoside

triphosphate (dNTP) mix, and an RNase inhibitor, with (+RT) or without (-RT) reverse transcriptase. A thermocycler, Mastercycler® RealPlex^4, was used (Eppendorf) to reverse transcribe the RNA to cDNA by incubating the samples at 25°C for 10 minutes, extending at 37°C for 120 minutes, heat-inactivating the reverse transcription enzyme at 85°C for 5 minutes, and a final hold step at 4°C.

To check for contamination, PCR was run, using DreamTaq Green Master Mix (Thermo Fisher Scientific); the templates were either the +RT and -RT cDNA samples, gDNA as a control, and a non-template control (NTC) as a negative control; the primer pair *succinate dehydrogenase complex flavoprotein subunit A* (*Sdha*) was used (Table 2.1). The following thermocycler conditions were used for this step: initial denaturation at 95°C for 3 minutes; 30 cycles of the following 3 steps: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds; a final extension step at 72°C for 15 minutes, the samples were then held at 4°C, and stored at -20°C.

2% agarose (Fisher Scientific) gels were made and placed in a 1X Tris-borate-EDTA (TBE) solution (EMD Millipore); the samples from the cDNA synthesis were loaded. After running the gel, it was then stained with ethidium bromide (Bio Rad, Hercules, CA) to allow for the bands to be detected. The products from the gDNA were expected to be at 728 bp, while the cDNA was at 127 bp. If the gDNA band was not present in the +RT samples, and the -RT samples and NTC did not have any bands, the cDNA was deemed usable for real-time qPCR.

Real-time qPCR was done to quantify the mRNA levels. Primers for *Ubiquitin-conjugating enzyme* (*Ubc-9*), used as a loading control were found in a previously published paper.[74] Primer-BLAST from National Center for Biotechnology Information (NCBI) was used to design the remaining primers: *tryptophan hydroxylase 1* (*Tph1*), *tryptophan hydroxylase 2* (*Tph2*),

acetylserotonin O-methyltransferase (*Asmt*), and *aralkylamine N-acetyltransferase* (*Aanat*) (Table 2.1). PowerUp SYBR Green (Applied Biosystems) was used for the master mix. The gene expression changes are represented as the mean (±SEM) of the fold changes observed in the proliferating and differentiating cells. For both subsets of cells, fold changes were calculated by relative comparison of each of the treatments (6 µM curcumin, 1.5% OPP, and dual-treatment) to the control groups. A minimum of three replicates of RNA from each subset of cells and treatments were used for this experiment.

Table 2.1: Primers used for gene expression analysis

Gene	Primer orientation	Primer sequence (oriented 5' to 3')
Sdha	Forward	GCA GAA GAA GCC ATT TGC GG
	Reverse	AGC ACA GTC AGC CTC ATT CAA
Ubc-9	Forward	TCG TAC CTT TCT CAC CAC AGT ATC TAG
	Reverse	GAA AAC TAA GAC ACC TCC CCA TCA
Tph1	Forward	ATA CTG TGG CTA TCG GGA AGA C
	Reverse	ATG GAA AAC CCT GTG CGT TC
Tph2	Forward	CCG ACC ACC CAG GAT TCA A
	Reverse	TCC CGA AAC ACA ACA CCC CA
Asmt	Forward	TTC TTC CGT TCC CGC CT
	Reverse	ACC ATC AGC CCA GTC GT
Aanat	Forward	CAC ATC CTT CTC TGC CCA AC
	Reverse	TGA CCC TTA CCT CTC TAC TCC AC

2.4 Western blotting analysis

To determine the level of TPH1 and TPH2 proteins present within the cells, whole RN46A-B14 cell protein extracts were subjected to western blotting analysis. The cells came from the same 150 mm Petri dishes that the cells were obtained for the RNA extraction mentioned in section 2.1;

however, 4 million cells were used in this step. The cells were pelleted and resuspended in PBS two times, to wash the cells from any residual media. 1X RIPA buffer and protease inhibitor were added to the pellet cells, resuspended, and shook on a shaker at 4°C for 15 minutes. The solution was then centrifuged at 14,000 g for 15 minutes. After this, the supernatant, containing the protein extract, was carefully removed as to not disturb the pellet containing cell debris, and placed into a new 1.5 ml tube (Eppendorf), then placed in the -20°C freezer for storage; the remaining cell debris was discarded.

On the first day of the western blot, the total protein concentration of the samples was determined by conducting a bicinchoninic acid (BCA) assay (Thermo Scientific). Briefly, eight known concentrations of bovine serum albumin (BSA) were prepared. 10 µl of each standard and unknown protein sample were pipetted into a 96-well plate. 200µl of working reagent was then added to each well. The plate was incubated at 37°C for 30 minutes, and then read with the plate reader at 570 nm. Normalized protein samples were then prepared, using 25 µg of protein per sample, with 2-Mercaptoethanol (βME) (Sigma Aldrich) and 1X lane marker reducing sample buffer (Thermo Fisher Scientific). Samples were loaded into a precast 4–15% Mini-PROTEAN® TGX Stain-Free™ (Bio Rad) polyacrylamide protein gel; after separation the blots were transferred onto Trans-Blot® Turbo™ Mini polyvinylidene fluoride (PVDF) membranes (Bio Rad). The membranes were blocked overnight at 4°C, washed, incubated overnight with the primary antibody for the target proteins at 4°C, washed, and incubated on a shaker with the secondary antibody at room temperature for one hour. The protein bands were then detected with Clarity™ enhanced chemiluminescent (ECL) substrate and read using ChemiDoc™ XRS and Image Lab software (Bio Rad). The membranes were re-blocked for one hour, washed, re-probed

with the primary antibody, targeting the loading control protein, overnight at 4°C, washed, and incubated on a shaker with the secondary antibody at room temperature for one hour, then detected.

The primary antibodies used were TPH1 (1:1000) (Invitrogen), TPH2 (1:500) (Invitrogen), and β-actin (Cell Signaling); the secondary antibody used was goat-anti-rabbit (H + L) horseradish peroxidase (HRP) conjugate (1:3000) (Bio Rad), with StrepTactin HRP Conjugate (1:5000) (Bio Rad). Three replicates were used for this experiment.

2.5 Metabolomics

To collect the exo-metabolome and the endo-metabolome from the RN46A-B14 cells, the same procedure was followed as is discussed in section 2.1. On the day of sample collection, 2 ml of media was collected and centrifuged at 4°C, 2,000 g for 5 minutes; 1 ml of the supernatant was then transferred to a new tube, snap-froze in liquid nitrogen, and stored at -80°C for future analysis. The following steps were all conducted on ice: the remaining media from the plate was removed, and the plate was rinsed two times with PBS. Ice cold methanol/water (80:20), pre-chilled at -80°C overnight, was then added to the plate, and immediately after the plate was scraped vigorously to collect the cell extract, which was placed in a 2 ml tube. An additional volume of ice-cold methanol/water (80:20) was added to collect any residual cell extract and then also added to the same 2 ml tube. The tube then was vortexed and incubated in an ice/ethanol water bath for 5 minutes. The tubes were centrifuged at 4°C, 5,725 g for 5 minutes; the supernatant, containing the cell extract, was removed and transferred to a new 1.5 ml tube; the 2 ml tube contained the protein pellet. All tubes were then snap-froze in liquid nitrogen and stored at -80°C; the following day the cell extract was freeze-dried using the CentriVap benchtop vacuum concentrator (Labconco), snap-froze, and stored at -80°C until further analysis.

The day prior to conducting nuclear magnetic resonance (NMR) metabolomic analysis, a 0.5 mM solution of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in deuterium oxide (D_20) was prepared. A reference was prepared with the DSS/D_20 solution; samples were prepared by adding the DSS/D_20 solution to reconstitute the dried cell extract powder; the samples were centrifuged and transferred to 5 mm Aldrich® ColorSpec® NMR tubes (Sigma Aldrich) using Pasteur pipettes (Fisher Scientific).

The Agilent DD2 600 MHz NMR spectrometer was used to acquire spectra for each of the samples for ^1H at a temperature of 25°C at a frequency of 599.73 MHz, 64 scans, and nuclear Overhauser effect spectroscopy (TNNOESY) was used for the pulse sequence. The spectra were then imported into the Spectrus Processor (ACD/Labs 9.0), where the free induction decay (FID) files were Fourier transformed. Batch-processing of the spectra allowed for auto-phasing and correction of the baseline, using the DSS peak as a reference to set the spectra to 0 ppm; manual phasing and baseline corrections were done to individual spectrum if needed. Intelligent bucketing was used to divide the spectra into integrals; the resulting table of common integrals was exported, as values. This table was imported into soft independent modeling of class analogy (SIMCA 15.0) (Umetrics) to allow for multivariate analysis.

To identify and quantify metabolites of interest, the FID files were imported into the Chenomx suite (Chenomx 8.4), Fourier transformed, and then they were individually phased, baseline corrected, and had the chemical shape and shift indicator (CSI) calibrated to the DSS peak in the processor application. The profiler application was used to fit the peaks and clusters of each individual spectra, using DSS as the reference for concentration purposes, and utilizing Chenomx's 600 MHz library, which is a database of metabolites they have profiled in their system. At least four replicates were used for each condition of both the proliferating and differentiating cells.

2.6 Statistical Analysis

Statistical analyses were performed using the unpaired t-test with GraphPad Prism software 8.00 (La Jolla, CA); p values of less than 0.05 were considered statistically significant.

CHAPTER 3

Results

Aim 1: To determine the appropriate concentrations or dose of curcumin and OPP treatment in the proliferating and differentiating neuronal cells using a cell viability assay on the proliferating cells.

3.1 Determination of the acceptable concentrations for curcumin and oil palm phenolics

The RN46A-B14 cell line had not been used in this lab prior to this work. So it was important to first learn about how the cells grow, and what treatment ranges were acceptable. In cancer cells, determining the viability of cells is done to see at what point growth inhibition occurs; this is the desired effect as the focus of cancer treatments is to stop the proliferation of cells. However, for this study, the purpose was to find a working concentration of curcumin and OPP in the proliferating cells that would not be overly toxic.

Initially, an MTS assay for curcumin was conducted, using a range of concentrations from 0-20 µM (Figure 3.1). The findings indicated that the addition of 1 µM of curcumin significantly increased the cell viability in relation to the control while at 15 and 20 µM the growth of the cells was significantly decreased. At 2-7.5 µM, there was no significant change in cell viability observed. Even though at 10 µM the curcumin did not have a significant impact on the viability of the cells, the viability of the cells was less than fifty percent of what the control had. From this, it was determined that a working range between 1-7.5 µM of curcumin would be appropriate.

An MTS assay was also done for OPP, using concentrations of 0-10% (from a stock solution of 1500 ppm GAE) (Figure 3.2). Although the viability of the cells was significantly decreased at all but one concentration of OPP (0.5%), only the 5, 7.5 and 10% samples had cell viabilities that were around or less than fifty percent of the viability of the control. Due to this, a

working range of 0.5-3.75% was deemed a suitable range of concentrations for further experiments.

Aim 2: To examine changes in the gene expression of serotonin pathway-related genes, *Tph1, Tph2, Aanat,* and *Asmt* and investigate if there are protein level changes of TPH1 and TPH2 due to treatment of curcumin and OPP.

3.2 Identify serotonin pathway-related genes regulated by curcumin and OPP

To see if supplementing the proliferation and differentiation media with curcumin and OPP would impact the serotonin pathway, the expression of serotonin pathway-related genes was determined. Tryptophan is metabolized to 5-hydroxy-L-tryptophan by tryptophan hydroxylase (TPH1 or TPH2), dopa decarboxylase (DDC) decarboxylates the 5-hydroxy-L-tryptophan to serotonin. The acetylation of serotonin to N-acetyl serotonin occurs via arylkylamine N-acetyltransferase (AANAT). Lastly, N-acetyl serotonin is methylated by N-acetylserotonin O-methyltransferase (ASMT), resulting in the synthesis of melatonin. The data from real-time qPCR showed that there was no significant change in gene expression of *Tph1*, *Tph2*, or *Asmt* in either the proliferating or differentiating cells (Figure 3.3). A significant increase in *Aanat* was seen in proliferating cells treated with curcumin, yet in the differentiating cells, there was a significant decrease in *Aanat* with the addition of OPP as well as dual treatment of curcumin and OPP. This suggests curcumin is able to regulate the expression of *Aanat* within the proliferating cells, while OPP is able to regulate the expression of *Aanat* within the differentiating cells. Furthermore, curcumin and OPP affect *Aanat* in an opposite way. These data indicate that curcumin and OPP may impact the serotonin pathway through influencing *Aanat*.

3.3 Level of proteins involved in the metabolism of tryptophan to serotonin

For this experiment, the TPH1 and TPH2 proteins were looked at more closely, as TPH is the rate-limiting step for the synthesis of serotonin. Consistent with what was seen from the gene expression data involving *Tph1* and *Tph2*, no alterations in the protein levels of TPH1 or TPH2 was observed in either the proliferating or differentiating cells (Figure 3.4). Taken together, these data suggest that there is no effect of OPP and curcumin on TPH.

Aim 3: To conduct metabolomic analysis on the cell extracts, to evaluate differences between the proliferating and differentiating cells, as well as any differences in each subset between curcumin and OPP treatments; identify and quantify which metabolites are responsible for the differences in metabolic pathways.

3.4 Identification of metabolomic profiles of proliferating and differentiating cells, under different treatment conditions

Using the spectra obtained from ^{1}H NMR analysis to conduct the metabolomics approach, it is possible to ascertain any differences metabolically. As expected, utilizing the RN46A-B14 proliferating cells and inducing differentiation resulted in cells exhibiting different metabolic profiles (Figure 3.5). In this instance, clear separation between the subtypes of cells is seen, even with using an unsupervised approach, principal component analysis (PCA), indicating the RN46A-B14 proliferating cells and inducing differentiation resulted in cells which exhibit different metabolic profiles (Figure 3.5). From the PCA plot, it is evident that the proliferating and differentiating cells have different metabolic profiles, but the effect of treatment of curcumin and OPP is not able to be seen.

The main focus of this experiment was to observe if treatments had an effect on the metabolic profiles of the cells. In order to see the effect of treatment, the subtypes of cells had to

be separated, so that only the proliferating or the differentiating cells were being examined. Along with examining the subtypes of cells individually, a supervised approach, partial least squares discriminant analysis (PLS-DA) was also done. For this, each treatment was identified as a class, causing the differences between treatments to be shown, if applicable. In the proliferating cells, this led to there being a clear separation between the control and curcumin-treated cells, and the cells treated with OPP and dual-treatment (Figure 3.5A). Even though the separation was less evident in the differentiating cells, a similar pattern was observed (Figure 3.5B). These data suggest that OPP, as a single or dual treatment, may be responsible for the majority of the alteration of metabolic profiles, with respect to the control. Given that the dual treatment cluster is closer to the OPP group as compared to the curcumin set, it is possible that OPP, not curcumin, may be responsible for the majority of the alteration of metabolic profiles in the dual treatment samples.

Next, orthogonal partial least squares discriminant analysis (OPLS-DA) was used to get a better comparison between the control and each treatment. Figures 3.7-3.9A show the OPLS-DA scores plots for the proliferating cells and 3.10-3.12A for the differentiating cells: control vs. curcumin, control vs. OPP, and control vs. dual treatment respectively. Based on the scores plot, it was observed that every treatment, from both the proliferating and differentiating cells, exhibit clear separation from the control. This suggests that the treatments do have some effect on cellular metabolism. To identify the spectral regions that significantly varied between the control and treatment groups in both the proliferating and differentiating cells, the corresponding S-plot was generated from the OPLS-DA model (Figures 3.7-3.12B).

These regions were then analyzed further in Chenomx to identify the metabolites that contributed to the separations, resulting in Table 3.1. Overall, the metabolites that were changed in either the control or treatment conditions in both proliferating and differentiating cells included

ATP, alanine, betaine, dimethylamine, glucose, glutamate, glycine, isoleucine, lactate, o-phosphocholine, proline, sarcosine, taurine, threonine, sn-glycero-3-phsophocholine, and β-alanine. Venn diagrams for the proliferating and differentiating cells were generated to further examine the relationship between treatment conditions and the metabolites responsible for the alterations (Figure 3.13). Statistically significant metabolites ($p<0.05$) in both subsets included dimethylamine, sarcosine, lactate and glycine, while in the proliferating cells sn-glycero-3-phosphocholine, ATP, and betaine were significantly impacted, and in the differentiating cells β-alanine and glutamine were significantly impacted. In both subsets of cells, only the curcumin (both single and dual treatment) treated cells had an alteration in metabolic profile due to sarcosine and dimethylamine. Lactate was significantly impacted in all treated proliferating cells, while only the OPP treated differentiating cells were significantly impacted. Significant changes in glycine were seen in the proliferating cells treated with OPP (single and dual treatment), while in the differentiating cells a significant impact was only seen in curcumin treated cells. OPP also induced a significant change in sn-glycero-3-phosphocholine and ATP in the proliferating cells, and β-alanine in the differentiating cells; dual treatment of curcumin and OPP significantly impacted betaine and glutamine in the proliferating and differentiating cells, respectively. This data suggests that, while the same metabolites are responsible for the alterations in metabolic profiles in both the proliferating and differentiating cells, the metabolites are not necessarily involved in similar ways.

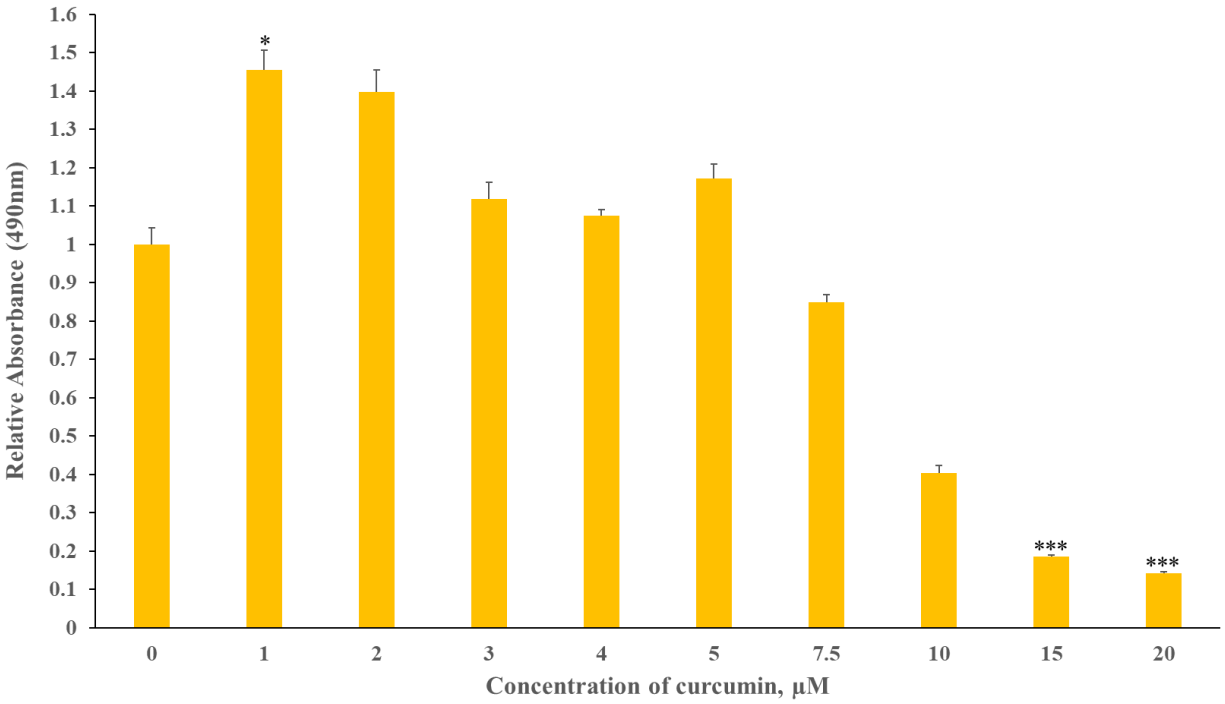


Figure 3.1: Determination of cell viability with varying treatment doses of curcumin. An MTS assay to determine a working range for curcumin was conducted with proliferating RN46A-B14 cells. They were seeded at a density of 5,000 cells per well and treated with varying concentrations of curcumin, from 0-20 µM, for 72 hours prior to the addition of the MTS solution. After incubation for one hour, the absorbances were read via a plate reader. Results are the average of six technical replicates. The vertical axis shows the relative absorbance/viability of the cells as a percentage, in relation to the control ± standard error of the mean (SEM), shown through the error bars. The statistical analysis results compare treated RN46A-B14 cells to the control. Significant differences are shown with * for $p < 0.05$, and *** for $p < 0.001$.

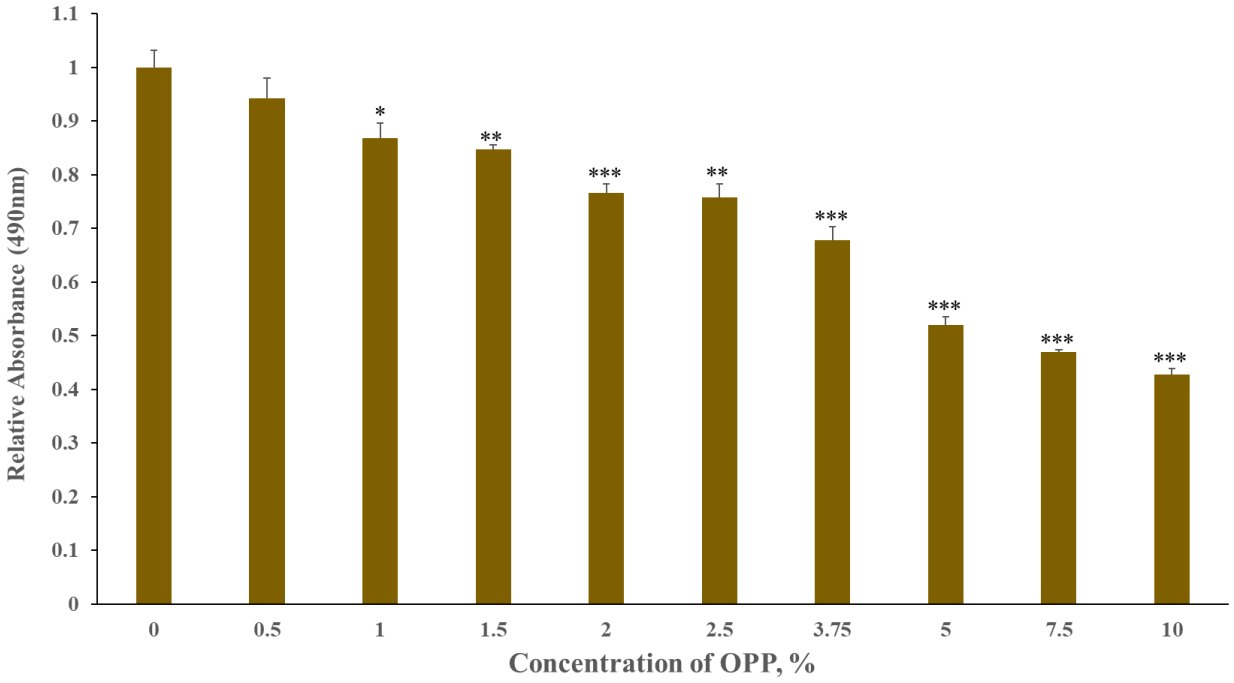


Figure 3.2: Determination of cell viability with varying treatment concentrations of OPP. An MTS assay to determine a working range for curcumin was conducted with proliferating RN46A-B14 cells. They were seeded at a density of 5,000 cells per well and treated with varying concentrations of OPP, from 0-10% OPP (from a stock solution of 1500 ppm GAE), for 72 hours prior to the addition of the MTS solution. After incubation for one hour, the absorbances were read via a plate reader. Results are the average of six technical replicates. The vertical axis shows the relative absorbance/viability of the cells as a percentage, in relation to the control ± SEM, shown through the error bars. The statistical analysis results compare treated RN46A-B14 cells to the control. Significant differences are shown with * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

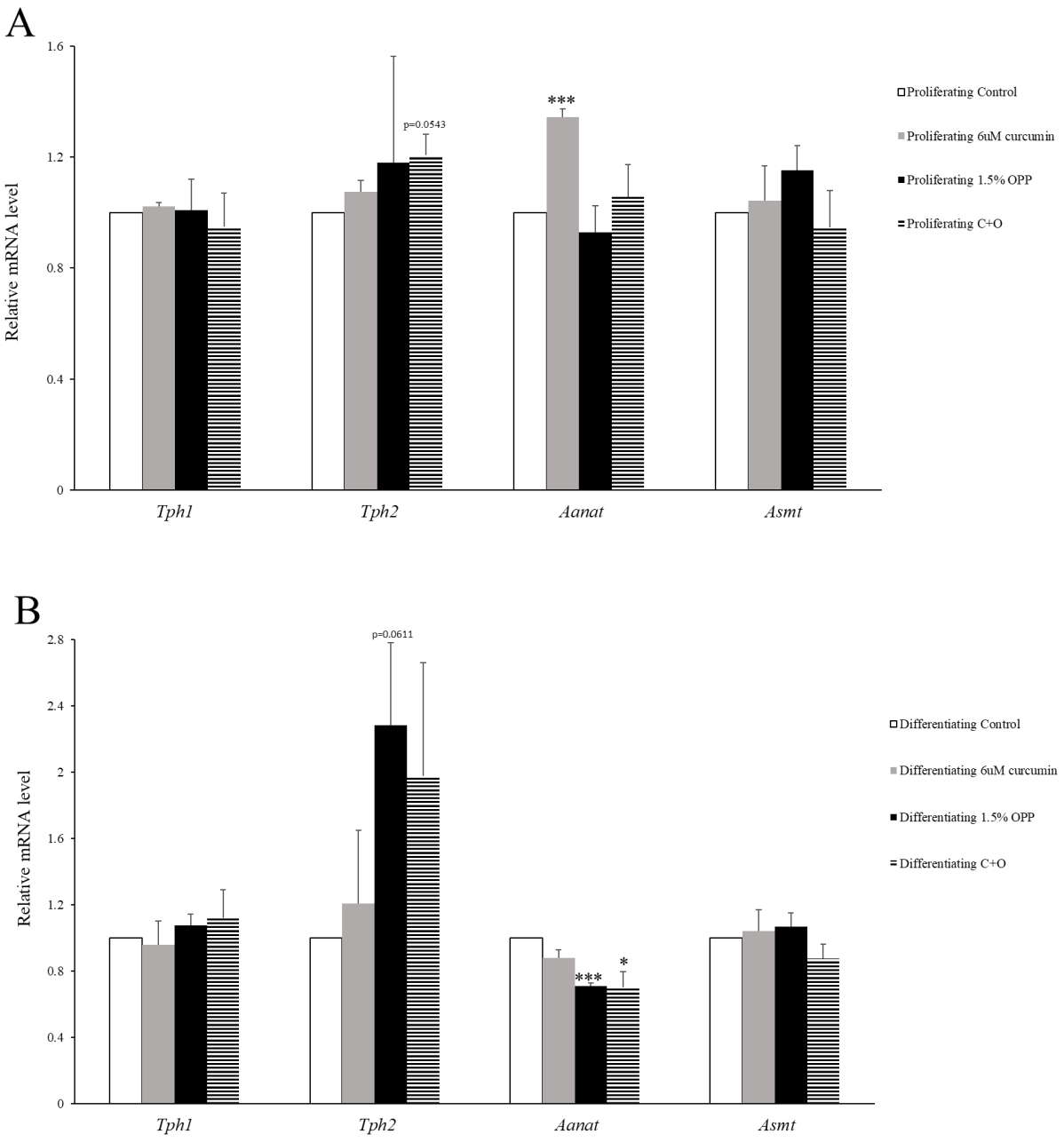


Figure 3.3: Effect of curcumin, OPP, and dual treatment on the expression of serotonin-pathway metabolic genes. Real-time qPCR analysis of total RNA extract from RN46AB14 cell-line, (A) proliferating and (B) differentiating cells, treated with 6 µM curcumin, 1.5% OPP (1500 ppm GAE), and dual-treatment of 6 µM curcumin and 1.5% OPP for 48 hours prior to sample collection. All proliferating samples are compared to the proliferating control cells, whereas all differentiating samples are compared to the differentiating control cells. *Ubc9* was used to normalize gene expression levels. The results are the average of three biological replicates. The vertical axis is indicative of the relative mRNA level ± SEM. Statistically significant differences are indicated with * for $p < 0.05$, *** for $p < 0.001$.

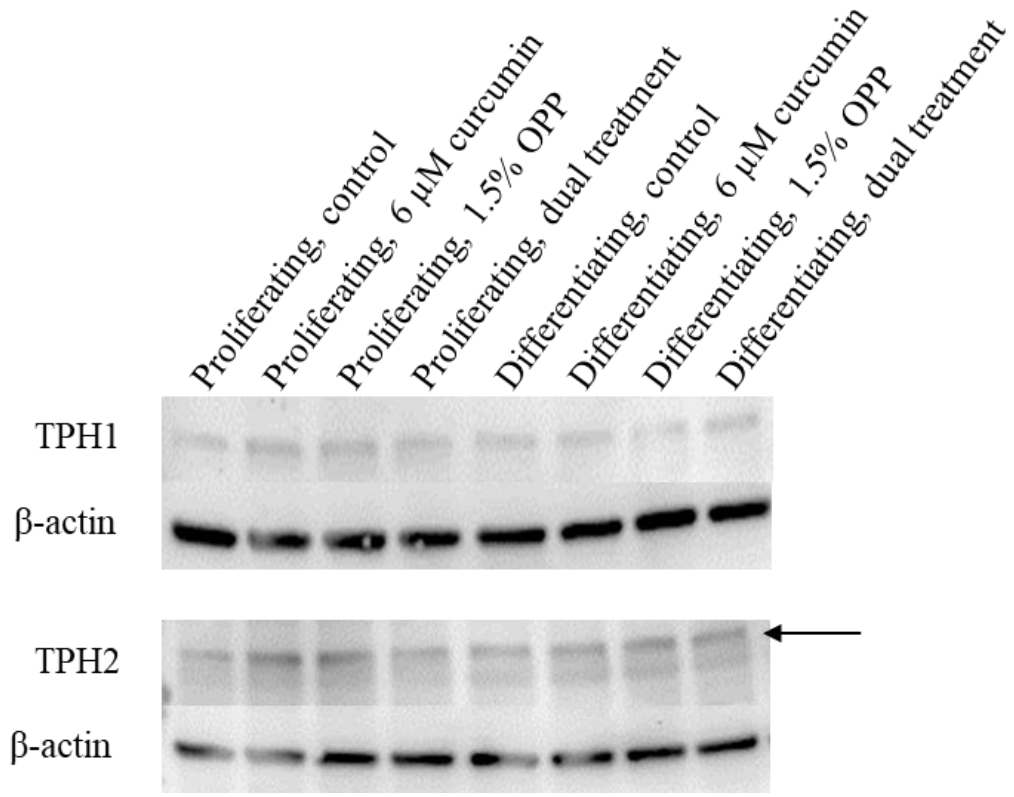


Figure 3.4: Effect of curcumin and OPP on the protein levels of TPH1 and TPH2 in proliferating and differentiating RN46A-B14 cells. Total cellular protein was extracted from RN46A-B14 proliferating and differentiating cells, treated with 6μM of curcumin, 1.5% OPP (1500 ppm GAE), and dual-treatment of 6 μM curcumin and 1.5% OPP for 48 hours prior to sample collection, and then subjected to western blot analysis. TPH1 and TPH2 were the target proteins, and β-actin was used as a loading control. The arrow denotes the protein band of interest for TPH2.

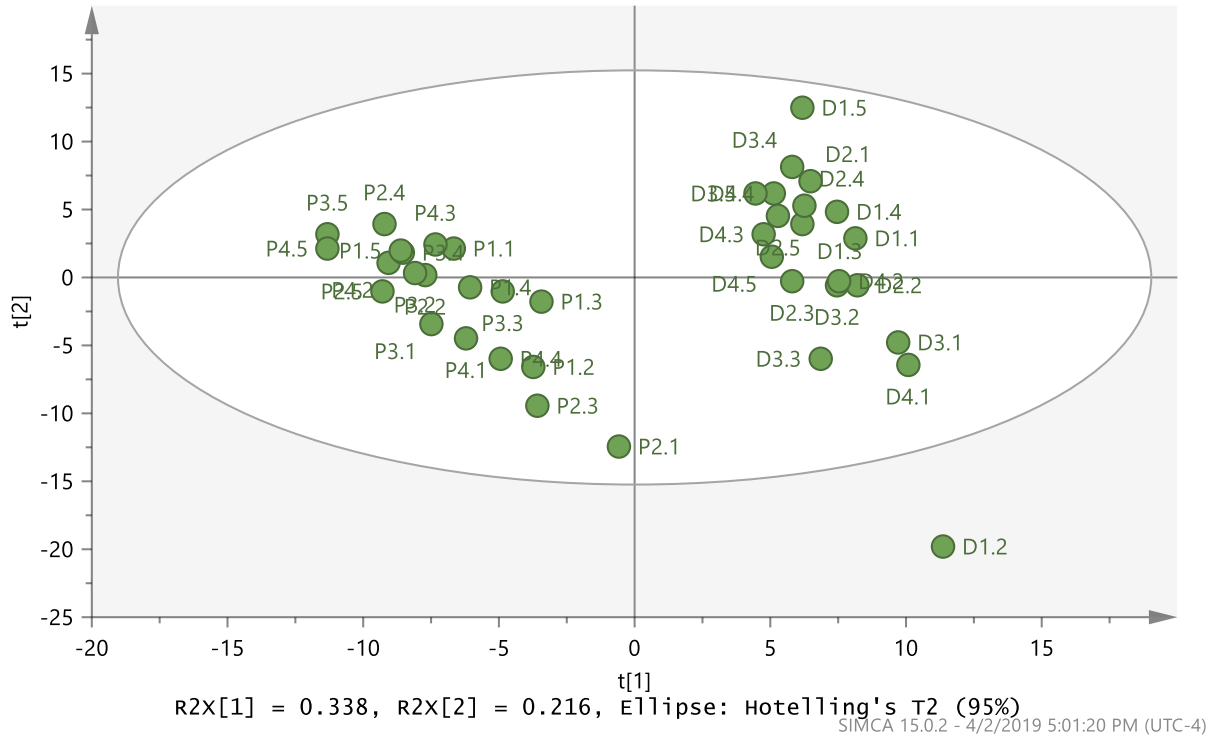


Figure 3.5: PCA scores plot of proliferating and differentiating cells, under all treatment conditions. One spot is representative of one sample: all samples labeled (P) are from proliferating cells, while (D) are from differentiating cells. The numbers are representative of the treatment (where 1 is control, 2 is curcumin, 3 is OPP, and 4 is dual treatment of curcumin and OPP) and replicate number (up to 5 replicates possible per treatment condition), respectively; for example, P2.4 is from proliferating cells, treated with curcumin, and is the fourth replicate. At least four biological replicates were used for each treatment condition. All samples were treated with 6 µM of curcumin, 1.5% OPP, or dual-treatment of curcumin and OPP for 48 hours prior to sample collection. There is a clear separation seen between all of the proliferating (P) samples and the differentiating (D) samples.

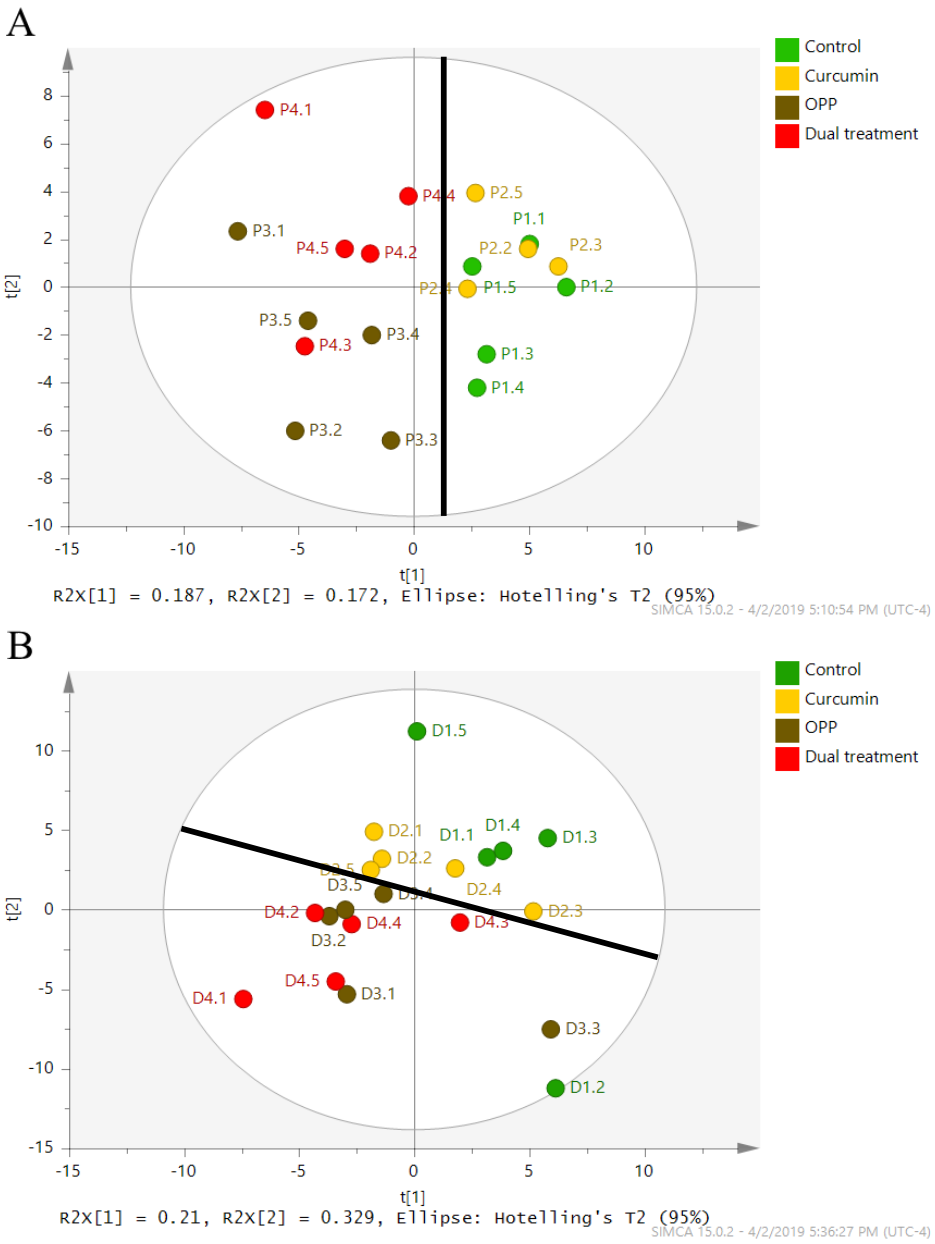


Figure 3.6: PLS-DA scores plot of the subtypes of RN46A-B14 cells. One spot is representative of one sample: all samples in A) are from proliferating cells, while B) are from differentiating cells. Treatments are indicated by color and number: green/1 are the control samples, yellow/2 are the curcumin-treated, brown/3 is the OPP-treated, and red/4 is the dual treatment of curcumin and OPP. The numbers are representative of the treatment and replicate number (up to 5 replicates possible per treatment condition), respectively. At least four biological replicates were used for each treatment condition. A) There is a clear separation seen over the t[1] (vertical) axis, separating the control and curcumin-treated proliferating cells from the cells that were treated with OPP and dual treatment of curcumin and OPP. B) There is also a separation seen, this time not quite as well defined, over the t[2] (horizontal) axis, separating most of the control and all of the curcumin-treated differentiating cells from the cells that were treated with OPP and dual treatment, of curcumin and OPP.

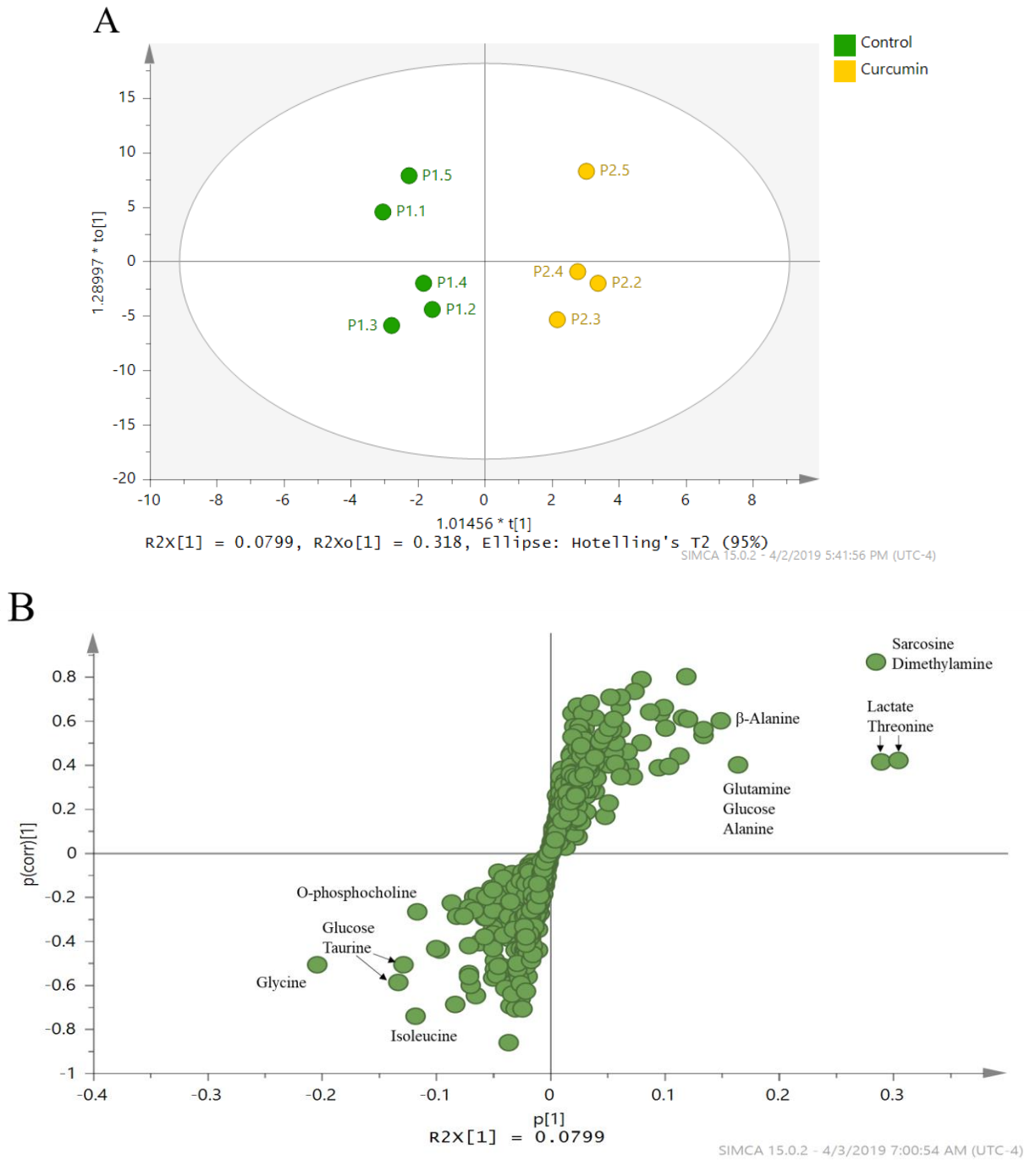


Figure 3.7: OPLS-DA scores plot of the proliferating RN46A-B14 cells, control vs. curcumin. A) There is a clear separation seen over the t[1] (vertical) axis, separating the control and curcumin-treated proliferating cells. B) The corresponding S-plot shows which areas within the NMR spectra may be responsible for the differences in the metabolic profiles; these points represent metabolites. The points deviating from mid-line in the upper right quadrant are responsible for alterations in the curcumin-treated cells, while the lower left quadrant shows changes that may be seen within the control cells. The key metabolites that changed are marked on the S-plot: glycine, o-phosphocholine, glucose, taurine, isoleucine, sarcosine, dimethylamine, lactate, threonine, β-Alanine, glutamine, and alanine.

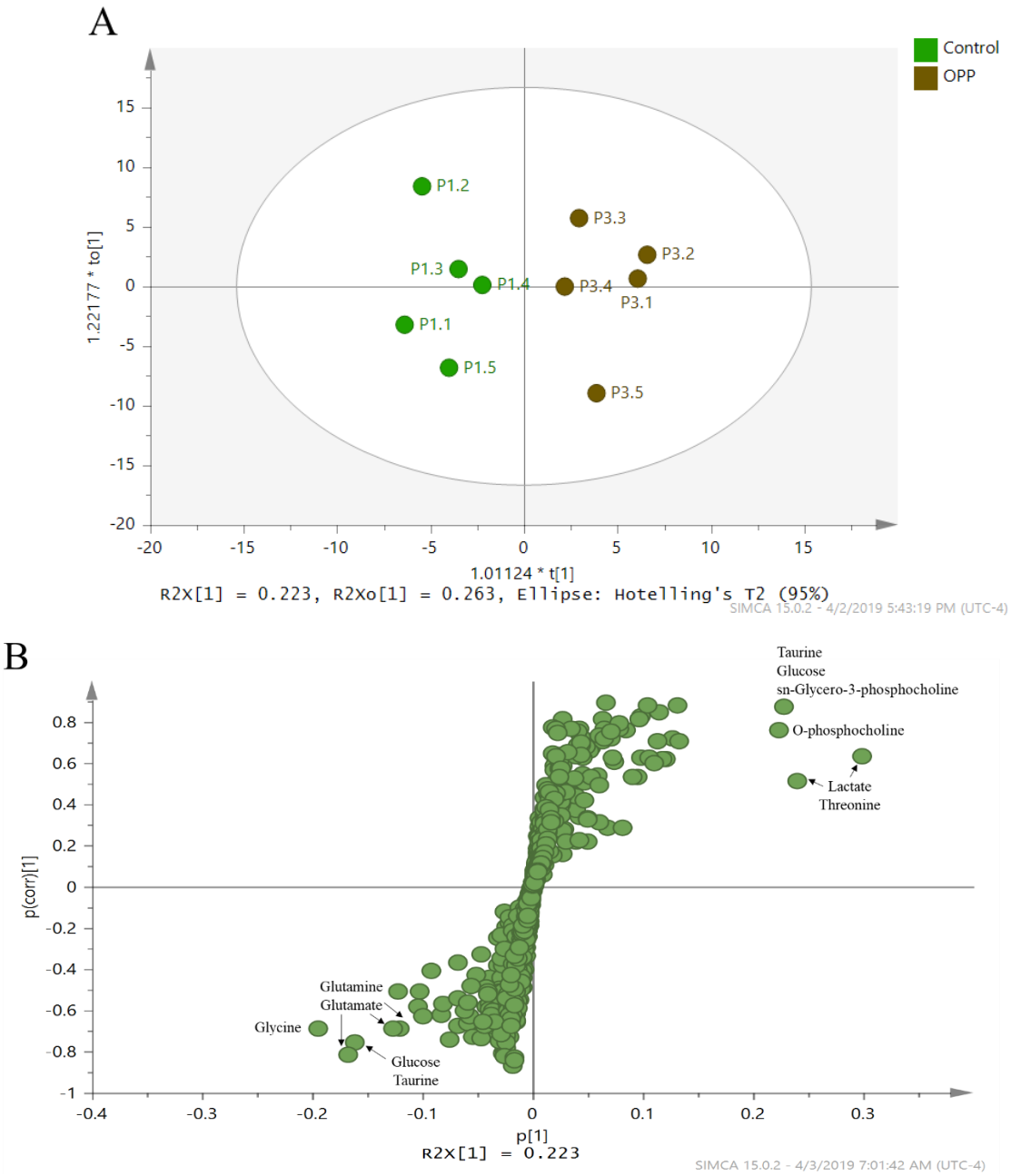


Figure 3.8: OPLS-DA scores plot of the proliferating RN46A-B14 cells, control vs. OPP. A) There is a clear separation seen over the t[1] (vertical) axis, separating the control and OPP treated proliferating cells. B) The corresponding S-plot shows which areas within the NMR spectra may be responsible for the differences in the metabolic profiles; these points represent metabolites. The points deviating from mid-line in the upper right quadrant are responsible for alterations in the OPP treated cells, while the lower left quadrant shows changes that may be seen within the control cells. The key metabolites that changed are marked on the S-plot: glycine, glutamine, glutamate, glucose, taurine, sn-glycero-3-phosphocholine, o-phosphocholine, lactate, and threonine.

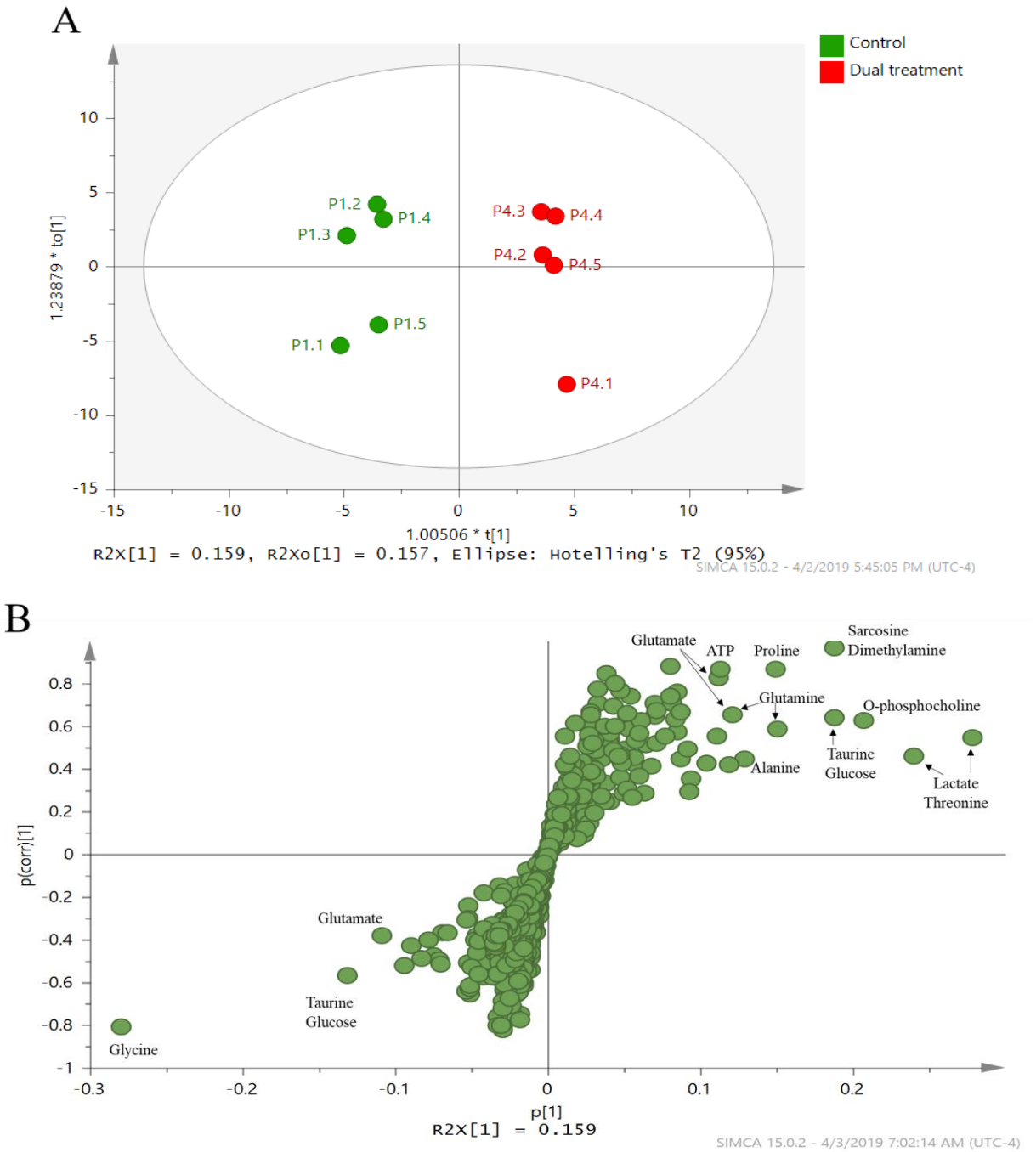
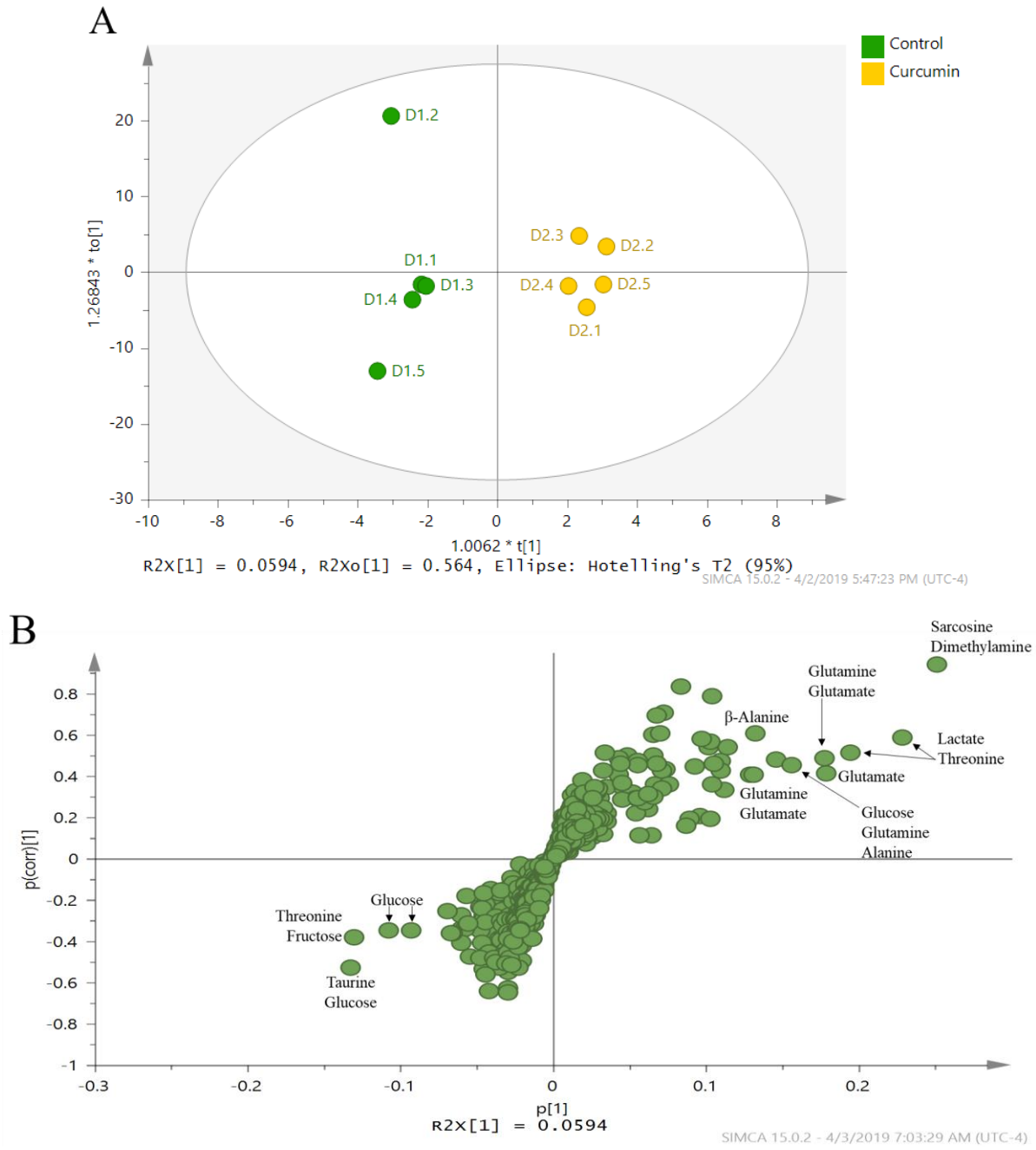


Figure 3.9: OPLS-DA scores plot of the proliferating RN46A-B14 cells, control vs. dual treatment. A) There is a clear separation seen over the t[1] (vertical) axis, separating the control and dual treatment of curcumin and OPP treated proliferating cells. B) The corresponding S-plot shows which areas within the NMR spectra may be responsible for the differences in the metabolic profiles; these points represent metabolites. The points deviating from mid-line in the upper right quadrant are responsible for alterations in the cells receiving the dual treatment, while the lower left quadrant shows changes that may be seen within the control cells. The key metabolites that changed are marked on the S-plot: glycine, glutamate, glucose, taurine, ATP, proline, sarcosine, dimethylamine, o-phosphocholine, alanine, lactate, and threonine.



OPLS-DA scores plot of the differentiating RN46A-B14 cells, control vs. curcumin. A) There is a clear separation seen over the t[1] (vertical) axis, separating the control and curcumin-treated differentiating cells. B) The corresponding S-plot shows which areas within the NMR spectra may be responsible for the differences in the metabolic profiles; these points represent metabolites. The points deviating from mid-line in the upper right quadrant are responsible for alterations in the curcumin-treated cells, while the lower left quadrant shows changes that may be seen within the control cells. The key metabolites that changed are marked on the S-plot: threonine, glucose, taurine, β-Alanine, glutamine, glutamate, alanine, sarcosine, dimethylamine, lactate, and threonine.

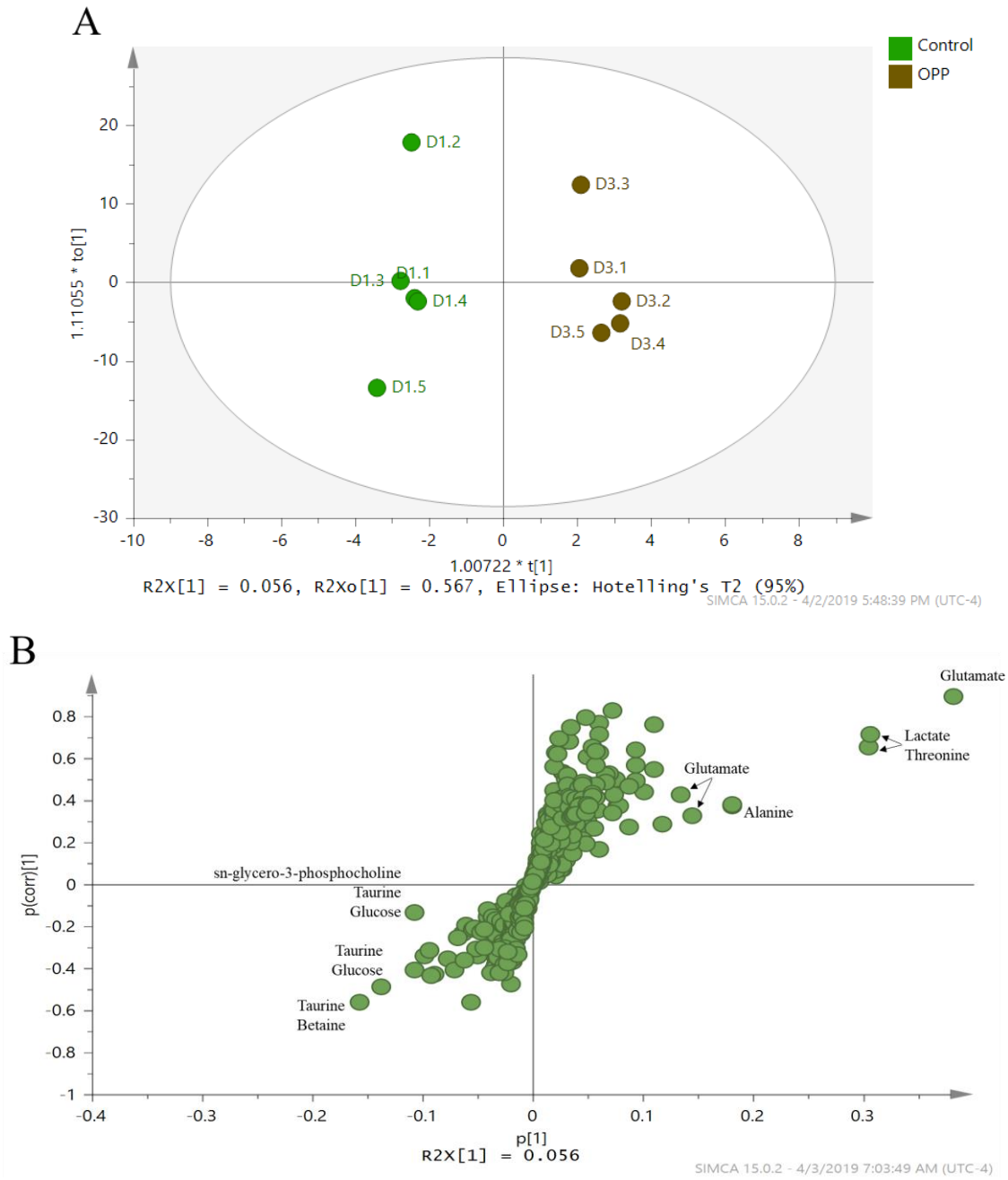


Figure 3.11: OPLS-DA scores plot of the differentiating RN46A-B14 cells, control vs. OPP. A) There is a clear separation seen over the t[1] (vertical) axis, separating the control and OPP treated differentiating cells. B) The corresponding S-plot shows which areas within the NMR spectra may be responsible for the differences in the metabolic profiles; these points represent metabolites. The points deviating from mid-line in the upper right quadrant are responsible for alterations in the OPP treated cells, while the lower left quadrant shows changes that may be seen within the control cells. The key metabolites that changed are marked on the S-plot: sn-glycero-3-phosphocholine, taurine, glucose, betaine, glutamate, lactate, threonine, and alanine.

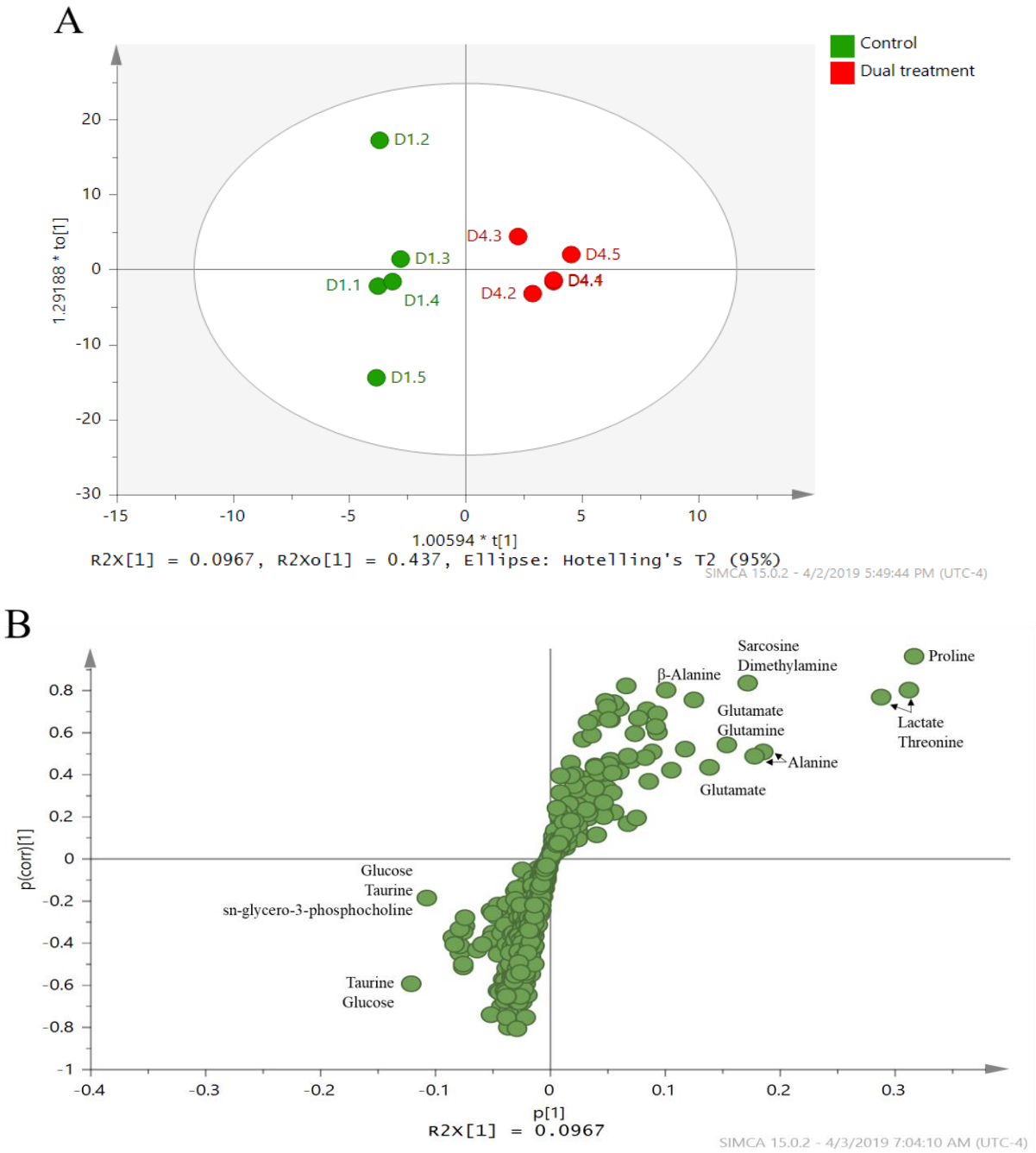


Figure 3.12: OPLS-DA scores plot of the differentiating RN46A-B14 cells, control vs. dual treatment. A) There is a clear separation seen over the t[1] (vertical) axis, separating the control and dual treatment of curcumin and OPP treated differentiating cells. B) The corresponding S-plot shows which areas within the NMR spectra may be responsible for the differences in the metabolic profiles; these points represent metabolites. The points deviating from mid-line in the upper right quadrant are responsible for alterations in the cells receiving the dual treatment, while the lower left quadrant shows changes that may be seen within the control cells. The key metabolites that changed are marked on the S-plot: glucose, taurine, sn-glycero-3-phosphocholine, β-Alanine, sarcosine, dimethylamine, glutamate, glutamine, alanine, proline, lactate, and threonine.

Table 3.1: Metabolite concentrations of RN46A-B14 proliferating and differentiating cells. List of concentrations of metabolites determined from ^{1}H NMR spectra of RN46A-B14 A) proliferating cells and B) differentiating cells, treated with 6 μM, 1.5% OPP, or dual-treatment of curcumin and OPP for 48 hours prior to collection of cell extract. p-values of less than 0.05 were considered statistically significant.

A)

Metabolite Name	Control vs. Curcumin		Control vs. OPP		Control vs. Dual Treatment	
	p-value	Fold change	p-value	Fold change	p-value	Fold change
ATP	0.4045	0.90	0.0019	1.48	0.3737	1.18
Alanine	0.3163	0.88	0.0843	0.78	0.4185	1.29
Betaine	0.6210	1.17	0.1531	1.62	0.0317	4.53
Dimethylamine	0.0033	16.55	1.0000	1.00	0.0040	22.60
Glucose	0.9703	1.01	0.7582	0.96	0.7072	0.93
Glutamate	0.9281	0.98	0.4555	0.87	0.2864	0.73
Glutamine	0.4818	0.88	0.1885	0.74	0.1179	0.63
Glycine	0.0823	0.83	0.0289	0.59	0.0002	0.30
Isoleucine	0.7804	0.96	0.0564	0.81	0.4161	1.24
Lactate	0.0097	1.61	0.0053	1.74	0.0012	1.92
o-phosphocholine	0.6113	0.92	0.3190	1.22	0.6709	1.07
Proline	0.0505	0.78	0.1019	0.59	0.2838	1.65
Sarcosine	0.0049	11.82	1.0000	1.00	0.0169	12.02
Taurine	0.1966	0.70	0.3883	1.19	0.8068	0.94
Threonine	0.2245	1.33	0.5544	1.18	0.0944	2.13
sn-glycero-3-phosphocholine	0.4188	0.80	0.0408	1.63	0.0804	1.83
β-Alanine	0.7413	0.96	0.3080	1.18	0.3737	1.30

B)

Metabolite Name	Control vs. Curcumin		Control vs. OPP		Control vs. Dual Treatment	
	p-value	Fold Change	p-value	Fold change	p-value	Fold change
ATP	0.3843	0.86	0.0540	1.23	0.9590	0.99
Alanine	0.1246	0.69	0.4583	1.10	0.5975	0.89
Betaine	0.7878	0.90	0.5500	0.77	0.9654	0.98
Dimethylamine	0.0220	13.18	1.0000	1.00	0.0421	7.13
Glucose	0.7133	1.07	0.6805	1.07	0.5760	0.90
Glutamate	0.0521	1.42	0.0518	1.53	0.0978	1.50
Glutamine	0.4744	0.92	0.1873	0.81	0.0295	0.69
Glycine	0.0188	0.64	0.0926	0.79	0.0520	0.67
Isoleucine	0.6569	0.94	0.5850	1.09	0.9799	1.00
Lactate	0.1345	1.40	0.0082	1.65	0.2449	1.30
o-phosphocholine	0.4934	1.20	0.1101	1.42	0.7141	0.91
Proline	0.1009	1.51	0.2818	1.28	0.7400	1.14
Sarcosine	0.0189	20.74	1.0000	1.00	0.0213	13.35
Taurine	0.6665	0.95	0.5089	1.13	0.7371	1.07
Threonine	0.3022	0.79	0.5158	1.16	0.5653	1.21
sn-glycero-3-phosphocholine	0.7216	0.92	0.5869	1.12	0.9373	0.98
β-Alanine	0.0698	1.47	0.0119	1.71	0.1288	1.65

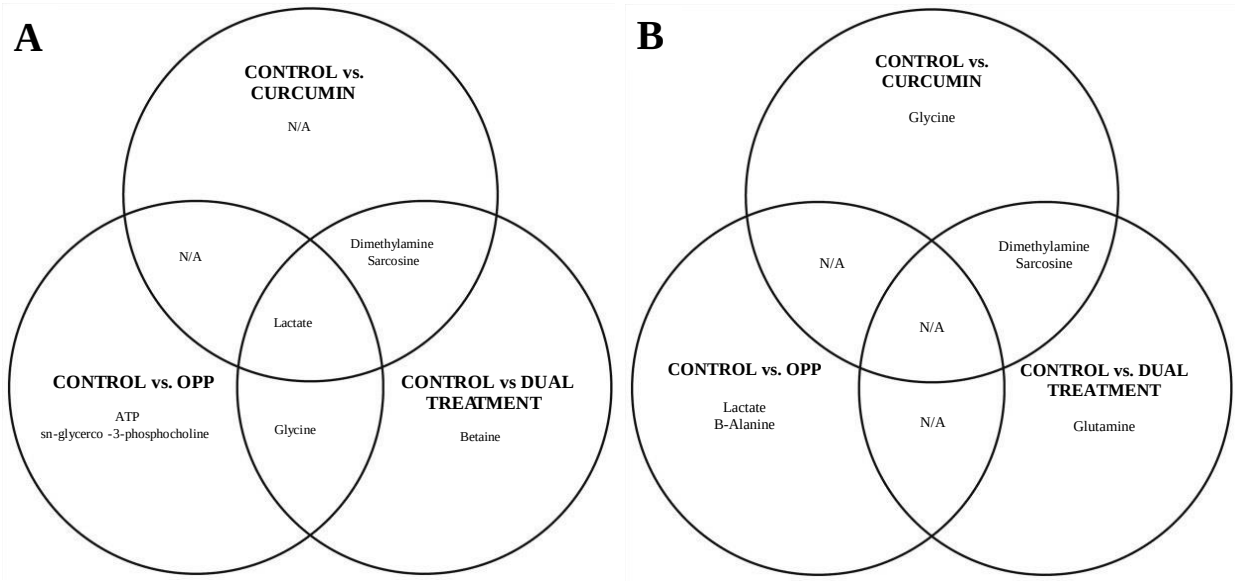


Figure 3.13: Venn diagrams of significantly impacted metabolites. Metabolites that were significantly impacted in A) the proliferating and B) differentiating cells are shown. For the proliferating cells lactate is seen in control vs. all three treatment conditions; dimethylamine and sarcosine are seen in both the control vs. curcumin and control vs. dual treatment; glycine is seen in both control vs. OPP and control vs. dual treatment; ATP and sn-glycero-3-phosphocholine are significantly impacted in control vs. OPP; and betaine is significantly impacted in control vs. dual treatment. For the differentiating cells dimethylamine and sarcosine are seen in both the control vs. curcumin and control vs. dual treatment; glycine is significantly impacted in control vs curcumin; lactate and β-alanine are significantly impacted in control vs. OPP; and glutamine is significantly impacted in control vs. dual treatment.

CHAPTER 4

Discussion

4.1 Discussion

People of all ages can suffer from depression; the overall quality of life is decreased because the disorder can affect how individuals are able to function on a daily basis, it is also affected by the high risk for relapse after treatment. The overall health burden is also present in multiple facets of life, including individually, in the workplace, and within the family. From an individual perspective, residual symptoms of depression may linger even after treatment has been administered. Within the workplace, it has been reported that 27 days per year per depressed employee are lost, due to either the calling in sick, or being at work but not functioning at an efficient level. Depression can also alter family structures, causing instability, and possibly completely disrupting the home life.[75] Overall, we did see the potential antidepressant activity OPP may have within neuronal RN46A-B14 cells in the serotonin pathway; we also saw that there are additional metabolic changes that have occurred, which need to be further elucidated.

Cell viability is an important aspect to consider when determining treatment doses. In certain instances, such as with cancer cells, decreased cell viability is preferred; however, in neuronal cells treatment should not be overly toxic. We found, through the MTS assay, what range of treatment dosage would be appropriate to use for both curcumin and OPP. Interestingly, treatment dosages of 1-5 µM curcumin enhanced the viability of the RN46A-B14 proliferating cells, with respect to the control, although the mechanism is not fully understood. Concentrations at 10, 15, and 20 µM reduced the viability of the cells by 60, 81, and 86% respectively, as compared to the control. The half maximal inhibitory concentration (IC50), or the concentration at which treatment inhibits cell proliferation by half, is between 7.5-10 µM, but closer to 10 µM. Having

viability less than the control is understandable, as an additional component is being added to the media, which may affect the overall cell viability; however, for this experiment we wanted to minimize the adverse effects while maximizing the beneficial properties. Because of this, we determined that a working range up to 7.5 µM curcumin could be tolerated. For OPP, we observed that every dose of OPP decreased the viability of cells in relation to the control. A 5% dose of OPP resulted in a 52% decrease in cell viability, while 7.5% and 10% dosages resulted in 53 and 57% reduction in cell viability, respectively. The IC50 for OPP is around 5%; because of this we believed that doses up to 3.75% would be acceptable for this study.

It has been suggested that a decrease in *Asmt* activity, with a subsequent decrease in synthesis of melatonin, accumulation of *Aanat* may occur, leading to increased synthesis of serotonin.[76; 77; 78] In our case, we believe that the reduction of expression of *Aanat* along with no changes seen upstream in *Tph1 and Tph2* or downstream in *Asmt*, results in a similar mechanism.[76] Based on this mechanism, in addition to our data, we proposed a model, demonstrating what we believe is the mechanism of action for OPP based on what was observed. We supplemented the media of the neuronal cells with OPP, which caused a decrease in the expression of *Aanat*. Because of this, we postulate that the expression of serotonin will increase, as there were no other upstream or downstream alterations observed. This causes a “pooling” of serotonin, which may result in decreasing the occurrence of depression. The mechanism is still not fully understood, but may be an important novel treatment for those suffering from depression.

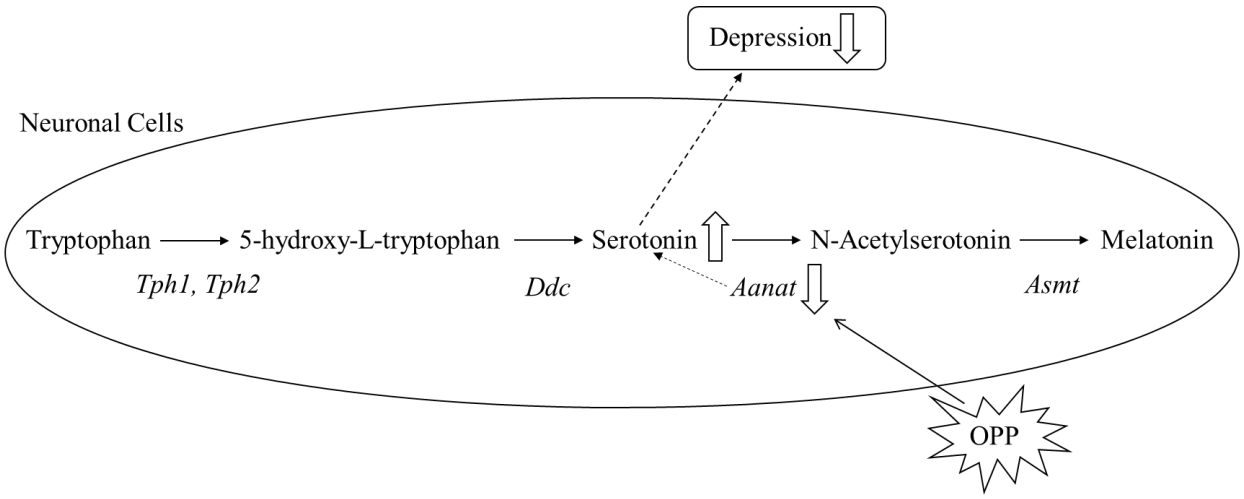


Figure 4.1: Proposed model for the activity of OPP on serotonin pathway-related genes.
Tph1: tryptophan hydroxylase 1; Tph2: tryptophan hydroxylase 2; Ddc: dopa decarboxylase; Aanat: aralkylamine N-acetyltransferase; Asmt: acetylserotonin O-methyltransferase.

The polyphenolic nature of both curcumin and OPP led us to believe that both compounds may similarly affect the mechanism of depression. From a metabolomic standpoint, we did not see any impact in the tryptophan pathway – the concentrations of tryptophan, serotonin, N-acetylserotonin and melatonin were too low to be calculated. Also, serotonin is typically excreted, so may be present in the exometabolome/growth media. We have collected this, but further analysis needs to be conducted. We observed that curcumin did not have any effect on the regulation of serotonin metabolism in the differentiating cells. This could be because the scope of our experiment was too focused, and we did not examine the pathway(s) that curcumin impacts.

The metabolomics data firstly showed that the metabolic profiles between proliferating and differentiating cells differ; this is consistent to what we thought would be seen, as even though the cells have the same origin, they express different morphologies. To better understand how the treatments affected each subset of cells, PLS-DA was done, in which each treatment condition was assigned a class. Both the proliferating cells and differentiating cells showed a similar pattern with respect to the effect of treatment – the control and curcumin-treated cells displayed more similar

metabolic profiles, while cells treated with OPP and dual treatment of OPP and curcumin exhibited similar profiles. We further examined the effect of treatment, by comparing each treatment condition of both subsets of cells to the control; this data showed that every treatment does have some alterations in the metabolic profile, with respect to the control. However, based on the R^2 and Q^2 values, where R^2 is indicative of how well the data fits the model and Q^2 estimates the predictive ability of the model, the models treated with OPP or dual-treatment of curcumin and OPP exhibited better fitting of data as well as predictive abilities. We observed, based on class segregation through OPLS-DA, that every treatment – curcumin, OPP, and dual treatment of curcumin and OPP – exhibited different metabolic properties as compared to the control. Although this is true, it is unlikely that curcumin had much of an impact on the metabolic profiles of the proliferating or differentiating cells; the response seen in the dual treatment of OPP and curcumin was mostly related to the effect of OPP. This is because the predictive nature of the models with curcumin-treatment only was very poor, while the models involving OPP and dual treatment of curcumin and OPP had increased predictive abilities.

Our main focus in this study was to examine the effects of treatment within the serotonin pathway and determine if antidepressant properties may be present. From the metabolomic data, we may be able to broaden the scope to focus on overall mood, anxiety, and other related disorders such as schizophrenia or bipolar disorder. Sarcosine, which was responsible for alterations in all metabolic pathways with treatment of curcumin (single and dual treatment) is of interest because it is a competitive inhibitor of the type I glycine transporter, and is an N-methyl-D-aspartate receptor (NMDAR) co-agonist, which is believed to enhance NMDAR function, which is believed to be low among schizophrenics.[79] Sarcosine has been reported to have few or no side effects with administration in humans of 2 grams/day for one week, exhibits antidepressant and

antipsychotic properties and may impact cognition in schizophrenia.[79; 80] Further studies may be done to better examine the relationship between curcumin administration and changes in sarcosine levels.

β-alanine may also be an important metabolite to target, as significant impacts of this metabolite were only seen in the differentiating cells treated with OPP. Research has indicated that a diet supplemented with β-alanine can result in anxiolytic effects, as well as cause alterations in the concentration of 5-hydroxyindoleacetic acid – possibly related to anxiety, carnosine – acts as an antioxidant and may have the ability to suppress ischemia in the brain, and BDNF, which is believed to be related to both depression and anxiety.[81; 82] Further, one study reported that supplementation with β-alanine for 30 days reduced anxiety, and increased expression of BDNF within the hippocampus of both younger and older rats.[83] This indicates that OPP may result in similar findings, and needs to be further explored.

4.2 Conclusion/Future Direction

The utilization of cell culture allowed for there to be no issue with adherence to treatment regimen, while also maintaining a controlled environment. Also, the biological replicates are more likely to be almost identical to each other, as opposed to the heterogeneity that would be seen within animals. While cell culture may allow for a certain mechanism to be elucidated, it does not allow for a systemic understanding of changes that are occurring. This may cause changes that were seen *in vitro* to not happen *in vivo* due to the presence of other signaling pathways and intercellular connections or regulatory systems in place, to ensure that there is the maintenance of homeostasis. Future *in vivo* studies need to be conducted in order to better understand how supplementation with curcumin and/or OPP affects an organism, as a whole system.

For future analysis, studies can be done to examine the effect of curcumin, OPP, and dual treatment of curcumin and OPP have on other pathways of interest, possibly illuminating other therapeutic options for depression, as well as potentially discovering biomarkers for depression. Analyzing the growth media that was collected can be done, to see if there are further metabolic changes from cellular excretions that were not seen intracellularly. As we used total OPP for this experiment, further studies could be done utilizing different fractions of OPP, which are made up of different polyphenolic compounds. This may demonstrate that certain polyphenolic compounds have a larger impact on overall metabolism, and result in a more potent nutraceutical. Treating the cells with OPP and then inducing a stressor, or adding a stressor and then supplementation with OPP can be done to examine whether the OPP is exhibiting preventative or is able to attenuate the effects of stress, which can be present at an animal or human level with the presence of oxidative stress or inherent day-to-day stressors. Also, this can show whether the antioxidative properties of OPP, in addition to its other potential properties, are enhanced by the presence of oxidation within the cells as compared to normal conditions. As the concentrations of metabolites within the tryptophan pathway – including tryptophan, serotonin, and melatonin – were low, we could add additionally tryptophan as a substrate to the growth media. We believe this will allow for more tryptophan to be available for the synthesis (and subsequent excretion) of serotonin, allowing for higher concentrations of these compounds to be present. Also, further investigation into the effect OPP has on the expression of *Aanat* needs to be conducted, to determine whether this does increase the synthesis of serotonin, and consequently can help to decrease the symptoms of depression, perhaps by altering overall mood or emotions.

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ABSTRACT

THE EFFICACY OF CURCUMIN AND OIL PALM PHENOLICS, AS POTENTIAL ANTIDEPRESSANTS, ON THE SEROTONIN PATHWAY IN A NEURONAL RAT CELL LINE

by

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Depression can be experienced by individuals of all ages, and can result in alterations of mood, decreased ability to function, and fatigue. Current treatment involves pharmaceuticals, which can result in adverse side effects, and there is a risk of relapse. Nutraceuticals are of interest because they may be able to work as efficiently as current treatments, and will cause fewer adverse effects. Curcumin and oil palm phenolics (OPP) are polyphenolic compounds that exhibit beneficial properties, including anti-oxidant and anti-cancer, and may assist in the regulation of diabetes. For this experiment, we utilized a neuronal rat cell line, RN46A-B14, which exhibits proliferative and differentiating properties, to study the effect of supplementation of curcumin and OPP on the regulation of the serotonin pathway as well as the metabolic profile, to discover potential antidepressants. Real-time quantitative PCR was done to determine if curcumin and OPP had any impact on the regulation of serotonin pathway-related genes. It was found that the expression of *Aanat* in the differentiating cells was decreased. Western blot analysis was done to examine the protein levels of tryptophan hydroxylase (TPH1 and TPH2), in which no significant changes were seen within any treatment condition. ^{1}H NMR analysis was also done, in which the

metabolomics approach was used to determine if there were any changes in metabolic profiles of treatment conditions; it was found that all treatment conditions did differ from the control, but the OPP and dual treatment of curcumin and OPP exhibited the largest changes in metabolic profiles. Overall, the study showed the potential serotonin enhancing ability of OPP within differentiating cells.

AUTOBIOGRAPHICAL STATEMENT

Melanie Hutchings obtained her Bachelor of Science degree in Nutrition and Food Science from Wayne State University, Detroit, MI in May, 2013, where she made the Dean's list five times. She then spent two years working hand-in-hand with dietitians at a hospital in Lubbock, Texas to garner experience within a practical setting. In August, 2016, she moved back to Michigan to begin work toward a Master of Science in Nutrition and Food Science, also from Wayne State University; she plans to complete her degree in May of 2019. She received the Graduate Professional Scholarship for the 2017-2018 school year, and worked as an instructional assistant for 3 semesters. Concurrently, she is doing supervised practice within the Coordinated Program of Dietetics at Wayne State University to further her knowledge of dietetics, and to become a credentialed dietitian. She also plans to begin coursework and research within the PhD program, to further expand her knowledge within the field of nutrition while also increasing her skills as a researcher.