Anti-Carcinogenic Effects of *Morinda citrifolia* (Noni): Identifying Signal Transduction Pathways using ER Positive and ER Negative Human Breast Cancer Cell Lines

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Master of Science

in

Molecular Biosciences and Bioengineering

August 2012

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We certify that we have read this Thesis and that, in our opinion, it is satisfactory in scope and quality as a Thesis for the degree of Master of Science in Molecular Bioscience Bioengineering.

Thesis Committee:

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<tr>
<td>Dulal Borthakur</td>
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<td>Scot Nelson</td>
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Dedication

To my brother, Andy Hwang.
Acknowledgments

Thanks to Dr. Pratibha Nerurkar for all her support and guidance. I would never be able to complete my project if she had not accepted me into her lab and taught me everything.

I would like to express my gratitude to Dr. Dulal Borthakur and Dr. Scot Nelson for their counsel and guidance.

I would also like to thank our lab members for all their moral support. Especially my colleagues who trained me.

Last but not least, thanks to my friends and family for all the emotional support and understanding.
Abstract

It is predicted that one in every eight women in the United States will be diagnosed with breast cancer in her lifetime, making breast cancer the second most prevalent cancer in the nation. Native Hawaiians have the highest incidence as well as mortality rates compared to other ethnic groups in Hawaii. An increase in the use of alternative medicine, specifically by breast cancer patients prompted us to study the anti-cancer effects of Hawaiian traditional medicine, *Morinda citrifolia* (noni). Estrogen receptor positive (ER +ve) cells, MCF-7 and estrogen receptor negative (ER −ve) cells, MDA-MB-231 were initially treated with fNJ at varying concentrations for up to 96h. 10% fNJ demonstrated cell death in 30 – 40% of the cells and 15% fNJ demonstrated cell death in 50 – 60% of the cells. In addition, when non-carcinoma breast cells, MCF-10A underwent the same treatment, 15% fNJ only demonstrated 10 – 15% cell death in MCF-10A. Cell proliferation assays suggest that fNJ is toxic to breast cancer cell lines, MCF-7 and MDA-MB-231 but not normal breast cell line, MCF-10A. Apoptosis array data have shown a significant decrease of a class of proteins called inhibitors of apoptosis proteins (IAPs) such as survivin, Bcl-2, cIAP-1, and cIAP-2 in fNJ treated MCF-7 and MDA-MB-231 cells. There was also a significant decrease in proteins that assist with cell replication such as claspin and phospho-Rad17. This demonstrates that fNJ induces cell selective apoptosis and inhibits cell proliferation in breast cancer cell lines, MCF-7 and MDA-MB-231. [Grants: NCCAM (R21AT003719), USDA-CREES (2004-34135-15182)]
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<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer type 1</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and alternative medicine</td>
</tr>
<tr>
<td>CDH1</td>
<td>Cadherin 1</td>
</tr>
<tr>
<td>CHEK2</td>
<td>Dysfunctional check point homolog 2</td>
</tr>
<tr>
<td>cIAP-1</td>
<td>Cellular inhibitor of apoptosis protein 1</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>eIF-2α</td>
<td>Eukaryotic translation initiation factor-2α</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptors</td>
</tr>
<tr>
<td>ER-ve</td>
<td>Estrogen receptor negative</td>
</tr>
<tr>
<td>ER+ve</td>
<td>Estrogen receptor positive</td>
</tr>
<tr>
<td>Fas/TNFRSF6</td>
<td>Fibroblast associated tumor necrosis factor receptor super family member 6</td>
</tr>
<tr>
<td>fNJ</td>
<td>Ferment noni fruit juice</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human laryngeal carcinoma</td>
</tr>
<tr>
<td>HSP-60</td>
<td>Heat shock protein 60</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of apoptosis</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>JNK-1</td>
<td>c-Jun N-terminal kinase-1</td>
</tr>
<tr>
<td>LAN5</td>
<td>Human neuroblastoma</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
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<tr>
<td>LLC</td>
<td>Lewis lung carcinoma</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast ER+ve carcinoma</td>
</tr>
<tr>
<td>NAG-1</td>
<td>Nonsteroidal anti-inflammatory activated gene-1</td>
</tr>
<tr>
<td>NfkB</td>
<td>Necrosis factor-kappa beta</td>
</tr>
<tr>
<td>NJ</td>
<td>Noni fruit juice</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Noni-ppt</td>
<td>Noni fruit precipitate</td>
</tr>
<tr>
<td>phospho-Rad17</td>
<td>Phosphorylated Rad 17</td>
</tr>
<tr>
<td>PK-B</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF related apoptosis-inducing ligand</td>
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Chapter 1: Introduction

1.1 Breast Cancer

1.1.1 Impact

Breast cancer is the third highest cause of cancer-related mortality in Hawaii [1].

Coming in behind prostate cancer, it is the second most prevalent cancer in the United States [2]. An estimated number of 226,870 women in the United States will be diagnosed with breast cancer by the end of 2012. Of these women, 1,120 will be from Hawaii [1]. Although breast cancer mortality seems to remain relatively constant for the past 30 years, the incidence rates have been slowly increasing from 1975 – 2008 [3].

1.1.2 Factors and Causations

Cancer is characterized as uncontrolled growth of damaged or abnormal cells in the body. Unlike normal cells that proliferate and die and under apoptosis, these malignant (cancerous) cells do not undergo cell cycle arrest and will continue to rigorously grow in its abnormal state (www.cancer.org). Sometimes the cancer metastasizes and travels to distant organs. The cancer is named after the body part where the tumor originates [3]. Thus, breast cancer originates from the breast tissue. Some breast cancers are formed within the ducts or lobules of breasts and are therefore referred to as ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), respectively (figure 1). Most breast cancers are invasive and travel from ducts and lobules to nearby breast tissues [3]. Many
factors including age, sex, family history, physical fitness, and diet influence the type of breast.

Fig. 1. Ductal and Lobular Carcinoma In Situ [source: www.virtualmedicinecentre.com]

Aside from being female, age is the biggest risk factor for breast cancer. Invasive breast cancer is found in 1 out of 8 women under the age of 45, whereas it is found in 2 out of 3 women over the age of 55 [4]. A woman at the age of 30 has a 0.43% risk of developing breast cancer, compared to a woman at the age of 60 who has a 3.45% risk of developing breast cancer [3]. This evidence provided by the American Cancer Society suggests that the probability of developing breast cancer increases with age. It is believed that the increased likelihood of developing breast cancer at an older age may be due to a longer life expectancy in modern times. Other factors such as menopausal hormone use, changes in reproductive patterns, rising obesity rates, and improved detection and screening methods, also contribute to this increase.

Family history and genetics influence a woman’s likelihood of developing breast cancer. Studies have shown that 5 – 10% of diagnosed breast cancer is thought to be
hereditary [4, 5]. The more closely related a woman is to a family member who has
breast cancer, the greater her chance of developing breast cancer [6]. Studies have shown
that a number of individuals with genes such as breast cancer type 1 (BRCA1) and
BRCA2 [7] and dysfunctional check point homolog 2 (CHEK2) [8], tumor protein 53
(TP53) [9], ataxia telangiectasia mutated (ATM) [10], and cadherin 1 (CDH1) [11] genes
have a higher risk of developing breast cancer.

In light of current studies on lifestyle choices and cancer, obesity, physical fitness [12,
13], and nutrition have been implicated as risk factors (figure 2) [14, 15]. Garnet
Anderson’s study showed a 70% increase risk factor of breast cancer in obese
premenopausal women compared to normal weight premenopausal women [16]. This
suggests that women with higher than normal body mass index (BMI) have an increased
risk of developing breast cancer. Besides having a high BMI, women with higher than
normal central adiposity (measured by waist circumference) also have a higher risk of
developing breast cancer [17]. Obese individuals tend to have higher concentrations of
pro-inflammatory markers circulating in the blood causing the person to be in a chronic
inflammatory state [18-20]. An activated pro-inflammatory marker, necrosis factor-
kappa beta (NF-κB), has been observed in mammary adipose tissues obtained from high
fat diet fed mice [21] and obese women with breast cancer [22]. These findings show
that breast tissues with high adipocyte density have elevated levels of NF-κB.
Furthermore, the correlation between adipocyte size, BMI, and inflamed mammary
glands supports the idea that obesity is associated with an increased risk of breast cancer
on a cellular level. In 2010, 35.8% of the women in the United States were considered
obese (www.cdc.org), indicating that 40.6 million American women have a high risk of
developing breast cancer.

**Figure 2.** Modifiable risk factors that affect cancer [source: Food, nutrition, physical activity and the prevention of cancer: a global perspective. World Cancer Research Fund/American Institute for Cancer Research, 2007: p. 30-46]
Whether or not an individual’s diet affects their risk of developing breast cancer is still a controversial topic. A number of studies carried out to discover the correlation between foods and the onset of breast cancer have yielded conflicting results[3, 15]. Foods such as bitter melon [23], curcumin[24], resveratrol [25], and other foods high in antioxidants and fiber [26, 27] have been shown to decrease the onset of breast cancer. Whereas high temperature cooked meats [28], red meats[29], and high fat foods [21] have been shown to increase the risks. More research is needed to accurately determine the effects of dietary consumption on breast cancer. Other factors such as periods and amount of food consumption, family history, food-food and food-drug interactions might also play an important role in the development of breast cancer and need to be further analyzed.

1.1.3 Estrogen Receptor Negative vs. Estrogen Receptor Positive Breast Cancer Cells

Estrogen is a steroid hormone that is commonly found in women to regulate pregnancy, bone density, menstrual cycle, liver function, breast and uterine cancer. Estrogen receptors (ER) are molecules that bind to estrogen hormones and initiate a number of signaling pathways [30]. The presence of intracellular ER indicates the cell’s receptiveness to estrogen hormones (figure 3) [30, 31]. Consequently, elevated levels of circulating estrogen increases the risk of developing breast cancer for individuals with estrogen receptor positive (ER+ve) breast cells[32-34].
Due to the lack of estrogen receptors, estrogen receptor negative (ER-ve) breast cells are unresponsive to selective hormonal therapy compared to ER+ve breast cells [35]. Consequently, studies have shown that since the amount of ER present on each cell varies, not all ER+ve breast cells respond well to hormonal treatment. As the amount of ER decreases, so does the efficacy of the treatment [19, 36]. This raises a problem because 15% of the breast cancer patients in the United States are ER-, leaving approximately 450,000 women incurable [37]. Unfortunately, only 30% of breast cancer patients have overexpressing ER that is highly susceptible to selective treatment [35], leaving the rest of the women to rely on other non-selective cancer treatments such as chemical and radiation therapy. Side effects from these non-selective cancer treatments such as hair loss, lowered immune system, fatigue, nausea and abnormal digestive processes (www.cancercare.org) are incentives to further investigate novel selective breast cancer treatments.

**Figure 3.** General transcription mechanisms of estrogen receptor positive and estrogen receptor negative breast cancer cells [source: Food, nutrition, physical activity and the prevention of cancer: a global perspective. World Cancer Research Fund/American Institute for Cancer Research, 2007: p. 30-46]
1.2 Complementary and Alternative Medicine

1.2.1 Usage in the United States

Complementary and alternative medicine (CAM) covers a spectrum of medical and health care practices that range from traditional to modern methods to prevent or cure certain diseases and ailments [38, 39]. Complementary medicines are used alongside with other conventional medicines. Whereas, alternative medicines are used to substitute conventional medicines [39]. There are two types of CAM therapies: practitioner therapies and self-care therapies [40]. Commonly used practitioner-type CAM therapies include deep breathing, chiropractic treatments, massage therapy, acupuncture, and hypnotism therapy. Commonly used self-care therapies include natural product consumption, diet based therapy, meditation, and yoga [41, 42].

The use of CAM in the United States has been increasing over the past decade. In 2002, 36.0% of American adults reported that they used CAM. In 2007, this number jumped to 38.3% [38, 42]. With over a third of the American adults and 11.8% of the American children using CAM, the total out-of-pocket expenses spent on CAM healing philosophies, therapies and products was $33.9 billion [40]. 44% of the out-of-pocket costs were spent on non-vitamin, non-mineral products indicating the popularity of natural products (figure 4) [40]. Unfortunately by definition, CAM is not considered a conventional medicine due to the lack of evidence to prove how safe and effective they are [43]. Due to the increasing popularity of natural products in the United States, additional research is needed to inform the public of their effectiveness and side effects, if any.

1.2.2 Complementary and Alternative Medicine as Cancer Treatment

CAM treatments have been used for certain diseases and conditions such as head or chest cold, anxiety, digestive illnesses, high cholesterol, and cancer [41, 42]. Many cancer patients find additional ways to improve their diseased state or to prevent side effects caused by conventional medicine [44]. An international survey showed that 35.9% of cancer patients used CAM [45]. In a 2005 study conducted by Molassiotis et al., 44.7% of the breast cancer patients studied (n=956) used CAM to either treat the disease or increase physical and emotional well-being [46]. For a multitude of reasons, some cancer patients turn to natural CAM products for treatment. 40.1% of the cancer patients that utilize CAM rely on natural products [45]. Therefore, a solid investigation into the effectiveness of CAM against cancer will be of great benefit to patients who utilize it.
1.3 Morindacitrofolia (Noni)

1.3.1 History

*Morindacitrifolia* (noni), also known as Indian Mulberry, nono, or cheese fruit, is a small shrub that is native to South Asia and grows prominently in the tropics [47]. Noni shrub grows about two to six meters tall, with either rounded, elliptic or long leaves and globular fruits that range from three to 20 cm long [48, 49]. Noni has been traditionally used in Hawaii, Polynesia and Southeast Asia as either a dye, famine food, [50] and is considered the most important medicinal plant based on reports of usage across Polynesian cultures [48, 51, 52].

*Figure 5.* Noni fruit  [source: www.resorthealth.com]
1.3.2 Traditional and Modern Uses

Traditional healers from Hawaii, Samoa, Rotuma, and Fiji have been known to utilize different parts of the plant to treat wounds, infections, menstrual cramps and bowel irregularities[48, 53]. Table 1 summarizes the traditional uses of noni prior to the 1800s. Contrary to popular belief, traditional healers do not commonly utilize the ripe fruits. The most commonly used part of the noni plant was the leaf. Traditional healers would heat noni leaves over a small fire then use it as a poultice by wrapping it around the wounded or infected area [54]. Yet the use of noni fruits is popular as a modern dietary supplement [48].

<table>
<thead>
<tr>
<th>Noni plant part</th>
<th>Traditional treatment for</th>
</tr>
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<tbody>
<tr>
<td>Leaves</td>
<td>Topical burns, headaches, fevers, neonatal inability to breathe, bone fractures, menstrual cramps, back pain*, insect infestations, boils, rheumatic pain, ulcers, gout, internal bleeding, ringworm[48, 50, 55-57]</td>
</tr>
<tr>
<td>Fruit</td>
<td>Sores in mouth, peeling or cracking of toes, boils, pimples, blood impurities*, kava intoxication, insect infestations, blotchy skin*, heart trouble, stomach pain*, menstrual cramps*, heartburns, sore throat [48, 55, 56, 58]</td>
</tr>
<tr>
<td>Bark</td>
<td>Stonefish poisoning, topical infections, asthma*, [55, 57, 58]</td>
</tr>
<tr>
<td>Root</td>
<td>Topical infections, stomach pain* [55]</td>
</tr>
<tr>
<td>Stem</td>
<td>Hernia [49]</td>
</tr>
<tr>
<td>Seeds</td>
<td></td>
</tr>
<tr>
<td>Flower</td>
<td>Sore eyes, topical burns [57]</td>
</tr>
</tbody>
</table>

Table 1. Traditional uses of the noni
In the 1980s, commercial noni fruit juice (NJ) gained popularity fueled by Ralph Heinicke’s claim that it contains Proxeronine, a precursor for Xeronine. Heinicke suggested that Xeronine is an enzyme that can modify dysfunctional proteins to its appropriate conformation[48, 53, 59]. In a study conducted in 1999, noni ranked the second most commonly used complementary medicine[57]. The Nutrition Business Journal reported that noni juice is the number one sales of single herbs in American in 2005 (www.nutritionbusiness.com). Noni was commonly used topically to treat ailments up until the 1900s, when people began consuming noni products for its medicinal properties. Fermented noni juice is popularly known as the “traditional” method of preparing noni. It is believed that the Chinese, which composed a significant proportion of the population in Hawaii, influenced fermenting the fruit. Reports have shown that in the 1930s fermented noni juice was commonly prepared and consumed by households in Hawaii. Furthermore, studies show an increase of demand for noni fruit from early to late 1990’s[57]. Since then, multimillion-dollar companies have found ways to package and sell this plant.

Due to the rising popularity of noni, many studies have been conducted to test its efficacy as a medicine and have shown its anti-cancerous[60-62], anti-diabetic[63], anti-tuberculosis[64], anti-viral[65], and anti-bacterial[66-68] effects. These studies were already reviewed by Pawlus, Wang, Blanco, and Brown[49, 53, 69, 70]. The up to date modern uses of noni are summarized in Table 2.
<table>
<thead>
<tr>
<th>Noni plant part</th>
<th>Treatment for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Tuberculosis, helmintic infections, oxidative stress, open wounds, hyperlipidemia [64, 71-73]</td>
</tr>
<tr>
<td>Fruit</td>
<td>Bacterial infections, cancer, pain, memory impairment, reduced cerebral blood flow, diabetes, nausea, gastric ulcers, liver disease, low humoral immunity, neuronal damage, stress-induced cognitive impairment, helmintic infections, lung cancer, oxidative stress, inflammation, post surgery nausea, hyperglycemic, liver diseases [62-64, 66-68, 72, 74-88]</td>
</tr>
<tr>
<td>Bark</td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>Viruses, hypotension, colorectal cancer, NF-kB associated inflammation, hyperlipidemia, diabetes, pain [62, 65, 71, 73, 89, 90]</td>
</tr>
<tr>
<td>Stem</td>
<td></td>
</tr>
<tr>
<td>Seeds</td>
<td>Hyperlipidemia, oxidative stress, melanoma cancer [91-93]</td>
</tr>
<tr>
<td>Flower</td>
<td></td>
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</tbody>
</table>

Table 2. Modern uses of noni
From skincare products to supplement capsules and tea, noni has been packaged in multiple ways with claims to a better and healthier lifestyle. In 1992, a Maui massage therapist encapsulated the dry powder form of noni fruit and sold it as supplement[57].

In 1996, John Wadsworth and Stephen Story introduced Tahitian Noni Juice as a natural healthy supplement to the United States (http://www.tahitian-juice.com). Other noni juice companies such as, Virgin Noni Juice, Healing Noni, and Noni Maui, sprouted since then with their own line of noni juices. These bottles juices are sold either non-fermented or fermented.

![Encapsulated freeze-dried noni fruit](source: www.estatenoni.com)

1.3.3 Fermented Noni Fruit Juice

To present date, there are two popular methods to prepare noni fruit juice: fermented and non-fermented. In the fermented method, ripe yellow fruits are picked, washed and left in glass air sealed containers for about two weeks to two months. The juice is extracted and the pulp is removed. Some fermented juices are pasteurized. Pasteurized noni has a distinctively different taste as well as different pH. To prepare non-fermented noni juice, fresh ripe fruits are pressed through a juicer. The juice can be consumed pure, or diluted with water or other fruit juices. In some juices, the starchy
pulp of the noni is mixed with either apple or grape fruit juice to increase palatability [94].

Most noni fruits are consumed as a naturally fermented juice [95]. It is suggested that in Hawaii, this type of noni preparation might have originated from the prominent Chinese ethnic influence in the mid-1800s [57]. Based on local records, Hawaii residents commonly picked, fermented, and consumed noni fruits before meals to treat diabetes, heart trouble and blood pressure [96, 97]. Recent literature suggests that the probiotic properties of most fermented foods are beneficial to your health [98, 99]. The beneficial effects include improved lactose digestion, controlled gastrointestinal infection, reduced cholesterol levels and stimulated immune system [100]. Besides the difference in biotic cultures found in fermented foods compared to non-fermented foods, the chemical constituents may vary as well. In 2007, Shu-Chuan Yang et al demonstrated that the chemical composition of fermented noni juice (fNJ) differed from that of non-fermented NJ. The fNJ yielded higher phenolic compounds, condensed tannins, flavonoids and scopoletin than non-fermented NJ. Additional results from the study showed that fNJ has higher anti-oxidative activity as compared to non-fermented NJ [101].

1.4 Noni and Cancer

1.4.1 In vitro studies

In 1999, Hiramizu and Furusawa tested the antitumor properties of a polysaccharide-rich ethanol extract of noni fruit precipitate (noni-ppt). They found that noni-ppt suppressed the growth of Lewis lung (LLC) peritoneal carcinoma cells by up
regulating several mediator and murine effector cells[61]. In 2006, Arpornsuwan and Punjanon also conducted a study investigating the effects of noni fruit extract against cancer cells. They observed cytotoxic effects against human laryngeal carcinoma (HepG2), human breast ER+ve carcinoma (MCF7), and human neuroblastoma (LAN5) cell lines[102]. An interesting study conducted in 2003 by Hornick et al. looked at the anti-angiogenic effects of noni juice in human breast tumor explants by using a three-dimensional fibrin clot matrix model. The commercial noni juice used demonstrated anti-angiogenic and degenerative effects against the tumor explants[103].

Aside from investigating various noni fruit extracts, several studies have shown the anti-cancerous effects of chemical extractions from other noni plant parts. In 1993, Hiramatsu et al. demonstrated that the damnacathal and anthraquinones extracted from noni root inhibited ras functions in rat kidney cells [104]. Later in 2011, Nualsanit et al. also utilized damnacanthal and anthraquinone extracts from the root and found that the extracts exhibited pro-apoptotic effects by inducing caspase activity in human colorectal cancer cells [105]. New chemical compounds such as 6-O-(beta-D-glucopyranosyl)-1-O-octanoyl-beta-D-glucopyranose and asperulosidic acid extracted from noni fruit juice also displayed anti-cancerous properties against mouse epidermal cells[106].

1.4.2 In vivo studies

In vivo studies play a crucial role in determining the efficacy of the plant on humans. Table 3 is a table compiled by Brown that summarizes the in vivo studies conducted since 1994 to determine the effects of noni fruit juice against cancer. For all the studies mentioned in Table 3, subjects that received noni juice displayed a significant
amount of anti-cancerous effects. Taskin et al.’s Ehrlich ascites tumor injected Balb-c mice showed a reduction in tumor size in mice that have received oral dosages of noni juice as compared to control. Consequently, the same results can be seen in Stoner et al.’s study done on rats. A reduction in tumor size has been observed in rats that received noni juice as a part of their diet, compared to control.
<table>
<thead>
<tr>
<th>Author, year</th>
<th>Subjects – type and number</th>
<th>Treatment</th>
<th>Measurable outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Himazumi et al., 1994</td>
<td>Not provided</td>
<td>Mice inoculated IP with Lewis lung peritoneal carcinoma (LLC)</td>
<td>Control – 15 days mean survival time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1) Control</td>
<td>Noni – about 35 days mean survival time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) Original noni juice of 6–15 mg sold per 0.2 mL volume</td>
<td>9/22 mice survived 50+ days (41%)</td>
</tr>
<tr>
<td>Himazumi et al., 1996</td>
<td>Control – 5 mice</td>
<td>Sarcoma 180 ascites tumor</td>
<td>Control – Ascites in 5/5 mice</td>
</tr>
<tr>
<td></td>
<td>Treatment – 5 mice</td>
<td>Noni ppt (500 μg/mouse administered QOD for five days.</td>
<td>Noni ppt – Suppressed ascites in 5/5 mice</td>
</tr>
<tr>
<td>Himazumi and Furusawa, 1999</td>
<td>55–68 mice</td>
<td>Body weight to detect ascites measured for 2 weeks</td>
<td>Antitumor activity effects from 6–15 mg crude noni juice per mouse. Prolonged lifespan by more than 75%</td>
</tr>
<tr>
<td></td>
<td>Inconsistent number of mice per treatment group</td>
<td>Control: Lewis lung peritoneal carcinoma (LLC)</td>
<td>Group mg/mouse # survivors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1) Noni crude juice inoculations (3, 6, 12, 15, 20 mg/mouse)</td>
<td>Control 0.55 0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) Noni ppt fractions (insoluble)</td>
<td>1) Crude Juice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) Noni soluble</td>
<td>3 1/10 10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 4/18&lt;sup&gt;a&lt;/sup&gt; 22%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12 4/17&lt;sup&gt;b&lt;/sup&gt; 24%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 9/22&lt;sup&gt;c&lt;/sup&gt; 41%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 2/11 18%</td>
</tr>
<tr>
<td>Wang and Su, 2001</td>
<td>Rats (# not provided)</td>
<td>Cancer (DMBA – 7,12-dimethylbenz(a)anthracene) was given intragastrically on day 8. Three rats from each group killed after 24 h</td>
<td>DMBA adducts measured in various organs. TNJ reduced DMBA adducts 30% (60%) in heart, 41% (50%) in lung, 42% (70%) in liver, and 80% (90%) in kidneys of female (and male) rats, respectively</td>
</tr>
<tr>
<td>(corporate funding)</td>
<td></td>
<td>Control: Water for 1 week</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment group:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% Tahitian noni&lt;sup&gt;a&lt;/sup&gt; juice (TNJ) replaced drinking water for 1 week</td>
<td></td>
</tr>
<tr>
<td>Furusawa et al., 2003</td>
<td>Mice</td>
<td>8 small experiments with Asbestos tumor cells (S180)</td>
<td>Noni-ppt produced a survival rate of 25–45%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control:</td>
<td>Interferon worked better than noni by increasing survival rate even more (71–100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment:</td>
<td>Cure rate abolished with macrophage inhibitors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.5 mg in 0.1 mL water; 4.0 – 6.5 mg per mouse)</td>
<td>(2-chloroadenosine), T cells (cyclosporine, or natural killer (NK) cell (anti-asialo GM1 antibody)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1) Noni ppt from ripe fruit juice (Hawaii)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) Noni ppt from Tahitian noni&lt;sup&gt;a&lt;/sup&gt; juice (TNJ)</td>
<td></td>
</tr>
<tr>
<td>Author, year</td>
<td>Subjects – type and number</td>
<td>Treatment</td>
<td>Measurable outcomes</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Palu et al., 2008</td>
<td>Control-5 mice</td>
<td>Control: Water for 16 days; Treatment groups: Tahitian noni juice (TNJ) (1% or 1 mg/mL of commercial product; -unknown noni concentration) for 16 days; Noni fruit juice concentrates (NFJC) (5% or 5 mg/mL from noni fruit puree)</td>
<td>Decreased IL-4, but increased interferon gamma in mice study; Spleenocyte and peritoneal exudate cells treated with TNJ or NFJC resulted in activation of CB&lt;sub&gt;2&lt;/sub&gt; (cannabinoid) receptors, while inhibiting CB&lt;sub&gt;1&lt;/sub&gt; receptors</td>
</tr>
<tr>
<td>Li et al., 2008</td>
<td>CD marker profile study</td>
<td>Mice were intraperitoneally injected with fermented noni extract (500 µL FNE/mouse/day) (from fruit grown in Kawaihae on Hawaii’s South Kohala coast)</td>
<td>CD marker profile study; Increased peripheral blood granulocytes and natural killer (NK) cells; Increased peritoneal total leukocyte counts</td>
</tr>
<tr>
<td></td>
<td>Prevention study</td>
<td>Injected with carcinogen after FNE injection (prevention study) or before FNE injection (treatment study).</td>
<td>Prevention study; &gt; 85% of nude mice were tumor free 1.5 month after tumor inoculation vs 100% death of control mice. Beige mice have no functional NK cells and all died within 20 days</td>
</tr>
<tr>
<td></td>
<td>4 nude mice/group</td>
<td></td>
<td>Prevention study; Prolonged survival of the nude mice, but they eventually all died</td>
</tr>
<tr>
<td></td>
<td>4 beige mice/group</td>
<td></td>
<td>Prevention study; The supernatant contains the anti-tumor substance</td>
</tr>
<tr>
<td></td>
<td>Fractionation study</td>
<td></td>
<td>Tumor diameters about 40–50% smaller than those in control group; Due to induction of apoptosis</td>
</tr>
<tr>
<td>Taskin et al., 2009</td>
<td>31 Mice (Balb-c)</td>
<td>Four groups of mice induced with Ehrlich ascites tumor; Oral noni (Aloni&lt;sup&gt;®&lt;/sup&gt; Hanoju Europe Ltd, Dinxperlo, The Netherlands); Doxorubicin (potent anticancer agent); Oral noni + doxorubicin</td>
<td>Oral noni (0.9% NaCl)</td>
</tr>
<tr>
<td></td>
<td>8 mice/treatment group</td>
<td></td>
<td>Noni may be useful in the treatment of breast cancer; Percent noni in Aloni&lt;sup&gt;®&lt;/sup&gt; not specified, so conclusion suspect</td>
</tr>
<tr>
<td></td>
<td>7 mice/control</td>
<td></td>
<td>All seven berry types were equally capable of inhibiting tumor progression in the rat esophagus</td>
</tr>
<tr>
<td></td>
<td>10 groups of 15 rats each</td>
<td>Rat esophagus induced with carcinogen N-nitrosomethylbenzylamine (NMBA) for 5 weeks, then placed on diets containing 5% of either black or red raspberries, strawberries, blueberries, noni, acai or wolfberry</td>
<td>Tumor incidence: 60–75% in berry groups; 60% in noni group; 95% in carcinogen; 0% in control group</td>
</tr>
</tbody>
</table>

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1.4.3 Molecular Mechanisms

Although there have been many studies conducted on the efficacy of noni against cancer, many of them do not explain the molecular mechanisms behind the anti-cancerous effects. Table 4 summarizes studies done thus far that demonstrate signal transduction pathways caused by noni. In 2007, Takashima et al. studied the apoptotic effects of noni leaf extract via tumor necrosis factor (TNF) and TNF related apoptosis-inducing ligand (TRAIL)[107]. Other studies done by Hirazumi and Furusawa also examined the apoptotic effects of noni through TNF. In addition, they also demonstrated that increased levels of interleukin-1β (IL-1β), IL-10, IL-12, interferon-γ (IFN-γ), and nitric oxide (NO) may have contributed to the anti-cancerous effects [61]. Other studies demonstrate how noni induces apoptosis by affecting the immune system. In 2008, Li et al. showed that fermented noni juice increased levels of granulocytes and natural killer (NK) cells in the peripheral blood, peritoneum, and spleen therefore contributing to the decrease in tumor size of mice[108]. Soon after, Stoner et al. demonstrated that noni fruit powder inhibited tumorigenesis by reducing the levels of cytokines in rats [109].

Although many studies have demonstrated anti-cancerous effects of noni, most fail to identify the molecular mechanisms involved. Thus far, only eight mechanisms have been identified and additional studies are warranted. Identifying the proteins and signal transduction pathways involved in noni’s anti-cancerous properties may aide in determining the benefits of noni against cancer.
<table>
<thead>
<tr>
<th>Type of Noni Extract</th>
<th>Model used</th>
<th>Molecules observed and measurable outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Damnacathal and anthraquinone extracted from noni roots using chloroform</td>
<td>Ras transformed normal rat kidney cells</td>
<td>Inhibited ras function [104]</td>
</tr>
<tr>
<td>Noni-ppt</td>
<td>Lewis lung carcinoma cells subcutaneously injected in C57BL/6 mice</td>
<td>TNF-α, IL-1β, IL-10, IL-12, IFN-γ, and NO levels increased [61]</td>
</tr>
<tr>
<td>6-O-(beta-D-glucopyranosyl)-1-O-octanoyl-beta-D-glucopyranose and asperulosidic acid extracted from noni fruit juice</td>
<td>Mouse epidermal JB6 cells</td>
<td>Suppressed 12-O-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor (EGF) induced activator protein 1 (AP1) transactivation[106]</td>
</tr>
<tr>
<td>Anthraquinone compound extracted from noni leaves using methanol</td>
<td>Immortalized human T-lymphocyte Jurkat cells</td>
<td>Increased TRAIL induced cytotoxic activity [107]</td>
</tr>
<tr>
<td>Fermented noni exudate</td>
<td>C57BL/6J mice</td>
<td>Increased levels of granulocytes and NK cells in the peripheral blood, peritoneum, and spleen[108]</td>
</tr>
<tr>
<td>Noni fruit powder</td>
<td>Rats</td>
<td>Inhibited N-nitrosomethylbenzylamine (NMBA) induced tumorigenesis in the rat esophagus and reduced cytokine, IL-5 and GRO/KC (rat homologue for human IL-8 serum levels) [109]</td>
</tr>
<tr>
<td>Damnacanthal extracted from noni roots</td>
<td>Human colorectal carcinoma cells</td>
<td>Induced nonsteroidal anti-inflammatory activated gene-1 (NAG-1), caspase, and transcription factor CCAAT/enhancer binding protein β (C/EBPβ) [105]</td>
</tr>
<tr>
<td>Noni fruit juice</td>
<td>Human lung carcinoma A549 cells</td>
<td>Induced phosphorylation of protein kinase B (PKB), extracellular regulated protein kinase 1/2 (ERK-1/2), c-Jun N-terminal kinase-1 (JNK-1), and eukaryotic translation initiation factor-2α (eIF-2α)[110]</td>
</tr>
</tbody>
</table>

Table 4. Summary of molecular mechanisms involved in the effects of noni against cancer
Chapter 2: Hypothesis and Aims

2.1 Hypothesis

Based on our preliminary data and literature review, we hypothesize that fermented noni fruit juice (fNJ) will prevent proliferation and induce programmed cell death (apoptosis) in MCF-7, and ER–veMDAMB-231 cancer cells by modulating cell cycle arrest proteins as well as inhibiting cell death proteins called “inhibitors of apoptosis” (IAP).

Figure 7. fNJ induces apoptosis through hypothesized pathways
2.2  Specific Aims

2.2.1  Specific Aim 1

To investigate the effects of fNJ in normal human breast, MCF10A and cancerous human breast cells, MCF-7 and MDAMB-231.

**Objective 1:** Identify dosage of fNJ that are non-toxic in normal cells, but will induce cell death in MCF-7 and MDAMB-231 cells.

**Approach:** Toxicity of varying doses of fNJ will be measured in cells after 24, 48, 72 and 96 h by using commercial 96 well non-radioactive cell proliferation (MTT) assay kit.

**Objective 2:** Identify the apoptotic proteins present in fNJ-treated human breast cancer cells that affects proliferation or causes cell death in MCF-7 and MDAMB-231.

**Approach:** Apoptosis will be measured using an apoptosis array in cells treated with two doses of fNJ that will achieve about 60-80% cell death.

2.2.2  Specific Aim 2

To identify the anti-proliferative and apoptotic mechanisms induced by fNJ in MCF-7 and MDAMB-231 cells.

**Objective 1:** Identify fNJ-induced apoptotic proteins in MCF-7 and the apoptotic signaling mechanisms.
**Approach:** Proteins will be identified from apoptosis array and confirmed by western blotting.

**Objective 2:** Identify fNJ-induced apoptotic proteins in MDAMB-231 and the apoptotic signaling mechanisms.

**Approach 1:** Proteins will be identified by apoptosis array will be confirmed by western blotting.

**Approach 2:** Nuclear and cytosolic proteins of fNJ treated MCF-7 and MDAMB-231 cells will be extracted and be confirmed by western blotting.
Chapter 3: Materials and Methods

3.1 Preparation of fNJ with endotoxins removed

*Morindacitrofolia*(noni) fruits were obtained from noni trees on the University of Hawaii ManoaMagoon facility, Honolulu, Hawaii. The fruits were rinsed, dried, and left to ferment in airtight jars placed in indirect sunlight for 14 days. The fermented juice was extracted and centrifuged at 3000 rpm at room temperature for 15 minutes. The supernatant was filtered with a 0.45μm filter then a 0.20μm filter. The pH of the fermented noni juice (fNJ) was adjusted to pH 7 using 10mM NaOH.

Endotoxins were removed from the fNJ using Detoxi-Gel Endotoxin Removing Columns (Pierce Biotechnology, Rockford, IL, U.S.A) as per manufacturer’s instructions. The fNJ with removed endotoxins were then aliquoted into 1.5 mL tubes and store in -80°C, then thawed once for cell treatment.

3.2 Cell Culture

Estrogen receptor positive (ER +ve) human breast cancer cells, MCF-7 were generously donated by Dr. June Panee. MCF-7 cells were maintained in Dulbecco’s modified eagle medium (DMEM) (ATCC, Manassas, VA, U.S.A.) with 10% heat inactivate fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10mg/ml insulin.

Estrogen receptor negative (ER -ve) MDA-MB-231 cells were generously donated by Dr. June Panee. MDA-MB-231 cells were maintain in Minimal essential medium eagle (MEME) (ATCC, Manassas, VA, U.S.A)) with 10% heat inactivate fetal bovine serum (FBS) and 1% penicillin/streptomycin.
3.3 MTT assay
Cell viability was determined and analyzed by the CellTiter96 Non-Radioactive cell proliferation assay (MTT) (Promega, Madison, WI, U.S.A.). MCF-7 and MDA-MB-231 cells were cultured and plated on to 96-well plates at a density 100,000 cells/ml for 48 h prior to the first treatment. The cells were treated with 2.5%, 5%, 10%, 15%, and 20% fNJ (v/v) for a total of 96 h. Dosages were calculated based on the recommended dose made by commercial noni juice companies. An average adult is recommended to consume two ounces twice a day, which equates to 59.14 milliliters twice a day. Since an average adult has a blood volume of 5 liters, the average adult would be consuming 1.18% of its blood volume per serving and 4.72% over the course of two days.

3.4 Human apoptosis antibody array
The expression profile of apoptosis-related proteins was detected and analyzed using a human apoptosis array kit (R&D Systems, Minneapolis, MN, U.S.A.) as per manufacturer’s instructions. This array contains duplicate spots of 35 apoptosis-related proteins. Briefly, the membrane containing immobilized apoptosis-related antibodies was blocked with bovine serum albumin for 1 h on a rocking platform at room temperature. The membrane was then incubated with MCF-7 or MDA-MB-231 cell lysate treated with or without fNJ along with detection antibody cocktail overnight at 2°C to 8°C on a rocking platform. The membranes were incubated with streptavidin-horseradish peroxidase conjugate followed by chemiluminescent detection reagent. The membranes
were scanned and pixel density was presented by quantifying the mean spot densities from two experiments.

### 3.5 Western Blot

After the in vitro experiments, protein extracts were prepared from the cells using special solubilizing buffer (SSB) containing 25 mM Tris-HCl (pH 7.4), 2 mM Na$_3$VO$_4$, 10 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 1 mM EGTA, 1 mM EDTA, and 1 mM PMSF. After protein was extracted, its concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, U.S.A). Equal amounts of protein extracts were separated on a 4–10% gradient polyacrylamide gel and then electrotransferred to polyvinyl diflouride (PVDF) membrane using a Bio-Rad mini-transfer tank. PVDF membranes were activated in 100% methanol for 1 minute. Membranes were incubated with primary antibodies overnight. The protein bands were detected with HRP-conjugated secondary antibodies and then imaged developed on a film using Pierce enhanced chemiluminescence (ECL) western blotting substrate (Thermo Scientific, Rockford, IL, CA, USA). Membranes were stripped with Re-blot (Millipore, Temecula, CA, USA) for 20 min minutes at room temperature and reprobed with antibodies to β-tubulin to control for differences in loading.

The primary antibodies used were survivin, claspin, cytochrome-c, and NFkB (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The secondary antibodies used was goat anti-mouse from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA. U.S.A.).

### 3.6 Statistical Methods
All tabulated data is presented as mean ± SEM. Analysis of statistical significance and graphing was done using GraphPad Prism 5. P-values of P< 0.05 were considered to be statistically significant. Means were tested for statistical significance difference using one-way ANOVA and Tukey’s post-test analysis.
Chapter 4: Results

4.1 fNJ decreases cell viability in breast carcinoma cells

MTT assays were performed on MCF-7 and MDA-MB-231 cells after treated once with 2.5%, 5%, 10%, 15%, and 20% fNJ (v/v) over the course of 48 hours. No cell death was observed in cells that received only one treatment. Therefore two treatments were used over the course of 96 hours (figure 10 and 11). In both cell lines, cell viability has been observed to be significantly lower in all the treatments except for 2.5% fNJ (v/v) as compared to control. For both breast carcinoma cell lines, 40% - 60% cell death was observed in the 15% fNJ treated as compared to control. 20% fNJ treated cells have been observed to have 70% - 85% cell death as compared to control. Based on these results, 15% fNJ was chosen for further experiments since higher doses induced cell death by 80%, while lower doses were non-toxic. Although MTT assay results show significant change in cell viability in treated as compared to control in both cell lines, visual observation shows that the cell death in treated MDAMB-231 cells are more apparent than treated MCF-7 cells. In order to determine the toxicity of fNJ against non-carcinoma cells, MCF10A, a normal breast epithelial cell line was treated under the same conditions as the breast carcinoma cell lines (figure 12). MTT assay results showed that there is no significant decrease of cell viability in any of the treatments compared to control in MCF-10A normal human breast epithelial cells.
4.2 Apoptotic proteins identified in fNJ treated breast carcinoma cells

An apoptosis array was used to determine the changes in apoptotic protein levels in 15% fNJ (v/v) treated breast cancer cells compared to control. The apoptosis array detects 48 different proteins that are related to the downstream or upstream down-regulation or up-regulation of apoptosis (figure 13). In 15% fNJ treated MCF-7 cells, a significant decrease of B-cell lymphoma 2 (Bcl-2), cellular inhibitor of apoptosis protein 1(cIAP-1), survivin, claspin, and phosphorylated Rad 17 (phospho-Rad17) has been observed compared to control (figure 14). Survivin and claspin resulted in a 60% decrease, which were the highest amount of decrease compared to other proteins observed. In addition, a significant 70% increase of heat shock protein 60 (HSP60) has been observed in treated compared to control.

In 15% fNJ treated MDA-MB231 cells, a significant decrease was observed in cIAP-1, cellular inhibitor of apoptosis protein 2 (cIAP-2), survivin, claspin, and fibroblast associated tumor necrosis factor receptor super family member 6 (Fas/TNFRSF6) as compared to control (figure 15). Fas/TNFRSF6 and cIAP-2 resulted in an 85% and 70% decrease, respectively, which were the highest amount of decrease compared to the other proteins. Subsequently, a 20% increase of clusterin has been observed in the treated samples compared to control.

4.3 fNJ inhibits survivin in MCF-7 (ER+ve) cells

Western blots were used to further verify the change in apoptotic proteins identified from the Apoptosis Array. In the apoptosis array, 15% fNJ treated breast cells, MCF-7 displayed significantly lower levels of survivin and cIAP-1 compared to control. Both survivin and cIAP-1 belong to a class of proteins called the inhibitors of apoptosis
(IAP) proteins. Western blot analysis demonstrated that there was a 40 – 60% decrease in survivin protein levels in 15% fNJ treated breast carcinoma cells as compared to control (figure 16).

4.4 fNJ up-regulates cytosolic levels of cytochrome c in MCF-7 (ER+ve) cells

In the apoptosis array, Bcl-2 levels were observed to be significantly lowered in the 15% treated MCF-7 cells compared to control. Since Bcl-2 is known to inhibit the release of cytochrome c from the mitochondria to the cytosol, western blots were used to detect the levels of cytochrome c in cytosolic protein extractions of MCF-7 cells in 15% fNJ treated and control. Western blot analysis demonstrated a significant 200% increase in the treated compared to control (figure 17).

4.5 fNJ does not affect cytosolic and nuclear levels of nFκB in MCF-7 (ER+ve) cells

Western blot analysis was used to determine cytosolic and nuclear protein levels of the pro-inflammatory and pro-survival transcription factor, nFκB (figures 18 and 19). No significant changes were observed in the 15% fNJ treated MCF-7 cells compared to control.

4.6 fNJ inhibits survivin in MDAMB-231 (ER-ve) cells

Western blots were used to further verify the change in apoptotic proteins identified from the Apoptosis Array. In the apoptosis array, 15% fNJ treated breast cells, MDAMB-231 displayed significantly lower levels of surviving, cIAP-1 and cIAP-2 compared to control. Survivin, cIAP-1 and cIAP-2, all belong to a class of proteins called the inhibitors of apoptosis (IAP) proteins. Western blot analysis demonstrated that
there was a 40 – 50% decrease in survivin protein levels in 15% fNJ treated breast carcinoma cells as compared to control (figure 20).

4.7 fNJ does not affect cytosolic levels of cytochrome c in MDAMB-231 (ER-ve) cells
In the apoptosis array, Bcl-2 levels were observed to have no significant difference in the 15% treated MDAMB-231 cells compared to control. Since Bcl-2 is known to inhibit the release of cytochrome c from the mitochondria to the cytosol, western blots were used to detect the levels of cytochrome c in cytosolic protein extractions of MDAMB-231 cells in 15% fNJ treated and control. Western blot analysis confirm the apoptosis array by showing no significant changes in the treated MDAMB-231 cells compared to control (figure 21).

4.8 fNJ does not affect cytosolic and nuclear levels of nFκB in MDAM-231 (ER-ve) cells
Western blot analysis was used to determine cytosolic and nuclear protein levels of the pro-inflammatory and pro-survival transcription factor, nFκB (figures 22 and 23). No significant changes were observed in the 15% fNJ treated MDAMB-231 cells compared to control.
4.9 Figures of results

**Figure 8.** Phase contrast photomicrographs of MCF-7 cells treated with fNJ for 96 hours (magnification x 200)

(A) Untreated control  (B) 15% fNJ (v/v)  (C) 20% fNJ (v/v)

**Figure 9.** Phase contrast photomicrographs of MDAMB-231 cells treated with fNJ for 96 hours (magnification x 200)

(A) Untreated control  (B) 15% fNJ (v/v)  (C) 20% fNJ (v/v)
Figure 10. Effects of fNJ on MCF7 cell viability

Two independent experiments were performed in replicates of seven. Data is represented as percentages of control and as mean ± SE (n=14). Mean values with common letters to control do not differ (p<0.05).
**Figure 11.** Effects of fNJ on MDAMB-231 cell viability

Two independent experiments were performed in replicates of seven. Data is represented as percentages of control and as mean ± SE (n=14). \(^{a, b}\) Mean values with common letters to control do not differ (p<0.05).
**Figure 12.** Effects of fNJ on MCF-10A cell viability

Two independent experiments were performed in replicates of seven. Data is represented as percentages of control and as mean ± SE (n=14).\(^a,b\) Mean values with common letters to control do not differ (p<0.05).
Figure 13. Human apoptosis array overlay
**Figure 14.** Effects of 15% fNJ (v/v) on apoptotic proteins in MCF-7 cells as compared to control.

Two independent experiments were performed in triplicate. Values are mean ± SE (n=6).

a, b Mean values with common letters do not differ (p<0.05).

**Figure 15.** Effects of 15% fNJ (v/v) on apoptotic proteins in MDA MB-231 cells as compared to control.

Two independent experiments were performed in triplicate. Values are mean ± SE (n=6).

a, b Mean values with common letters do not differ (p<0.05).
Figure 16. fNJ reduced survivin levels in MCF-7 cells

Figure 16A shows images of the blots. Figure 16B represents the relative intensity of the bands measured by densitometry units. Two independent experiments were performed in triplicate. Values are mean ± SE (n=6). a,b Mean values with common letters do not differ (p<0.05).
Figure 17. fNJ increases cytosolic cytochrome c in MCF-7 cells

Figure 17A shows images of the blots. Figure 17B represents the relative intensity of the bands measured by densitometry units. Two independent experiments were performed in triplicate. Values are mean ± SE (n=6). a,b Mean values with common letters do not differ (p<0.05).
Figure 18. fNJ did not affect changes in cytosolic nFkB levels in MCF-7 cells

Figure 18A shows images of the blots. Figure 18B represents the relative intensity of the bands measured by densitometry units. Two independent experiments were performed in triplicate. Values are mean ± SE (n=6). a,b Mean values with common letters do not differ (p<0.05).
Figure 19. fNJ did not affect changes in nuclear nFkB in MCF-7 cells

Figure 19A shows images of the blots. Figure 19B represents the relative intensity of the bands measured by densitometry units. Two independent experiments were performed in triplicate. Values are mean ± SE (n=6). a,b Mean values with common letters do not differ (p<0.05).
Figure 20. fNJ reduced survivin levels MDAMB231

Figure 20A shows images of the blots. Figure 20B represents the relative intensity of the bands measured by densitometry units. Two independent experiments were performed in triplicate. Values are mean ± SE (n=6). a,bMean values with common letters do not differ (p<0.05).
Figure 21. fNJ did not affect cytosolic levels of cytochrome c in MDAMB-231 cells

Figure 21A shows images of the blots. Figure 21B represents the relative intensity of the bands measured by densitometry units. Two independent experiments were performed in triplicate. Values are mean ± SE (n=6). a,b Mean values with common letters do not differ (p<0.05).
Figure 22. fNJ does not affect cytosolic nFkB in MDAMB-231 cells

Figure 22A shows images of the blots. Figure 22B represents the relative intensity of the bands measured by densitometry units. Two independent experiments were performed in triplicate. Values are mean ± SE (n=6). a,b Mean values with common letters do not differ (p<0.05).
Figure 23. fNJ did not affect nuclear nFkB levels in MDAMB-231 cells

Figure 23A shows images of the blots. Figure 23B represents the relative intensity of the bands measured by densitometry units. Two independent experiments were performed in triplicate. Values are mean ± SE (n=6). \(^a\) Mean values with common letters do not differ (p<0.05).
Chapter 5: Discussion

In both MCF-7 and MDAMB-231 breast carcinoma cell lines, cell death was observed in fNJ treated cells. Normal cells grown on a culture exhibit a behavior called contact inhibition where they limit themselves to grow in a single layer[1]. Cancer cells do not exhibit this behavior. Instead, cancerous cells tend to grow uncontrollably and overlap each other[1]. Visual observation of the control breast carcinoma cells confirms that behavior. MCF-7 and MDAMB-231 cells that were treated with fNJ cease to grow uncontrollably. Rounding of cells was observed in the fNJ treated group, indicating cell death possibly via apoptosis. 15% fNJ dosage was chosen for further experimentation because if caused a significant amount of cell death yet it was not severely toxic to the cells. Since an average adult has about 5 liters of blood, a 15% fNJ treatment would mean that an average adult would have to consume 375 milliliters of fNJ per day.

Previous studies conducted by West et al. demonstrated that noni juice is clinically safe even when consumed in amounts as high as 750 milliliters per day[111]. Resulting data from MTT assay demonstrates that cell viability decreases as fNJ treatment increases, which agrees with visual observations. This suggests that fNJ somehow causes cell death in MCF-7 and MDAMB-231.

Apoptosis is a form of intrinsic programmed cell death that can be characterized by several morphological features such as cell shrinkage, membrane blebbing, and nuclear DNA fragmentation [112]. Apoptosis plays a crucial role in regulating cell growth and tissue homeostasis. Therefore, an important factor to consider in anticancer
research, are the extrinsic and intrinsic signal transduction pathways of apoptosis. The intrinsic pathway of apoptosis can be activated via the mitochondria or extrinsic receptors such as TRAIL or FADD (figure 24)[113]. The mitochondrial apoptosis pathway initiates apoptosis through the release of cytochrome c, apoptosis inducing factor (AIF), or Smac/DIABLO. The release of these proapoptotic proteins into the cytosol activates cell death via the caspase cascade [113-115]. In cancer, the uncontrolled growth of abnormal cells may be induced by anti apoptotic proteins such as survivin, Bcl-2, XIAP, cIAP-1 and cIAP-2. By examining the effects of fNJ on a molecular level, a specific molecular approach to developing anticancer therapies can be developed.

In order to investigate the apoptotic mechanisms involved in cell death, an apoptosis array was used to view the changes of apoptotic protein levels between control and 15% fNJ treated MCF-7 and MDAMB-231 cells. In breast cancer cells, significant reductions in cIAP-1, cIAP-2, Bcl-2, survivin, and claspin were observed in the 15% fNJ group. A reduction of claspin has been observed in treated cells compared to control. Claspin is a protein involved cell cycle arrest [116]. When DNA damage occurs, the cells will either repair the damage or induce cell cycle arrest. ATR phosphorylates claspin then recruits Plx1 to claspin causing claspin to dissociate from the chromatin. The dissociation of claspin downregulates Chk1 which inhibits mitosis [116]. Thus, the reduction of claspin indicates the anti-proliferative effect of fNJ.

A common characteristic seen in cancer is the dysregulation of apoptosis[117]. Therefore proteins involved in pro or anti-apoptotic signals are of interest when it comes to cancer research. Survivin and cIAP-1 belong to a class of proteins call the Inhibitor of Apoptosis (IAP) proteins, which are known to suppress apoptotic cell death[118]. An overexpression of IAP proteins is commonly observed in human cancers and has been associated with tumor progression and treatment failure [119]. Thus, IAP proteins are potential targets for cancer therapy and drug development. Survivin is an IAP that is commonly expressed in breast cancer tissues and plays a big role in cell survival and chemoresistance by inhibiting caspases 3 and 9[120, 121]. Results show that MCF-7 and MDAMB-231 cells treated with 15% fNJ have approximately 50% less survivin than the non-treated control. This suggests that fNJ inhibits the expression of survivin in breast cancer cells. Previous studies have shown that IAPs such as, cIAP-1, cIAP-2, survivin, and XIAP, block apoptosis by inhibiting caspases 3 and 9 downstream of Bax, Bik, Bak,
and cytochrome c[115, 118, 122]. Thus suggesting that fNJ’s pro-apoptotic effects contribute to the inhibition of IAPs, which in return activates caspases 3 and 9.

Bcl-2 is a protein that is located mainly in the nuclear membrane, endoplasmic reticulum and mitochondrial membrane [123]. It is known to inhibit the release of cytochrome c from the mitochondria to the cytosol and therefore inhibiting caspases 3 and 9 induced apoptosis[114, 122, 123]. Results show that MCF-7 cells treated with 15% fNJ significantly reduces Bcl-2 levels by 40% compared to non-treated control. In addition, western blot results show an increase in cytosolic cytochrome c levels by 300% in treated compared to control. These results demonstrate the indirect correlation of Bcl-2 and cytosolic cytochrome c published by Kluck et al., indicating that fNJ inhibits Bcl-2 therefore allowing the release of cytosolic cytochrome c and activating apoptosis through caspases 3 and 9. These findings suggest the fNJ induces apoptosis in MCF-7 ER+ve cells through the mitochondrial apoptosis pathway. The same results were not found in MDAMB231 ER-ve cells. There were no significant changes in cytosolic cytochrome c levels in 15% fNJ treated MDAMB-231 cells compared to control, suggesting that fNJ does not induce apoptosis in MDAMB-231 cells through the mitochondrial apoptosis pathway.
The anti apoptotic proteins discusses earlier (Bcl-2, cIAP-1, cIAP-2, and survivin) are regulated by a pro-inflammatory transcription factor, NfκB[124]. NfκB works upstream and controls the gene expression of certain IAP proteins [118, 125]. Evidence suggests that NfκBmediate desensitization, chemoresistance, and radio resistance [126]. Consequently, Hirazumiet al. demonstrated that noni-ppt increased levels of TNF-α, which functionsextrinsically upstream of NfκB[61]. To further verify the role of NfκB, our results have demonstrated a significant decrease in tumor necrosis factor receptors (TNFR) in 15% fNJ treated MCF-7 and MDAMB-231 cells compared to control. Since inactive NfκB remain in the cytosol of cells [124], changes in NfκB levels wereexpected to be seen in nuclear and cytosolic protein extracts of fNJ treated MCF-7 and MDAMB-231. However, no changes in cytosolic and nuclear NfκB levels were observed in both breast carcinoma cells lines. Although previous studies have indicated the early
activating properties of NfκB[127, 128]. Since the proteins were extracted 96 hours after treatment, perhaps NfκB has already activated transcription and left the cell’s nucleus.

Many active chemical constituents in noni have been isolated and identified. Studies have been conducted to identify the anticancer signal transduction pathways induced by chemicals found in noni such as anthraquinone, scopoletin and damnacanthal. For example, damnacanthal isolated from noni have been demonstrated to induced apoptosis by activating caspase [105]. Our data has indicated that naturally fermented noni fruit juice as a whole is also as effective as the isolated components.
Figure 26. The role of NF-kB in apoptosis. [Source: Aggarwal, Bharat B., *Nuclear factor-kB: the enemy within.* Cancer Cell, 2006.6: p. 203-208.]

(A) NfxB is activated by inflammatory agents, carcinogens, infection, cytokines, and tumor promoters. (B) NfxB activates the expression of genes related to cell survival, proliferation, angiogenesis, and inflammation.
Chapter 6: Conclusion

Although several studies have shown the apoptotic effects of fNJ in in vivo models, to our knowledge, no studies have been conducted to identify the molecular mechanisms association with the anti-cancer effects of fNJ. Our studies have shown that fNJ induces apoptosis in human breast carcinoma, MCF-7 and MDAMB-231 cells through various intrinsic pathways. In both cell lines, fNJ is able to inhibit anti-apoptotic proteins thus allowing the breast cancer cells to undergo apoptosis via caspases 3 and 9. In addition, fNJ inhibited anti-apoptotic protein, Bcl-2 in treated MCF-7 cells. The inhibition of Bcl-2 allows the release of cytochrome c into the cytosol. High cytosolic levels of cytochrome c induced apoptosis by activating the caspase cascade.

Studies on the molecular mechanisms of breast cancer offer different and possibly more selective treatments. Although hormone treatments are available for women with ER+ve breast carcinoma, women suffering from ER-ve breast carcinoma will have to turn to non-selective treatments such as chemical or radiation therapy. fNJ delivers promising results as a selective treatment for ER+ve and ER-ve breast cancer.

6.1 Significance

Currently, the popular methods of cancer treatment are surgery, chemotherapy, radiotherapy and hormone therapy (www.breastcancer.org). Unfortunately each treatment has a side effect. More importantly, these treatments, besides hormone therapy, are systematic and will kill normal healthy cells therefore leaving the patients with a number of side effects, such as hair loss, lowered immune system, nausea, headache, and
possibly chemoresistance and radioresistance. fNJ can potentially offer a cost friendly and selective way of battling breast cancer.

6.2 Future studies

Since apoptosis is facilitated through extrinsic and intrinsic pathways [112], it is important to examine all aspects of it. Therefore, our lab will be not only further analyzing the intrinsic pathways by looking at the presence of caspase, but also the extrinsic pathway by looking at the presence of TNF-α and TNFR. In addition, our lab will be reexamining the pro-survival activites of early activating transcription factor, nFκB by extracting proteins 1-4 hours post treatment.

Anti-proliferation is an important factor to consider when looking at cancer therapies. Our studies contain preliminary data demonstrating the anti-proliferative effects of fNJ. Further studies will be conducted to verify it by looking in other proteins such as claspin, phosphorylated claspin, and chk1 in treated compared to control.

Many commercial noni fruit juice products are not fermented. Therefore a study on the comparison of the fermented and non-fermented fruit juices will be beneficial to their consumers. In addition, chemical fractionation and analysis is warranted to identify the constituents responsible for the anti-cancerous effects of noni fruit juice.
Reference:


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