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Discovery and Optimization of Anti-Cancer Properties of Traditional Herbal Medicines Using Zebrafish-Human Tumor Xenografts

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Biology

By
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Under the mentorship of Dr. Vinoth Sittaramane

ABSTRACT

Cancer is one of the most well-known diseases around the world. It hurts everyone in some way, whether they have it themselves or they know someone that is diagnosed. But the problem is not just this brutal disease, the problem is its invasive treatments. The most common treatments for cancer have harmful and painful side-effects that occur in most cases. As a solution to invasive cancer treatments, this experiment is testing herbal medications Neem, Nilavembu, Ashwagandha and Tulsi as potential nontoxic cancer treatments. First, the maximum tolerable dosage for each herbal agent was found. This dosage was used for the toxicity trials as well as the anti-cancer trials. The mortality rate, heart rate and hatch rate of the zebrafish during five days of incubation in solution provided the toxicity data. To test the anti-cancer effects of these herbal agents, a zebrafish-human tumor xenograft model was used. The zebrafish embryos were treated with the maximum tolerable dosage of each solution for five days; when the embryos were three days old, they were injected with human prostate cancer cell and were allowed to incubate in the solution for another two days. After the treatment, the zebrafish were died with acridine orange and imaged under the confocal microscope. Neem, Nilavembu, Ashwagandha and Tulsi all had herbal induced cell death with little to no toxicity. Therefore, presenting a strong case that this study can move forward to be tested on a human model.

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April 2020
Biology
University Honors Program
Georgia Southern University

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1. ACKNOWLEDGEMENTS

To begin, I would like to thank Georgia Southern Honors program for all of its amazing opportunities, resources and mentorship throughout my college career. The programs support has enabled me to be successful academically. I would also like to give a huge thank you to Dr. Vinoth Sittaramane for his mentorship throughout this study. His patience and enthusiasm really fueled my motivation to complete this project and sparked a newfound passion for research. I am also extremely thankful for his for his support inside and outside of the lab. He was not only a mentor to me during this project, but away from the project as well. Finally, I would like to acknowledge my lab partner, Maria Bryan. Our long hours in the lab did not seem as long with a friendly face working alongside of me. We tackled this experiment together and without her insight and extra hands, this experiment would not have been as successful.

2. INTRODUCTION

2.1 Cancer

2.1.1 Cancer overview

Cancer is a disease that has impacted everyone in some way; whether they are impacted directly by having the disease themselves, or indirectly by knowing someone who does. Cancer is a name given to a collection of diseases that have one thing in common: the abnormal and uncontrollable growth of cells. Normally the cells in our body grow, divide to form new cells, then die as they become damaged to be replaced. This cell cycle occurs all over the body and is repeated based on the need for new cells. However, cancer develops when this cell cycle is altered. In cancer, damaged or abnormal cells continue to survive and divide; they are able to do this because cancer cells are capable of ignoring signals that tell normal cells to stop dividing or go into apoptosis. These cancer cells become dangerous because of their uncontrollable growth and ability to invade other parts of the body. Some cancer cells are even able to influence normal cells around them to create a microenvironment, this environment helps to feed the cancer cells oxygen and nutrients by using blood vessels, thus further promoting growth.

2.1.2 Cancer Incidence

Cancer has a major impact on society, both in the United States and around the world. In the United States, 1 in every 3 Americans will be diagnosed with cancer in their lifetime [2]. And according to the most recent cancer statistics from 2018, there was an estimated number of 18 million cancer cases around the world [40]. The prevalence of cancer is staggering, and without a cure, it is expected to increase. In fact, by 2040, the number of new cancer cases is expected to grow to 27.5 million [13]. Because of the expansive societal impact of cancer, treatment of the disease is in increasingly high demand.

2.1.3 Cancer Treatment

There are many types of cancer treatment such as surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplant, and precision medicine [34]. The type of treatment largely depends on the type of cancer that needs to be treated and how advanced it is. The most common form of treatment is surgery, followed by chemotherapy and radiation [7]. The goal of surgery is to remove as much as the cancer as possible by cutting the cancer out of the infected part of the body. Many times, surgery is followed up with other treatments such as radiation or chemotherapy in attempt to completely remove the cancer. Chemotherapy uses drugs to kill cancer cells, while radiation therapy uses high powered energy of X-rays or protons [7].

The unifying characteristic of cancer treatments is not only their purpose, but also their extreme invasiveness. Each of the treatments not only kill cancer cells, but also neighboring healthy cells, especially radiation therapy. The invasiveness of the treatments is the reason for the extensive side effects that patients experience. To begin, some side effects of surgery include, pain, fatigue, appetite loss, swelling, bruising and risk of complications [28]. But the side effects of surgery are relatively minute compared to that of chemotherapy and radiation. Side effects of chemotherapy include, fatigue, hair loss, easy bruising and bleeding, anemia, nausea, vomiting, appetite changes and risk of infection [8]. In a self-reported chemotherapy study, about 86% of patients reported at least one side effect, with about 27% reporting a Grade IV (very intense) side effect [23]. The high incidence of side effects associated with chemotherapy is logical considering extremely potent toxins are continuously injected into the patient's body. The side effects of radiation are very similar but also largely dependent on the location of the cancer. The overall side effects include, fatigue, skin irritations, hair loss, then, depending on the location of

the cancer, there are also unique side effects because radiation also kills healthy cells in the area [25].

2.2 Call to Action

The treatments for cancer are almost as invasive as the cancer itself. Aside from the short-term side effects, the treatments often also have long term impacts associated with them such as, fatigue, permanent hair loss, reduced lung capability, muscles weakness, chronic pain and even secondary cancers [16]. Much of the world is focused on the cure of cancer, but an equally large problem is the invasiveness of the treatment. People suffering from a disease as life threatening as cancer should not be forced to undergo equally painful treatments. Alas, recent research hints that herbal medication may provide the necessary relief for suffering patients.

2.3 Herbal Medication

Improving human health and well-being is an area of research that will never fade. As long as illness and disease exist, research on treatment and prevention will continue. Plants and herbs have been used for medication and treatment since the beginning of written history. The oldest written evidence of herbal medication is over 5,000 years old, found on a Sumerian clay slab in Nagpur. The clay slab had 12 recipes that referred to over 250 various plants such as poppy and mandrake [24].

The early practice of herbal medicine existed in many different regions. The Chinese had a book written by their emperor in 2500 BC that contained 365 drugs which include herbs used today: ginseng, jimson weed, cinnamon bark, and ephedra [24]. The Indian holy books Vedas mentions herbal remedies using nutmeg, pepper and clove [33]. An Egyptian papyrus, known as

the Ebers Papyrus, dating back to 1550 BC, represents over 700 plant species used for drugs such as pomegranate, castor oil plant, aloe, senna, garlic, onion, fig, willow, coriander and juniper [24]. A Greek philosopher Threoprast founded botanical science around 225 BC. He developed the important idea that humans become accustomed to the agents by gradual increase in doses, he referred to pomegranate, castor oil plant, aloe, senna, garlic, onion, fig, willow, coriander, juniper and common centaury [24]. Herbal remedies continued to be the only form of medication throughout every region until the 18th century, when modern medication was developed.

Even modern medication utilizes herbal agents; there are many pharmaceutical drugs that originate from plants, such as aspirin from willow bark, digoxin from fox glove, quinine from cinchona bark, and morphine from opium poppy [37]. Although conventional medicine and herbal medicine have the same plant derived base, there are key differences. Unlike conventional medicine, herbal medicine uses plants that are generally unpurified. Also, herbal medication is primarily used to treat underlying causes of illness, chronic conditions and overall well-being, whereas conventional medicine focuses on treatment of specific illnesses and symptoms.

2.4 Complementary and Alternative Medication (CAM).

Despite the rapid growth of modern medicine, traditional techniques such as herbal medication are still used today as complementary and alternative medicine (CAM). Complementary and alternative medicine covers an array of unconventional approaches to prevent or treat a disease. Complementary medicine is typically used along with conventional medicine to aid in treatment, whereas alternative medication is used in place of conventional medicine. CAM treatments are often derived from ancient practices and are deemed “unconventional” because there is insufficient evidence of its effectiveness [10]. Despite the

controversiality among professionals, CAM has increased during the past decade with a generally positive attitude among the public [11]. The global prevalence is between 9.8%-76.0% with the overall prevalence of CAM reported as being 36% in the US (2007), 26% in the UK (2005), and 52% in Australia (2004) [3]. In the United States, CAM is most commonly used for musculoskeletal problems and most users are highly educated, middle aged women [10,11]. The vast majority of patients use CAM as a complement rather than an alternative for ailments ranging from sore throat and muscle fatigue to cancer and diseases effecting the cardiovascular system and lungs [10]. In fact, about 39.1 percent of cancer patients have reported using CAM treatments, herbal agents being most common followed by spiritual therapy [3]. Common CAM treatments include nonvitamin, nonmineral natural products, ayurvedic medicine, chiropractic techniques, yoga, meditation deep breathing exercises, massages, acupuncture, diet plans and many more [4]. In the United States, 4 out of 10 adults have used some type of CAM and nearly half of those who reported using CAM treatments, have used nonvitamin, nonmineral natural products such as herbal agents [10].

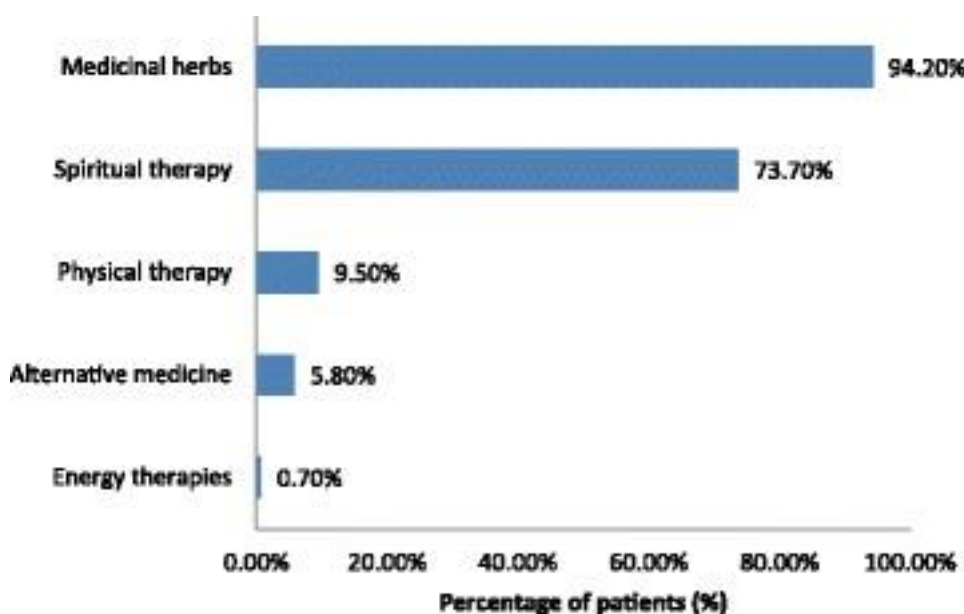


Figure 1: Types of CAM used by cancer patients in a cross-sectional, descriptive study conducted in 2017 (4).

2.5 Important Plant Derivatives

Plants can be processed in various ways so that they are in a form that is best suited for their desired function. Plants are often consumed medicinally as a tea, ointment, whole herb or as a rub. They contain a variety of compounds, many of which are secondary metabolites that are broken down into four major classes: polyphenols, alkaloids, glycosides, and terpenes. These metabolites have properties that are used to treat different illnesses and conditions.

2.5.1 Polyphenols

Phenol is an acidic organic compound that is toxic to consume on its own but used in many medications and household cleaners. Phenol is used in sore throat spray to numb the throat, in vaccines to prevent bacterial growth and in injections to treat muscle spasticity by preventing signals sent from the nerves to the brain. Phenol and its other derivatives have so many uses because of their

antioxidant properties, meaning they stop reactions of free radicals within the body by replacing the missing electron. Antioxidants are necessary because free radicals can be detrimental to molecules such as DNA. Phenols have also been used in cancer prevention. In a 2010 animal review, it was discovered that consuming plants rich in phenol helped to strengthen the immune system and makes cells more resistant to cancer throughout their life [38]. There has also been research in human studies that suggest phenol can help make cancer cells more receptive to chemotherapy treatment [27].

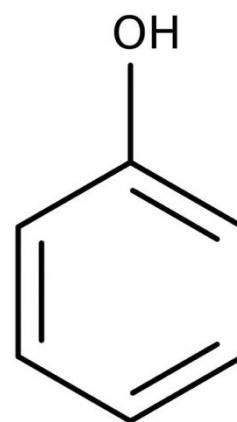


Figure 2: General structure of polyphenol an aromatic structure with an alcohol attached. Polyphenols have multiple rings in one compound. (ResearchGate).

2.5.2 Alkaloids

Alkaloids are a group of organic compounds that contain nitrogen. Most alkaloids contain one or more nitrogen atoms as a part of their cyclic structure [32].

The nitrogen atom makes these compounds extremely alkaline and causes them to act as a base in

chemical reactions. Alkaloids produce a huge diversity of physiological effects thus a wide diversity of medicinal properties. Alkaloids such as morphine and codeine are used for pain relief, some alkaloids are used for cardiac and respiratory stimulates such as quinidine found in plants of the genus Cinchona [32]. The alkaloid ephedrine, from plants in the genus Ephedra, are used to constrict blood vessels which is useful in treating blood hemorrhages, along with sinus discomfort from colds and asthma [32]. Also, two alkaloids, vincristine and vinblastine from *Vinca rosea*, are used as chemotherapeutic agents to treat many types of cancer [32].

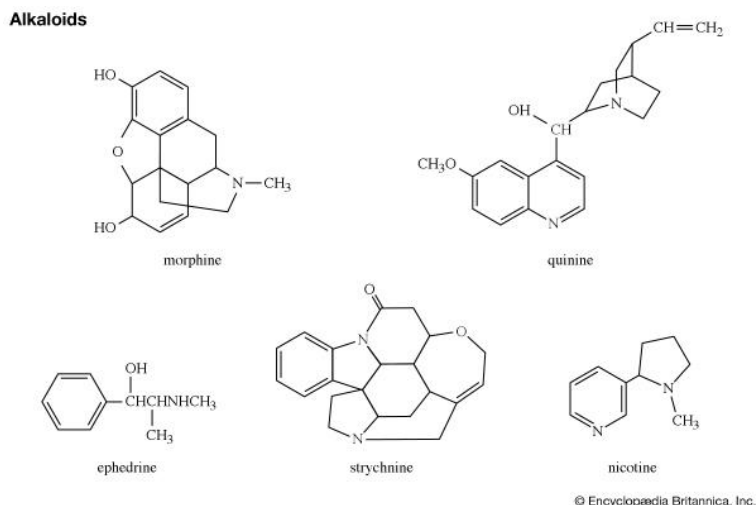


Figure 3: Common alkaloids Class of any naturally occurring organic nitrogen-containing bases. (Britannica).

2.5.3 Glycosides

Glycosides are compounds in which a sugar molecule is linked to a non-sugar molecule with a glycosidic bond. They give a permanent froth when shaken with water because the non-polar molecule does not dissolve. In reference to herbal medicine,

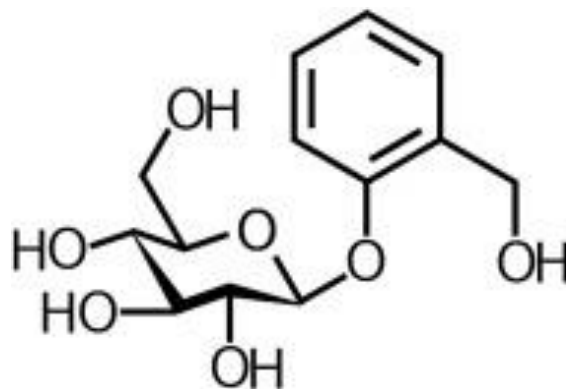


Figure 4: General structure of a glycoside. This particular glycoside is Salicin, a glycoside related to aspirin (ResearchGate).

glycosides are often broken down into subcategories of cardiac glycosides and cyanogenic glycosides. Cyanogenic glycosides have the cyanide compound which in large doses is deadly, but when used in small doses is an effective muscle relaxant [36]. Elderberry is a cyanogenic glycoside that is commonly used as a cough suppressant. On the other hand, cardiac glycosides have a strong effect on the heart along with anti-diuretic properties [36]. Glycosides such as those from the plants Foxglove and Lily from the Valley, effect the heart by increasing cardiac muscle strength and increasing rates of contraction. Other plants with cardiac glycoside compounds also have a diuretic effect that stimulates urine production which aids in excess fluid removal. Recently, some in vitro and in vivo experiments have revealed that some cardiac glycosides induce potent and selective anticancer effects [18]. Cardiac glycosides ouabalin and bufalin, have been shown to be potent inhibitors of cell growth while glycosides from other plants are still being tested [18].

2.5.4 Terpenes

Terpenes are a large group of volatile unsaturated hydrocarbons based on a cyclic molecule with the formula $C_{10}H_{16}$. Terpenes are primarily found in essential oils of plants. Terpenes have a wide range of uses because of

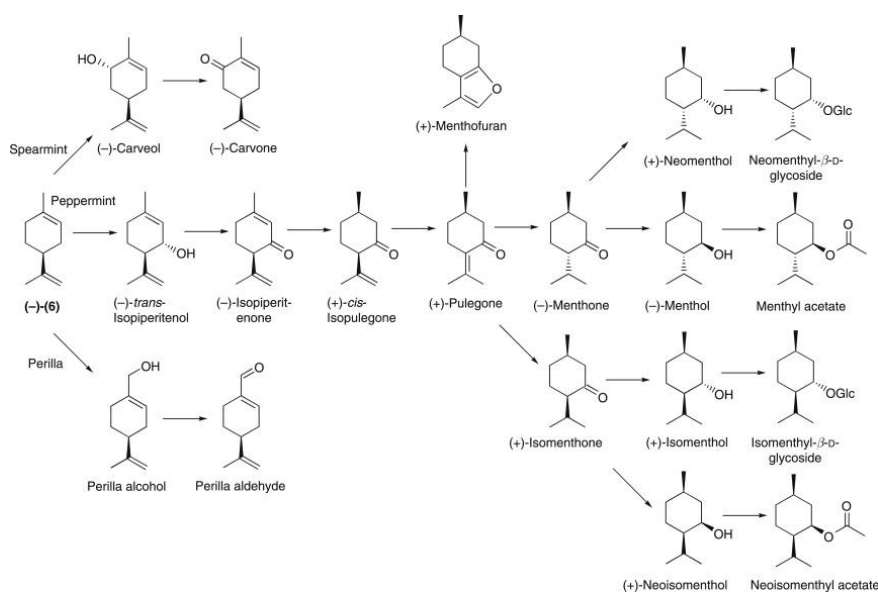


Figure 5: Common terpenes as shown above, terpenes can combine and produce a great variety of skeletons. Each skeleton can then be acted upon by enzymes to become functional (ScienceDirect).

its low acidity, including its use as a food additive and its use in cosmetic products. They are also used medicinally as anti-inflammatory, antioxidant, analgesic, anticonvulsive, antidepressant, anxiolytic, anticancer, antitumor, neuroprotective, anti-mutagenic, anti-allergic, antibiotic and anti-diabetic treatment [22]. The most popular medicinal terpene is cannabis which is primarily used for pain relief. But there are many more in vitro animal studies that are dissecting the medicinal properties of other terpenes.

2.6 Current Research

For many years, plants have been used for medication, yet there is very little proof of its effectiveness. So, to gain more knowledge on the effects of herbal agents, I have researched the immunomodulatory and anti-cancer effects of two herbal agents, Nilavembu and Neem, in zebrafish.

2.6.1 Nilavembu

Nilavembu is an herb cultivated in Southern and Southeastern Asia. It is a primary herb in Ayurvedic medicine, one of the world's oldest holistic healing systems developed in India [26]. Nilavembu is said to strengthen the body by healing the liver and alleviating fever. Its primary use is for diseases in which fever is the main symptom such as intermittent fever, malaria, chikungunya and dengue fever [29].



Figure 6: Image of Nilavembu plant Nilavembu is a herb originally cultivated in Southern and Southeastern Asia, but can be grown in other parts of the world (Image from The News Minute).

Nilavembu contains four major terpenoids:

Andrographolide (AP1), 14-deoxy-11, 12-didehydroandrographolide (AP3), Neoandrographolide (AP4), and 14-deoxyandrographolide (AP6) [29]. As discussed earlier, terpenes have a wide range of uses and are present in about 60% of plant-based products [15]. The most prominent terpene is Andrographolide, which comprises about 8-186 mg/g. Andrographolide is a diterpenoid that has a broad range of therapeutic applications including anti-inflammatory and anti-platelet aggregation. Additionally, andrographolide may have anti-cancer properties by induction cell arrest at G0/G1 phase and stimulation of lymphocytic proliferation and activation. These actions could result in decreased proliferation of and increased immunocytotoxicity against tumor cells [20]. Although Nilavembu has been promoted as a dietary supplement for cancer prevention/cure, and is largely composed of andrographolide, there is no evidence substantiating its cure in cancer [15].

1.6.2 Neem

The neem tree is an evergreen tree found in Asia that has been used to support healing for nearly 4,500 years [21]. It is a highly popular plant in Ayurvedic medicine because of its versatility. Every part of the neem tree, including the leaves, flowers bark and roots

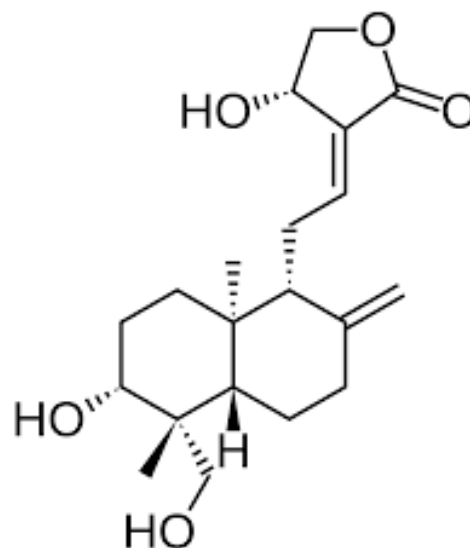


Figure 7: Chemical structure of Nilavembu (Researchgate).



Figure 8: Image of Neem plant. Neem is an evergreen tree found in Asia but also cultivated in other parts of the world (Gardening Knowhow).

is useful; parts of the neem tree are typically broken down into a powder or oil form for use. Traditionally, the Neem tree was commonly used for healthy skin and hair, for an aid in digestion and metabolism, and for aiding blood and boosting the immune system [39].

Today scientists have discovered more than 140 compounds that have been isolated from the neem tree and evaluated for use [35]. At least 35 are biologically active principles that have shown an important influence as tumor suppressors by interfering with the carcinogenesis process [19]. In a review of the anticancer

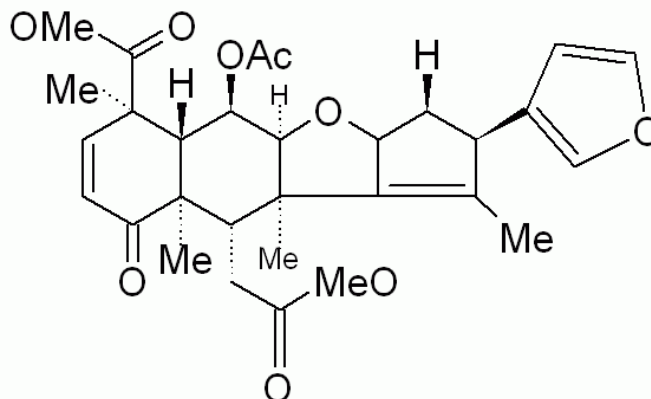


Figure 9: Chemical structure of Neem (Veera Andrade).

activity of neem in gynecological cancers, it was highlighted that although neem may not be great for cancer treatment, its potential as a cancer preventative has been recommended by many authors. Several in vitro experiments have been conducted and play a pivotal role in anticancer management through the modulation of various molecular pathways including p53, pTEN, NF- κ B, PI3K/Akt, Bcl-2, and VEGF [19]. These pathways include action involving the modulation of cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis processes. But to be able to more fully understand neem's anticancer properties, more in vivo studies need to be conducted.

1.6.3 Ashwagandha

Ashwagandha, also commonly known as Indian ginseng, is a plant found in India, Asia, and Northern Africa. It is commonly used in Ayurvedic medicine in many disease processes, especially those that are associated with the nerves.

Ashwagandha contains many useful secondary metabolites such as withanolides, flavonoids, alkaloids, steroids along with many other active functional ingredients [12]. Research regarding the metabolites provide a logical and scientific basis that Ashwagandha has the potential to be a medication for stress related diseases, neuronal disorders and cancers [30]. Other studies have also indicated that Ashwagandha has anti-cancer properties as well as immunomodulatory effects [31,35]. For example, a review published in 2000 cited that secondary metabolites isolated from Ashwagandha had promising antibacterial, antitumoral, immunomodulating and anti-inflammatory properties [5]. This review gave multiple examples of in-vitro studies as well as a few examples of in vivo studies using animal models. The results of another study in 2007 showed that Ashwagandha had an anti-tumor effect in Chinese hamster ovary cell carcinomas [31].



Figure 10: Image of Ashwagandha (Grow Organic).

1.6.4 Tulsi

Tulsi, also commonly known as Holy Basil, is yet another herb from India that is commonly used in Ayurvedic medicine. [9]. Its recommended uses include treatment for a range of conditions such as anxiety, cough, asthma, diarrhea, fever, dysentery,



Figure 11: Image of Tulsi (Plants with a Purpose).

arthritis, eye diseases, indigestion, vomiting, gastric, cardiac and genitourinary disorders, back pain, skin diseases, ringworm, malaria, and insect, snake and scorpion bites [9].

As the word spread about Tulsi's use to treat a wide range of conditions, scientists in the western hemisphere performed studies on the pharmaceutical actions of this "holy" herb. An analysis on the chemical make-up of Tulsi revealed that the herb has an excellent source of phytochemicals. Tulsi contains secondary metabolites such as carbohydrate, tannin, flavonoids, saponins, glycoside, terpenoid, fatty acids and phenol. In fact, the analysis revealed that Tulsi has an exceptionally high amount of phenol, reporting levels between 1.6% and 7.6%. The analysis also reported that Tulsi has a substantial number of alkaloids, ranging between 0.91% and 1.28% [4]. Both phenols and alkaloids were both previously discussed as secondary metabolites that have medicinal properties.

Scientific studies concluded that it can be used to strengthen the immune system against toxic chemicals, protect the body against pathogens with anti-microbial activity and stabilize the metabolism by controlling blood glucose levels [9]. There have also been studies proving that Tulsi has anti-cancer effects [17]. For example, A study on rats in 2008 showed that Tulsi

prevented the growth of cancer by reducing DNA damage and inducing apoptosis in precancerous and cancerous cells [17].

2.7 Research Question

The invasiveness of current cancer treatments proves it is necessary to find a less harmful alternative treatment. Based on the background research, Neem, Nilavembu, Ashwagandha and Tulsi have potential anti-cancer effects, but the research conducted on each of the herbal agents have only been performed on animal models. To take a step forward, the anti-cancer properties could of each herbal agent should be tested with a humanized xenograft transplant model on live zebrafish.

A human xenograft transplant is a model in which human pancreatic tumor cells (also referred to as P3 cells) are transplanted into a live animal model. This way, anti-cancer effects of the herbal agents can be tested on human cancer cells, without directly being tested on humans. Zebrafish were chosen as the research model because of their high fecundity and short life span. This allows more experiments to be done in a shorter period of time. Their external embryonic development also makes it easier to manipulate the zebrafish embryos. This research model will add value to the data because it will not only display whole animal toxicity, but it will also show anti-cancer effects on human cells without having to test humans.

2.7.2 Hypothesis and Predictions

I suspect that the herbal agents Neem, Nilavembu, Ashwagandha and Tulsi will not have toxic effects on zebrafish. But, based on previous studies conducted on the herbal medications, as well as studies about their plant derivatives, I expect that all of the herbal agents will show anti-cancer effects.

3. MATERIALS AND METHODS

3.1 Zebrafish (*Danio rerio*)

Zebrafish are a tropical fresh-water fish of the minnow family that originated in rivers and ponds on India. Zebrafish are now available in pet shops, but more importantly are becoming extremely popular in experiments where they are used for genetic human modeling. In contrast to rats and mice, Zebrafish have high fecundity and a short life span. This makes them a low-cost model that allows for maximized data collection. Zebrafish are also much easier to genetically manipulate because their embryos are laid and fertilized externally. Collectively, these characteristics made Zebrafish the best model to research the effects of Neem and Nilavembu.

3.2 Zebrafish Husbandry

All zebrafish were cared for according to the Institutional Animal Care and Use Committee of Georgia Southern. The breeding of the wild-type zebrafish occurred in groups that were set up in the evening of three males and three females. The embryos were collected the following morning and transferred to a petri-dish where they were allowed to incubate at 28.5° C for several hours until the zebrafish were sorted into wells and treated that evening.

3.3 Maximum Tolerable Dosage

To determine the toxicity and anti-cancer properties of Neem and Nilavembu, zebrafish were treated with the highest tolerable dosage of each herbal agent. To obtain the maximum tolerable dosage, a 10,000 parts per million stock solution of Neem and Nilavembu were made using 10 milligrams of the powder form of both herbs mixed with a milliliter of E3 solution. Then, in a 24-well plate, 10 embryos were treated per well with either 100ppm, 250ppm, 500ppm, or 1000ppm of each solution. The embryos were monitored for 5-days; each day the

solutions were changed to prevent the development of a toxic environment. The dosage in which the majority of the embryos survived became the maximum tolerable dosage.

3.4 Toxicity

To determine the toxicity of each herbal agent, embryos were treated with the maximum tolerable dosage of Neem and Nilavembu for five days and compared to the control in E3 solution. The mortality rate and hatch rate were collected every day of each trial, and the heart rate was recorded on the third day of each trial. At the end of the trial, all embryos underwent immunofluorescent antibody labeling on day 6 of the assay. All embryos were preserved in about 100 microliters of 4% paraformaldehyde (PFA). 50 milliliters of the detergent, incubation buffer (IB), was made from 25 milliliters of 2X phosphate buffered saline (PBS), 24.45 milliliters of double distilled H₂O (ddH₂O), 500 milligrams of Bovine Serum Albumin fraction IV, highest grade, 250 microliters of TritonX100, and 500 microliters of dimethyl sulfoxide (DMSO). Next, embryos were washed four times for 30 minutes each with 500 microliters of IB, using a rocking platform shaker. 1 milliliter of IB was mixed with 10 microliters of monoclonal anti-tubulin acetylated antibody. 2.5 milliliters of this mixture was then distributed to each embryo well sample and washed for 30 minutes. Diluted primary antibody was added to each embryo batch sample and incubated overnight. The next day, the samples were washed four times with 500 microliters of IB for 30 minutes each. 1 milliliter of IB was mixed with 10 microliters of monoclonal anti-tubulin acetylated antibody. 2.5 milliliters of this mixture was then distributed to each embryo well sample and washed for 30 minutes. Diluted secondary antibody was then added to each embryo batch sample and incubated overnight. The next day each sample was washed three times in 1X PBS for 5-10 minutes each. Each sample was then fixed in 4% PFA

overnight. The next day each embryo batch sample was washed 3 times with 1XPBS for 5-10 minutes each. Each batch sample was then transferred into wells in a 24-well plate. 1XPBS was replaced with 1 milliliter of 50% glycerol (in ddH₂O) and incubated for 10 minutes. 50% glycerol was replaced with 1 milliliter of 70% glycerol. Two embryos per slide per treatment group were then mounted on concave slides for confocal microscope imaging.

3.5 Anti-Cancer Properties

Similar to toxicity data collection, embryos were treated with the maximum tolerable dosage of Neem and Nilavembu for five days. There were two control groups involved; a control group that was injected with cancer and a control group that was not injected with cancer. When the embryos were about three days old, they were injected with cancer and continued to be treated for another two days. After the two days of treatment, the embryos in each group were washed three times and stained with acridine orange solution. and those with cancer present were separated from those with no cancer present. Then, two embryos from each group were randomly chosen for imaging.

4. RESULTS

4.1 Toxicity Results

There was a one hundred percent fatality rate for all of the herbal agents at 1000ppm, so that data was not shown. The maximum tolerable dosage for Neem, Nilavembu and Tulsi was a solution of 100ppm, whereas Ashwagandha had a maximum tolerable dosage at 250ppm. When treated with the maximum tolerable dosage for 5 days, the embryos from the maximum tolerable dosage of each herbal agent had similar mortality rates, hatch rates and heart rates when compared to the control, thus showing that the herbal agents are not toxic to the zebrafish. In addition, immunofluorescent imaging at the end of the toxicity trial showed that the herbal agents are not toxic to the fishes' health.

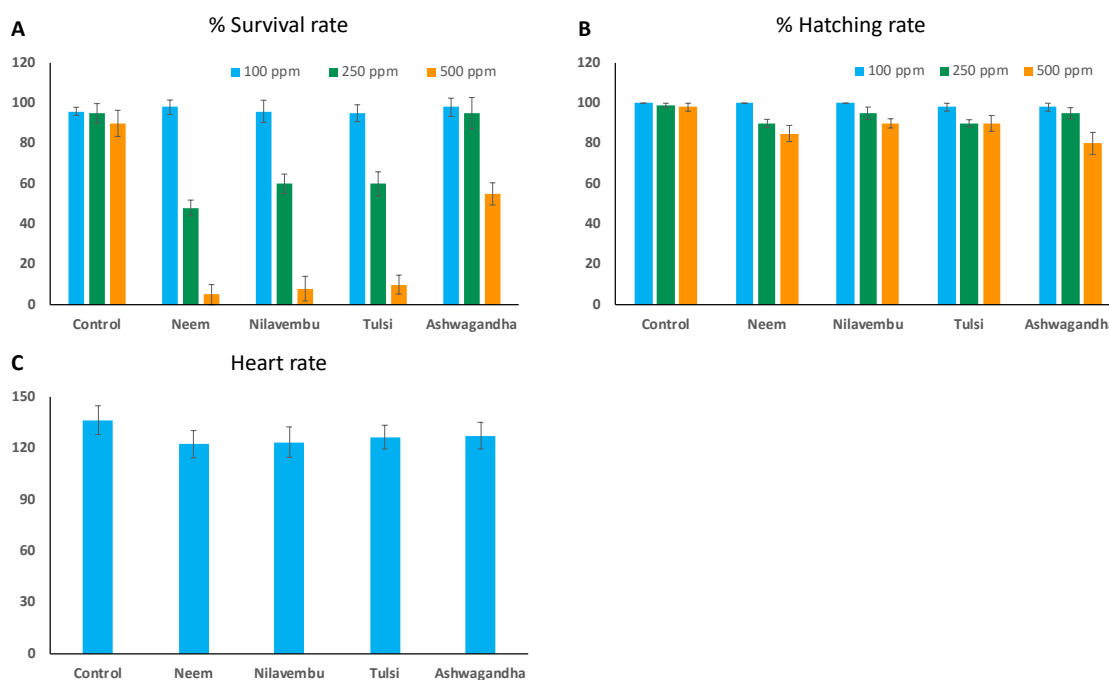


Figure 12

A: A graph of percent survival rate among the embryos. This graph shows the percent survival rate of each dosage. There is a 90% survival rate at the maximum tolerable dosage. Neem, Nilavembu, and Tulsi have a maximum tolerable dosage at 100ppm whereas Ashwagandha has a maximum tolerable dosage at 250ppm.

B: Graph of the percent hatching rate among embryos. The maximum tolerable dosage of each of the embryos have a comparable percent hatching rate to the control.

C: Graph of the average heart rate among the embryos. The embryos from each of the herbal agents have a comparable heart rate to the embryos of the control.

3.2 Anti-Cancer Results

Fluorescent staining showed significant anti-cancer effects of Neem, Nilavembu, Ashwagandha and Tulsi when compared to the control. As shown in Figure 13, the Dil staining revealed the cancer cells that were injected in each of the fish, the Acridine Orange reveal the dead cells in the fish, then the combination of the Dill and Acridine Orange reveal dead cancer cells. The control had cancer cells but no dead cells, thus showing no anti-cancer activity. The Neem had cancer cells and dead cells in the same location as the cancer cells, thus hinting that only the cancerous cells were dying. When the Dil and Acridine Orange merged, Neem had proof of anti-cancer activity. This is also a trend in Nilavembu, Tulsi and Ashwagandha. All of the herbal agents have dead cancer cells, but no evidence of dead normal cells. After counting the cells in each Z-stack and comparing the amount of cancer cells to the amount of dead cancer cells, it is evident that Neem has the most anti-cancer activity. There was 70% of cancer cell death in Neem, 50% cancer cell death in both Nilavembu and Tulsi, and about 35% of cancer cell death in Ashwagandha. This is percent of induced cancer cell death is significant when compared to the values of the control and can be seen in Figure 14.

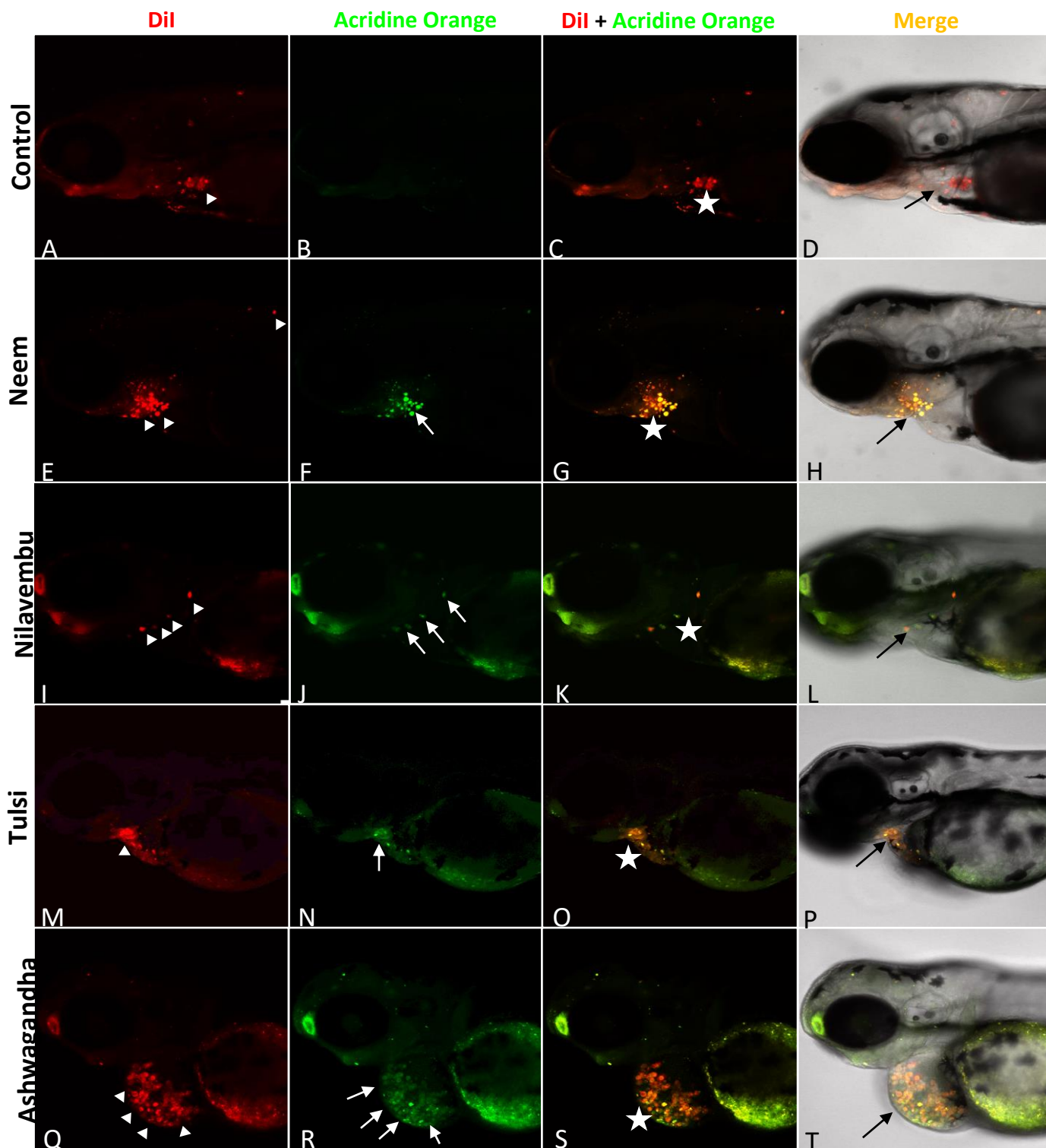


Figure 13: Imaging of each herbal and the control with cancer. The Dil staining revealed the cancer cells that were injected in each of the fish, the Acridine Orange reveal the dead cells in the fish, then the combination of the Dill and Acridine Orange reveal dead cancer cells.

Herbal Induced Cancer Cell Death (%)

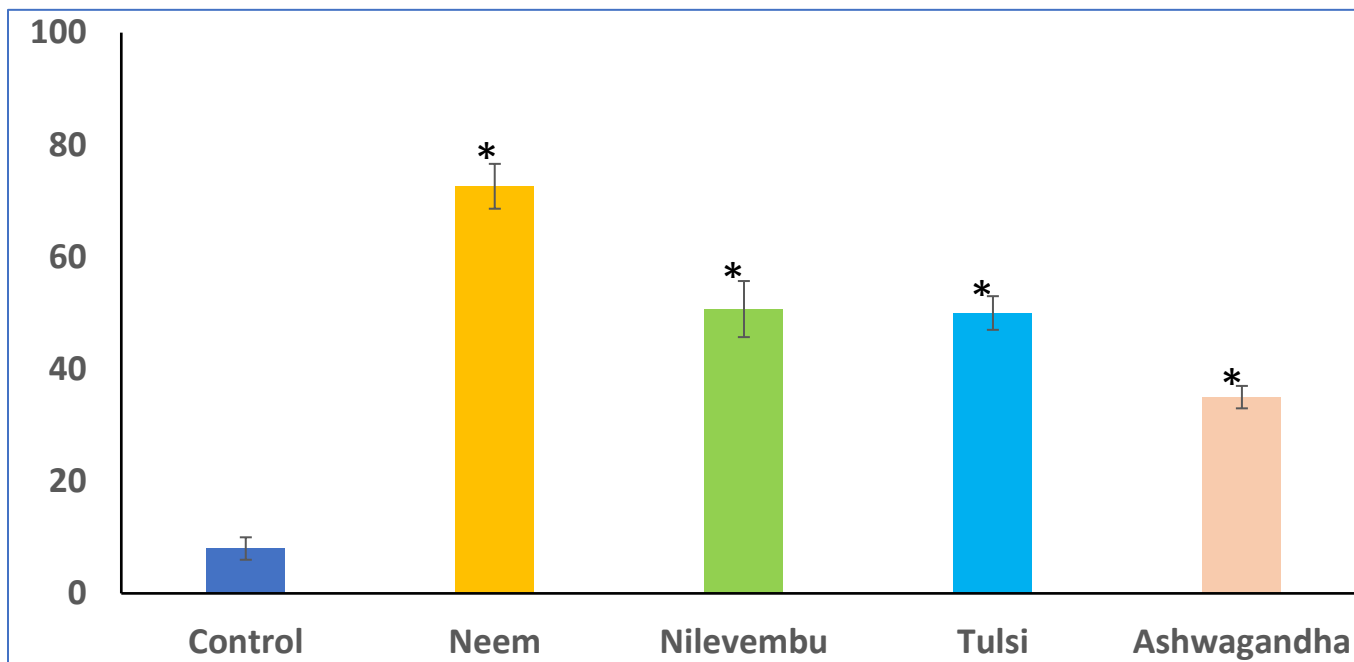


Figure 14: Chart of the herbal induced cell death of each herbal alongside the control. There was 70% of cancer cell death in Neem, 50% cancer cell death in both Nilavembu and Tulsi, and about 35% of cancer cell death in Ashwagandha. This is percent of induced cancer cell death is significant when compared to the values of the control.

5. DISCUSSION

As predicted, Neem, Nilavembu, Ashwagandha and Tulsi all had anti-cancer effects on the human prostate cancer cells and also had little to no toxic effects on the zebrafish. This aligns with the previous anti-cancer studies on each of the herbal agents

Neem had the greatest anti-cancer activity at 90% herbal induced cancer cell death. Ayurvedic medicine practitioners would expect this because the Neem tree is one of the most popular herbs that is used. The success of Neem is also not surprising considering the tree has more than 140 active compounds; one or more of these compounds is bound to have an effect cancer. Based on a previous study by Moga in 2018, we can infer that the anticancer property in

Neem occurs from one or more of the 35 tumor suppressing compounds he found in Neem. His experiment specifically highlighted the modulation of pathways p53, pTEN, NF- κ B, PI3K/Akt, Bcl-2, and VEGF [19]. Based on this study and our findings, we can speculate that Neem kills cancer cells by impacting cancer cells proliferation, differentiation, apoptosis, angiogenesis, and metastasis processes through these pathways.

Nilavembu's success also did not come as a surprise. Although it was primarily used to heal the liver and alleviate fever in Ayurvedic medicine, one of its other uses was to prevent cancer. Our findings also coincide with research done on one of the major terpenoids found in the herb, Andrographolide. This research has been thought to cause cancer cells to arrest in the G0/G1 phase and push them through apoptosis, an action that would not otherwise occur in cancer cells [20]. There was also evidence to believe that andrographolide stimulated lymphocytic proliferation and activation, which would activate the immune system against the cancer cells [20]. So, with this information and our findings, we can explain the herbal induced cell death caused from herbal induced apoptosis, or stimulation of the immune system against the cancer cells. Seeing that there was only 50% cancer cell death, it can be deduced that the death was occurring because of an activated immune system. This mechanism is logical because it would require more time to kill the cancer cells, and in the experiment, the cancer cells were only susceptible to the herbal agent for two days. Through this deduction, it is suspected that there would be a higher percentage of cancer cell death if the cancerous cells were treated with the herbal agent for a greater amount of time.

The herb Tulsi had the same percentage of cancer cell death as Neem. Similar to Neem, Tulsi had many uses in Ayurvedic medicine. Although its purpose is primarily immune system and metabolism related, one of its uses was to prevent tumorigenic activity [9]. The research

done on the effects of Tulsi, aligns with its anti-cancer effects in this experiment. According to the 2008 rat study, Tulsi prevented the growth of cancer by inducing apoptosis in cancerous cells [17]. Although the exact mechanism for the cell death is unknown, we can speculate that the mechanism is the same in both experiments. Other experiments on Tulsi reveal that it has an extremely large amount of phenols present [4]. As previously discussed, it was experimentally proven in 2010 that phenols have anti-cancer activity by strengthening the immune system and making cells more resistant to cancer [38]. Therefore, it is possible that the anti-cancer mechanism is through the immune system. The amount of cell death via an immune system mechanism would be smaller in a short period of time because an immune response would take more time than instant apoptosis. So, similar to Nilavembu, Tulsi could have a smaller amount of cell death because the mechanism triggers an immune response and the cancer was only introduced to the herbal agents for two days.

Unlike the other herbal agents, Ashwagandha's use in Ayurvedic medicine did not include cancer treatment or prevention. However, it is no surprise that Ashwagandha had anti-cancer effects based on previous research. Experiments on the secondary metabolites of Ashwagandha shown promising anti-cancer effects, specifically the metabolites withanolide (a metabolite specific to Ashwagandha) and alkaloids [5,32]. So, it can be assumed that the cancer cell death was caused by these metabolites. However, Ashwagandha had the least amount of cancer cell death despite its higher concentration of dosage, making it less successful than the other herbal agents.

Neem, Nilavembu, Ashwagandha and Tulsi all had anti-cancer effects on the human prostate cancer cells transplanted inside of the zebrafish when compared to the control. However, the level of success varied among the herbal agents. Based on the data, Neem was the most

successful herbal agent for treating cancer, while Ashwagandha was the least successful. In future experiments, the amount of time that the cancer is treated with the herbal agents can be lengthened to determine if Nilavembu, Tulsi and Ashwagnadha show an increase in herbal induced cell death.

6. CONCLUSION

The experiment presents a strong argument that Neem, Nilavembu, Ashwagandha and Tulsi have high anti-cancer efficiency with low toxicity. Neem had 90% herbal induced cancer cell death, Nilavembu and Tulsi had 50% herbal induced cancer cell death, and Ashwagandha had 35% herbal induced cancer cell death. Therefore, the evidence of the study presents a strong case that the herbal agents can be used as a non-toxic method of treating cancer. The scientific community can move forward by testing the herbal agents on a human model in future experiments.

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