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The Effects of Genistein, Thymoquinone, Epigallocatechin-3-gallate, S-Fluorouracil, and Low Level Laser Therapy on FaDu Hypopharyngeal and Laryngeal Carcinoma Cells

Osasu Adah

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The Effects of Genistein, Thymoquinone, Epigallocatechin-3-gallate, 5-Fluorouracil, and Low Level Laser Therapy on FaDu Hypopharyngeal and Laryngeal Carcinoma Cells

By:

Osasu Noel Adah

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

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Approved by:

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Abstract

The Effects of Genistein, Thymoquinone, Epigallocatechin-3-gallate, 5-Fluorouracil, and Low Level Laser Therapy on FaDu Hypopharyngeal and Laryngeal Carcinoma Cells

Osasu Noel Adah

Laryngeal and hypopharyngeal carcinoma are two types of head and neck squamous cell carcinomas. The location of the tumor makes it hard to detect without complex tests. Overall, these types of cancer are not common, and there are no simple screening tests for such cancers. Due to this issue, laryngeal and hypopharyngeal carcinoma are usually diagnosed when signs and symptoms occur, such as sore throat, trouble swallowing, and constant coughing. With the use of FaDu, a head and neck cancer cell line of the hypopharyngeal origin, experimentation can be performed in order to initiate possible treatments. The standard treatments for laryngeal and hypopharyngeal carcinoma include conventional chemotherapy, such as 5-fluorouracil and cisplatin, chemo-radiation, and surgery. The treatments used in this experiment include 5-Fluorouracil (5-FU), Genistein (G), Thymoquinone (TQ), Epigallocatechin-3-gallate (EGCG), and low level laser therapy (LLLT). 5-FU is one of the most commonly used drugs to treat several cancers, including head and neck cancers. G is an isoflavone metabolite of soy generated by gut floral bacteria and has been characterized as a possible prevention for cancer. TQ is an ingredient isolated from Nigella sativa and has been investigated for its anti-oxidant, anti-inflammatory, and anticancer activities in both in vitro and in vivo models. EGCG, one of the main flavonoids found in green tea, is considered as one of the most active molecules known for its anti-oxidant properties.
LLLT may enhance or decrease cell proliferation. LLLT has shown to induce apoptosis in cancer such as a human colon carcinoma cell line by increasing reactive oxygen species (Saenko et al 2016).

The goals of this experiment were to investigate the effects of LLLT on FaDu cells and to determine the effects of natural products such as G, TQ, and EGCG, as chemotherapeutic agents, and 5-FU (a conventional chemotherapeutic agent), combined with LLLT, on FaDu cells. Specifically, cells were treated with LLLT, TQ, G, EGCG, or 5-FU; or LLLT plus TQ, G, EGCG, or 5-FU. The cells were then harvested and cellular protein, intracellular glutathione levels, nitric oxide levels, and lactate dehydrogenase levels were evaluated. Due to the low level of anti-cancer efficacy, G was discontinued. The results show a significant decrease in protein levels and cell numbers following treatment with TQ, however, this trend was inconsistent throughout the study. There was a significant increase in nitric oxide and LDH levels following treatment with EGCG and EGCG + laser, and this trend remained consistent throughout the study. However, since EGCG displayed similar results as EGCG + laser, LLLT possibly does not have any additional effect when combined to EGCG. Overall, LLLT did not show significant effect on FaDu cells. EGCG alone was shown to be the most effective treatment, in terms of inducing the most cellular damage and toxicity in the cancer cells.
Acknowledgements

First, I am so grateful to God for His provisions for my life and for giving me the guidance and strength to accomplish the works that He has planned for me to do.

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<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
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<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>TQ</td>
<td>Thymoquinone</td>
</tr>
<tr>
<td>G</td>
<td>Genistein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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Introduction
Laryngeal carcinoma (cancer of the larynx) is the 2\textsuperscript{nd} most common cancer in the region involving the head and neck and is actually one of the more usual malignancies worldwide in men (Binshen et al 2011). Hypopharyngeal carcinoma is also a head and neck carcinoma that represents about 7\% of all cancers of the upper aerodigestive tract. The prevalence of laryngeal carcinoma is 4-5 times that of hypopharyngeal carcinoma (Quon, 2015). According to the National Institute of Cancer in 2016, there were an estimated 13,430 new cases and 3,620 deaths for laryngeal carcinoma. In contrast, an estimated 3,400 adults in the United States will be diagnosed with hypopharyngeal cancer according to the American Cancer Society (2016). The non-specific nature of nasal and aural symptoms increases the likelihood to misdiagnose and leads to laryngeal and hypopharyngeal carcinoma diagnoses at advanced stages (stages III-IV). Some of the symptoms of both of these cancers include hoarseness in voice, persistent coughing, difficulty swallowing, and ear pain. Treatment modalities often fail and are accompanied by short and long term side effects of the chemotherapeutic drug, 5-Fluorouracil (5-FU). Some of the side effects include neutropenia, anemia, tumor lysis syndrome, infertility, and less commonly, angina and heart failure (Cancer Research UK, 2016). The use of natural compounds, such as Genistein (G), Thymoquinone (TQ), and Epigallocatechin-3-gallate (EGCG), which stop cancer in different phases of the cell cycle, may provide an alternative to 5-FU drug regime due to possible less hazardous side effects and greater efficacy.

In order to study laryngeal and hypopharyngeal carcinoma, we used a head and neck cancer cell line known as FaDu. This cell line is specifically a hypopharyngeal carcinoma, however, it is sufficient to use in the investigation of laryngeal carcinoma due
to the closeness in structures, similar possible causes, signs, symptoms, and treatments. According to the ATCC, the FaDu line was established from a biopsy of a hypopharyngeal tumor removed from a patient in 1968. With the use of FaDu, experimentation can be performed in order to initiate possible treatments.

Anti-metabolites have been used to treat different types of cancers. Fluorouracil (5-FU) belongs to this class of chemotherapy drugs that is specifically a pyrimidine antimetabolite that usually acts on cells in the G1 to S phases of the cell cycle (Tohru et al 2013). 5-FU works by being incorporated into DNA and RNA replicating cycles and preventing further cell growth. The G1 (Gap 1) phase of the cell cycle is where the size of the cells increase and are being prepared for DNA replication. The S phase of the cell cycle is where DNA replication/synthesis occurs. According to results associated with combination chemotherapy with the DNA damaging cisplatin (CDDP) and 5-FU for laryngeal carcinoma, it is verified that CDDP and 5-Fu, along with radiotherapy, contributed to laryngeal preservation. The irreversible side effects of using CDDP and 5-FU include peripheral neuropathy and nephrotoxicity. The reversible side effects of using CDDP and 5-FU with chemotherapy include leukopenia, neutropenia, mucositis, and dermatitis (Furusaka et al 2013).

The use of isoflavones as a plausible treatment for laryngeal carcinoma has been investigated due to their cytostatic and cytotoxic effects on the body. Genistein (G) is one of the main soy isoflavones. G has been described to be a protein kinase inhibitor, which can block proliferation of squamous cell carcinoma and also arrest cell growth, especially at the S/G2-M phase of the cell cycle (Alhasan et al 1999). The G2 (Gap 2) phase of the cell cycle is where the cell is still growing and is getting prepared to divide.
Cell cycle arrest at the G2/M phase of the cell cycle was also shown when human intestinal colon cancer cells (Han et al, 2013) and human oral squamous carcinoma cells (Ye et al 2004) were treated with G. This isoflavone initiates programmed cell death (apoptosis) in the malignant cells (Alhasan et al 1999). Because of the absence of toxic effects and the natural biological influences of flavonoids in general, a diet rich in isoflavonoids, including genistein, can play a major part in the prevention of cancer (Alhasan et al, 1999).

TQ is a component isolated from *Nigella sativa* and has been considered for its anti-cancer activities in both in vitro and in vivo models since its first extraction in 1960s. Its anti-oxidant and anti-inflammatory effects have been reported in various disease models, including encephalomyelitis, diabetes, asthma, and carcinogenesis (Woo et al, 2011). TQ has also been found to induce cell death in human oral squamous carcinoma cells by caspase-activation dependent apoptosis (Chu et al, 2014). Caspase is a type of protease. The activation of this enzyme ensures that the cellular components are degraded in a controlled manner, which allows for apoptosis to take place with minimal effect to surrounding tissues (Rathore et al, 2015). TQ induces apoptosis in p53-dependent or p53-independent pathways in 6 human colorectal carcinoma cells. TQ arrests cell cycle progression in different types of tumor cells, specifically inducing G0/G1 arrest possibly through the increase in the expression of p16 and decrease in cyclin D1 in mouse papilloma carcinoma cells and other kinds of cancer, including acute lymphoblastic leukemia and prostate cancer cells (Hala et al 2004). Studies have also shown that TQ induces G2/M arrest in other cancer cells, including MNNG/HOS human osteosarcoma cells and MCF-7/DOX doxorubicin-resistant breast cancer cells (Woo et al 2011).
The anti-cancer effects of TQ are mediated through different modes of action: anti-proliferation, apoptosis induction, cell cycle arrest, ROS (Reactive Oxygen Species) generation, and anti-metastasis/anti-angiogenesis. TQ modulates multiple molecular targets, including p53, p73, PTEN, and STAT3. There are two mechanisms that might contribute to growth inhibitory activities of TQ in vitro: apoptotic cell death and interference with the cell cycle due to increased cyclin-dependent kinases and negative regulator involved in G1 cell cycle control (Shoeib et al 2003). In vivo studies have demonstrated that TQ decreases CDDP-induced side effects, including nephrotoxicity, without compromising its antitumor activity (Shoeib et al 2003).

EGCG is a flavonoid found in green tea and is known for its antioxidant properties (Legeay et al 2015). EGCG causes cell cycle arrest in the G1 phase in oral squamous cell carcinoma cells by upregulating B-cell translocation gene 2, which is a regulatory protein of the cell cycle (Lee et al 2015). Studies have also shown that EGCG inhibits the self-renewal capacity of head and neck squamous carcinoma cancer stem cells by suppressing their sphere-forming capacity, which is a measure of tumorigenicity (Lee et al 2013). EGCG inhibits enzyme activities, such as NFκB, in head and neck carcinoma cells, which result in suppression of cell proliferation, enhancement of apoptosis, and inhibition of cell invasion, angiogenesis, and metastasis (Chung et al 2012). NFκB is known for its role in inflammation, and EGCG blocks this enzyme from being activated (Zhe et al, 2004). Overall, EGCG seems to be one of the most promising naturally-derived compounds that demonstrate tumor suppressive effects on animal carcinogenesis models, mouse xenograft models, and several cancer cell lines (Muneyuki et al 2011).
Low level laser therapy (LLLT) involves exposing cells or tissue to low levels of red and near infrared (NIR) light, and is referred to as “low level” because of its use of light at energy densities that are low as compared to other forms of laser therapy that are used for ablation, cutting, and thermally coagulating tissue (Hamblin, 2008). From observation, it appears that LLLT has a wide range of effects at the molecular, cellular, and tissue levels. There are three major roles that LLLT is typically used for: wound healing, tissue repair, and inflammation relief (Hamblin, 2008). LLLT has been used clinically by physical therapists to treat a variety of musculoskeletal aches and pains, by dentists to treat inflamed oral tissues and to heal ulcerations, by dermatologists to treat edema, ulcers, burns, and dermatitis, by orthopedists to relieve pain and treat chronic inflammation and autoimmune diseases, and more (Hamblin, 2008). Within the cell, there is strong evidence to suggest that LLLT acts on the mitochondria to increase adenosine triphosphate (ATP) production, modulating reactive oxygen species (ROS). LLLT has shown promise for down regulating inflammation by reducing the presence of reactive oxygen species (ROS). However, there are limited studies on how LLLT affects cancer cells. Studies involving laser treatment on bone tumors have shown that a wavelength of 830nm increased osteoblast proliferation significantly and reduced osteosarcoma cells at an energy level of 10 J/cm$^2$ (Renno et al 2007). LLLT has limited systemic effects and may be implicated in toxic reduction in the body (Rhee et al 2012).

**Goals, Hypotheses, and Specific Aims**

In the literature, studies that tested the dual effects of LLLT and anti-cancer agents such as 5-FU, G, TQ, and EGCG are limited. Therefore, the goals of this study
were to 1) determine the effects of LLLT on FaDu cancer cells and to 2) determine the effects of natural products or a conventional chemotherapeutic agent, 5-FU, in combination with LLLT on cancer cell growth.

**Hypothesis I:** FaDu cancer cells exposed to laser treatments will show a reduction in cell number and viability.

**Specific Aim:** To determine the effect of 830 nm of laser light on FaDu cells, protein levels were measured as a correlate to cell number, and cell function was monitored via glutathione levels at 24, 48, and 72 hours post LLLT. LLLT effects were compared to 5-FU, G, EGCG, and TQ at their pre-determined, respective time-dependent IC$_{50}$’s.

**Hypothesis II:** FaDu cancer cells exposed to laser treatment in combination with single-dose cell cycle specific drugs/compounds will show a greater reduction in cell numbers than compared to the administration of separate treatments.

**Specific Aim:** To determine the effect of 830 nm of laser light on FaDu cells, cell function, and after 24, 48, and 72 hours in combination with chemotherapeutic drugs (5-FU) and other natural products at their time-dependent IC$_{50}$’s.

**Hypothesis III:** Treatment cell cycle specific drugs/compounds and additional treatment of LLLT will show a greater eradication or suppression cancer cell growth compared to single-dose treatment of LLLT.

**Specific Aim:** To determine the effects of additional treatment with 830 nm laser light in combination with chemotherapeutic drug (5-FU) and other compounds (TQ and EGCG) on FaDu cell numbers and cell function after 24, 48, and 72 hours.
Background of LLLT and treatment in study:

In phase 1, the cells were treated with each of the treatments (TQ, EGCG, and 5-FU). The cells were then placed in the incubator for 30 minutes. Next, the laser treatment was added to the designated cells. The cells were then harvested at 24, 48, and 72 hours. In phase 2, the cells were treated with each of the treatments (TQ, EGCG, and 5-FU). The cells were then placed in the incubator for 30 minutes. Next, laser treatment was added to the designated cells that were to be harvested at 24, 48, and 72 hours. The same day that the 24-hour cells were being harvested, laser treatment was done again for the 48 and 72-hour cells. The same day that the 48-hour cells were being harvested, laser treatment was done the third time for the 72-hour cells. The next day, the 72-hour cells were harvested.

The laser (830 nm) used in this study has three diodes on the face. The mean power of each diode is 30mW and the pulse duration lasts for 33 seconds. Therefore, a total of about 3 Joules of energy is emitted from the diodes and the duration lasts for 33 seconds. A total of 10 Joules is needed for this present study; therefore, the laser duration cycle was repeated once. Next, one of the diodes was covered to give a total 2 Joules, and then repeated once. Therefore, the total duration that the laser was treating the cells was about 132 seconds. Figure 1 shows a diagram of the laser used in the study.
**Figure 1:** Diagram of Low Level Laser (Microlight ML830)
Literature Review
Overview of Laryngeal Carcinoma

Laryngeal carcinoma (head and neck squamous cell carcinoma) is a malignancy that represents about 2-5% of cancers, but represents about 85-90% of squamous cell carcinomas (Wolf 2016). Symptoms of laryngeal and hypopharyngeal carcinoma include voice changes (hoarseness), constant sore throat, coughing, trouble or pain while swallowing, earache, trouble breathing, weight loss, and mass in neck (American Cancer Society, 2015). The exact etiological factors for laryngeal and hypopharyngeal carcinoma are unknown, but many co-factors, including tobacco smoking, alcohol consumption, poor diet, and depressed immune system, have presumed contributions (Mastronikolis et al 2012).

The 5-year survival rate for laryngeal carcinoma depends on the location of the cancer—glottis, supraglottis, or subglottis. Cancer in the glottis region, part of the larynx that include the vocal cords, has a 5-year survival rate of 90% for stage I, 74% for stage II, 56% for stage III, and 44% for stage IV. Cancer in the supraglottic region, part of the larynx that is above the vocal cords, has a 5-year survival rate of 59% for stage I, 59% for stage II, 53% for stage III, and 34% for stage IV. Cancer is the subglottic region, part of the larynx below the vocal cords, has 5-year survival rate of 65% for stage I, 56% for stage II, 47% for stage III, and 32% for stage IV. The 5-year survival rate for hypopharyngeal carcinoma (area around vocal cords) is 53% for stage I, 39% for stage II, 36% for stage III, and 24% for stage IV (American Cancer Society, 2016). Laryngeal and hypopharyngeal carcinoma are staged according to the Tumor-Node-Metastasis (TNM) classification system (stages 0-IV). This system takes into account the size and location of the tumor (T), lymph node involvement (N), and presence of distant metastasis (M) (Mastronikolis et al 2012). There are five stages of laryngeal carcinoma.
In Stage 0, the cancer cells are only growing in the inner lining layer of the larynx; there is no evidence the cancer has spread to the lymph nodes or beyond. In Stage I, the tumor is only in the vocal cords; there is no evidence the cancer has spread to the lymph nodes or beyond. In Stage II, the tumor is growing into the supraglottis/subglottis and/or the vocal cords do not move normally, but there is no evidence of lymph node involvement or distant metastasis. In Stage III, the tumor is in the larynx with vocal cord paralysis. The tumor could also be growing into the paraglottic space or into the inner part of the thyroid cartilage. Notably in stage III, the cancer is likely to have spread to a single lymph node on the same side of the neck as the tumor, and the node is $\leq 3$ cm across. It is possible that there is no evidence the cancer has spread to the lymph nodes, and it has not spread to distant sites. In Stage IV, either the tumor has grown through the thyroid cartilage and/or is growing into tissues beyond the larynx, or the tumor is growing into the tissue anterior to the spine in the neck (the prevertebral space), surrounds a carotid artery, or is growing down into the space between the lungs. The cancer could possibly spread to at least 1 lymph node that is larger than 6 cm across and possibly spread to distant sites (American Cancer Society, 2015). Survival decreases by more than one third when clinically positive lymph nodes are present. There is a five-year disease free survival of patients with supraglottic cancer is 80% for stages I-II, 70% for stage III and 40% for stage IV. Patients with glottic cancers have a better long-term prognosis. There is a five-year disease free for stages I-II is 85%-90%, for stage III is 75% and for stage IV is 45%-50%. However, in cases with an extremely advanced local and regional disease the overall 5-year survival is less than 5% (Mastronikolis et al 2012). There are also five stages for hypopharyngeal carcinoma. In stage 0, there is no evidence of primary tumor,
or any regional lymph node or distant metastasis. In stage I, the tumor is limited to one subsite of the hypopharynx, and there is no regional lymph node or distant metastasis. In stage III, the tumor is either greater than 4 cm in dimension from extension to the esophagus. The tumor could possibly be limited to 1 subsite of the hypopharynx and/or \( \leq 2 \text{cm} \) in greatest dimension or invade more than 1 subsite. Metastasis could be in a single lymph node \( \leq 3 \text{ cm} \) in greatest dimension and/or no distant metastasis. In stage IV, the tumor could invade the thyroid/cricoid cartilage, hyoid bone, thyroid gland, or central compartment soft tissue and could range from having no regional lymph node metastasis to metastasis in a lymph node \( >6 \text{cm} \) in greatest dimension. Distant metastasis is also a possibility (Stevenson, 2016). The larynx and pharynx are illustrated in figure 2.

![Anatomy of the Larynx and Pharynx](image)

**Figure 2:** Illustration of the larynx and pharynx showing labeled parts

**Cellular changes in Laryngeal and Hypopharyngeal Carcinoma:**

Common cellular changes that occur in laryngeal and hypopharyngeal carcinogenesis include those involving, the tumor suppressor p53, the proto-oncogene cyclin D1, and tumor suppressor p16 (Williams et al, 2015; Fu et al, 2007). p53 is a
nuclear phosphoprotein and tumor protein involved in gene transcription, apoptosis, DNA repair and synthesis, and cell cycle regulation (Mastronikolis, 2012). p53 is the gatekeeper of DNA integrity at the R checkpoint of the cell cycle. This protein is mutated in more than 50% of human tumors. p53 point mutations, deletions, and inactivation occur in the shift from the pre-invasive to the invasive state of laryngeal carcinoma (Mastronikolis, 2012). Mutations include loss of G1/S checkpoint control and the inability for apoptosis to regularly occur. The inactivation of p16 and p53 occur early in the development and progression of laryngeal carcinoma (Williams et al, 2015). Cyclin D1 is involved in cell cycle progression and interacts with cyclin-dependent kinases. When cyclin D1 gene amplification is observed in precancerous and cancerous lesions, it is always overexpressed. In this state of increased expression, cyclin D1 drives more cells into the G2/M phase of the cell cycle (Mastronikolis et al., 2012).

**Standard of treatment of laryngeal and hypopharyngeal carcinoma by stage:**

In stage 0 for laryngeal carcinoma, the cancer is usually in the glottic area. Vocal cord stripping, laser surgery, or radiation therapy are mainly used as treatment options. In stage I and II of laryngeal carcinoma, either radiation without surgery or partial laryngectomy usually takes place. Particularly, glottis cancers are usually treated by removing the vocal cords. Supraglottic cancers, the lymph nodes are usually removed. In stage III and IV of laryngeal carcinoma, treatment is usually some combination of surgery, radiation, and/or chemotherapy. Initial treatment usually includes surgery or chemotherapy (common chemotherapeutic drugs include cisplatin and 5-FU) with radiation. In stage I for hypopharyngeal carcinoma, surgery and/or radiation are usually
the main options for initial treatment. Surgery usually includes removing all or part of
the pharynx along with the lymph nodes on the sides of the neck. In stages II, III, IV for
hypopharyngeal carcinoma, the first option is usually to remove the pharynx, larynx, and
lymph nodes in neck area, accompanied by radiation or radiation with chemotherapy.

Treatment first with chemotherapeutic drug, such as cisplatin, along with radiation, is
another option (American Cancer Society, 2016).

Tables 1-5 below display different studies involving the treatments and their findings.

**Table 1: Studies on the Effect of Genistein**

<table>
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<th>Author(s)</th>
<th>Cell Line/Type</th>
<th>Major Findings</th>
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<td>1999</td>
<td>Alhasan et al</td>
<td>HN4 squamous cell carcinoma cell line</td>
<td>--genistein inhibits proliferation of a squamous cell carcinoma cell line --caused cell cycle arrest at the S/G2-M phase and induced apoptosis -- shown to inhibit cell proliferation and to induce cell cycle arrest at the G2-M phase in breast, prostate, and jurkat T cell leukemia cell lines.</td>
</tr>
<tr>
<td>2010</td>
<td>Tokkalov et al</td>
<td>p53-deficient cells (H1299, FaDu)</td>
<td>--genistein-based therapy may have antagonistic effects when combined with mitotic poisons. --proposed therapeutic strategy allows protection of p53 wild type cells from taxol and selectively increases apoptosis in p53-deficient cells.</td>
</tr>
<tr>
<td>2010</td>
<td>Johnson et al</td>
<td>SCC15 and SCC25 squamous cell carcinoma cell lines</td>
<td>--genistein inhibited SCC15 and SCC25 cell growth. --induced a decrease in phosphorylation of ERK and Akt at treatment concentrations of 20, 50 and 100 μM. (Akt accelerates tumor progression)</td>
</tr>
<tr>
<td>2013</td>
<td>Han et al</td>
<td>human intestinal colon cancer Caco-2 cells</td>
<td>--genistein significantly suppressed cell proliferation. --demonstrated to modulate cell cycle distribution through accumulation of cells at G2/M phase, with a significant decreasing effect of Cyclin B1 and</td>
</tr>
<tr>
<td>Year</td>
<td>Authors</td>
<td>footnote</td>
<td>Summary</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>2013</td>
<td>Zheng et al</td>
<td>SK-N-SH human neuroblastoma cells</td>
<td>-- Cells were arrested at the G2/M phase by genistein -- estradiol- or environmental endocrine disruptor-induced proliferation of human neuroblastoma cells is effectively abolished by genistein, likely in a cell cycle- and Akt pathway-dependent manner</td>
</tr>
<tr>
<td>2013</td>
<td>Hara et al</td>
<td>participants with gastric cancer</td>
<td>-- no association of plasma isoflavone concentrations with gastric cancer risk</td>
</tr>
<tr>
<td>2011</td>
<td>Ayhen et al</td>
<td>nasopharyngeal carcinoma cell line CNE</td>
<td>-- genistein induced a G2/ M cell cycle arrest --induces nasopharyngeal cancer cell early apoptosis rate -- characteristic morphological changes of apoptosis in CNE cells was observed after treated by genistein</td>
</tr>
<tr>
<td>2004</td>
<td>Ye et al</td>
<td>human oral squamous carcinoma line (SCC-25)</td>
<td>-- genistein inhibited SCC-25 cell growth via G2/M phase arrest. -- significant decrease of proliferating cell nuclear antigen expression in these cells after treatment</td>
</tr>
<tr>
<td>2001</td>
<td>Akimoto et al</td>
<td>human esophageal squamous cell cancer cell lines, TE-1 (p53, mutant) and TE-2 (p53, wild)</td>
<td>-- Genistein (30 microM) greatly enhanced radiosensitivity in cell lines by suppressing radiation-induced activation of survival signals, p42/p44 extracellular signal-regulated kinase and AKT/PKB</td>
</tr>
<tr>
<td>2000</td>
<td>Alhasan et al</td>
<td>HN4 squamous cell carcinoma of the head and neck cell line (HNSCC)</td>
<td>-- down-regulation of Cdk1, and CyclinB1, and up-regulation of the cyclin dependent kinase (Cdk) inhibitor p21WAF1--may be responsible for the induction of cell cycle arrest and apoptosis</td>
</tr>
<tr>
<td>2012</td>
<td>Li et al</td>
<td>Neuroblastoma (NB) cell line</td>
<td>--genistein, is an epigenetic modifier able to decrease hypermethylation levels of CHD5, and enhances the expression of CHD5 as well as p53 (tumor suppressor genes), which may contribute to inhibition of NB growth in vivo and tumor micro-vessel formation</td>
</tr>
</tbody>
</table>
Table 2: Studies on the Effect of Thymoquinone

<table>
<thead>
<tr>
<th>Year</th>
<th>Author(s)</th>
<th>Cell Line/Type</th>
<th>Major Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>Woo et al</td>
<td>Review: tumor xenograft mice models for colon, prostate, pancreatic and lung cancer</td>
<td>--review: anti-oxidant/anti-inflammatory and anticancer effects of TQ with a focus on its molecular targets,</td>
</tr>
<tr>
<td>2007</td>
<td>Roepke et al</td>
<td>human osteosarcoma cells</td>
<td>-- TQ induces p53-independent apoptosis in human osteosarcoma cells</td>
</tr>
<tr>
<td>2010</td>
<td>Gurung et al</td>
<td>Glioblastoma cells</td>
<td>-- TQ induces DNA damage, telomere attrition by inhibiting telomerase and cell death in glioblastoma cells</td>
</tr>
<tr>
<td>2003</td>
<td>Shoieb et al</td>
<td>canine osteosarcoma (COS31), human breast adenocarcinoma (MCF7), human ovarian adenocarcinoma (BG-1) and Madin-Darby canine (MDCK) cell lines</td>
<td>--TQ induced apoptosis of COS31 and decreased the number of COS31 cells in S-phase and increased cells in G1-phase, indicating cell cycle arrest at G1</td>
</tr>
<tr>
<td>2012</td>
<td>Lei et al</td>
<td>gastric cancer cells xenograft tumor mouse model</td>
<td>-- pretreatment with TQ significantly increased the apoptotic effects induced by 5-FU in gastric cancer cell lines in vitro</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-- TQ enhanced the 5-FU-induced killing of gastric cancer cells by mediating the downregulation of the anti-apoptotic protein bcl-2, the upregulation of the pro-apoptotic protein bax, and the activation of both caspase-3 and caspase-9</td>
</tr>
<tr>
<td>2015</td>
<td>Wilson et al</td>
<td>ID8-NGL mouse ovarian cancer cells</td>
<td>-- TQ reduced proliferation and increased apoptosis in ID8-NGL tumors after 10 and 30 day treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-- Prolonged TQ treatment did not significantly alter tumor number or mass compared to vehicle, but exerted an overall deleterious effect by stimulating ascites formation</td>
</tr>
<tr>
<td>2015</td>
<td>Parbin et al</td>
<td>MCF-7 breast cancer cells</td>
<td>- TQ elicited downstream effects of induction of the pro-apoptotic gene Bax, down regulation of the anti-apoptotic gene Bcl-2 and arrest of the cell cycle at the G2/M phase. --the result of a higher cytotoxicity of TQ towards MCF-7 breast cancer cells in comparison to normal cells indicates the potential of TQ to be an anticancer drug</td>
</tr>
<tr>
<td>2015</td>
<td>Harpole et al</td>
<td>SK-OV-3 Ovarian Cancer</td>
<td>- increase in nitric oxide following the administration of EGCG alone and in combination with TQ compared with TQ alone or untreated control cells with the difference being about three-fold higher</td>
</tr>
<tr>
<td>2015</td>
<td>Dehghani et al</td>
<td>human breast adenocarcinoma cell line (MCF7)</td>
<td>- Proliferation of MCF7 cells was significantly inhibited by TQ and nanothymoquinone in a concentration-dependent manner in defined times</td>
</tr>
<tr>
<td>Year</td>
<td>Author(s)</td>
<td>Cell Line/Type</td>
<td>Major Findings</td>
</tr>
<tr>
<td>------</td>
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<td>-------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2013</td>
<td>Ghiringhelli et al</td>
<td>myeloid-derived suppressor cells</td>
<td>-- 5FU-driven activation of the NLRP3 inflammasome in MDSCs promotes tumor angiogenesis by eliciting T&lt;sub&gt;H&lt;/sub&gt;17 responses that compromise anticancer immunity</td>
</tr>
<tr>
<td>2013</td>
<td>Zhang et al</td>
<td>Colorectal cancer</td>
<td>-- detection for G2194A, T85C and T464A could predict ~13% of 5-FU severe toxic side effects</td>
</tr>
<tr>
<td>2013</td>
<td>De la Cueva et al</td>
<td>Colorectal cancer-tumor derived cell lines/mouse xenograft models</td>
<td>-- both ChoKa and 5-Fu in combination display a synergistic anti-tumor effect due to ChoKa inhibitors-driven modulation of the metabolization of 5-FU.</td>
</tr>
<tr>
<td>2012</td>
<td>Chen et al</td>
<td>Hep-2/5-Fu Cell line</td>
<td>-- the drug resistance cell line had slower growth rate and larger size -- Hep-2/5-Fu cell line showed cross drug resistance to 5-Fu, cisplatin and vincristine</td>
</tr>
<tr>
<td>2013</td>
<td>Furusaka et al</td>
<td>Squamous cell carcinoma of the glottis larynx</td>
<td>-- synergistic effects of anticancer chemotherapy combined with chemoradiation therapy for squamous cell carcinoma caused marked cancer tissue degeneration -- Side effects included leukopenia, neutropenia, mucositis, and dermatitis</td>
</tr>
<tr>
<td>2011</td>
<td>Ma et al</td>
<td>FaDu cell line and resistant cell line, FaDu/T</td>
<td>-- multidrug resistant sensitivities of the FaDu/T cells to cisplatin (DDP), 5-FU, doxorubicin (Dox) and vincristine (VCR) were investigated -- percentages of FaDu/T cells in the G0/G1 and G2/M phases were increased while the cell percentage in the S phase decreased as compared with the percentages of FaDu cells. The anti-apoptotic ability increased prominently, as the index of apoptosis decreased</td>
</tr>
<tr>
<td>Year</td>
<td>Author(s)</td>
<td>Model/Cell Type</td>
<td>Major Findings</td>
</tr>
<tr>
<td>------</td>
<td>--------------------</td>
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</tr>
<tr>
<td>2013</td>
<td>Firat et al</td>
<td>diabetic rats</td>
<td>LLLT elicits a positive healing effect on palatal mucoperiostal wounds, and modulates the oxidative status; reduced numbers of inflammatory cells, and increased mitotic activity of fibroblasts, collagen synthesis, and vascularization</td>
</tr>
<tr>
<td>2013</td>
<td>Sperandio et al</td>
<td>cells (DOK) and oral cancer cells (SCC9 and SCC25)</td>
<td>LLLT (660 nm or 780 nm; 2.05, 3.07 or 6.15 J/cm²) can modify oral dysplastic cells (DOK) and oral cancer cells (SCC9 and SCC25) growth by modulating the Akt/mTOR/CyclinD1 signaling pathway; LLLT significantly modified the expression of proteins related to progression and invasion in all the cell lines, and could aggravate oral cancer cellular behavior</td>
</tr>
<tr>
<td>2009</td>
<td>Frigo et al</td>
<td>melanoma cells (B16F10) in mice</td>
<td>Irradiation should be avoided over melanomas as the combination of high irradiance (2.5 W/cm(2)) and high dose (1050 J/cm(2)) significantly increases melanoma tumor growth in vivo</td>
</tr>
<tr>
<td>2014</td>
<td>Gomes et al</td>
<td>tongue squamous carcinoma cell line (SCC25)</td>
<td>LLLT exerts a stimulatory effect on proliferation and invasion of SCC25 cells</td>
</tr>
<tr>
<td>2003</td>
<td>Kreisler et al</td>
<td>human periodontal ligament fibroblasts (PDLF)</td>
<td>809-nm diode laser; 1.96-7.84 J/cm-2; irradiated cells revealed a considerably higher proliferation activity than the controls. The differences were significant up to 72 h after irradiation</td>
</tr>
<tr>
<td>2002</td>
<td>Kreisler et al</td>
<td>human gingival fibroblasts (HGF) in vitro</td>
<td>809-nm semiconductor laser; 1.96-7.84 J/cm(2); irradiated cells revealed a considerably higher proliferation activity; differences were highly significant 24 hour after irradiation but decreased in an energy-dependent manner after 48 and 72 hour after irradiation</td>
</tr>
<tr>
<td>Year</td>
<td>Authors</td>
<td>Cells/Type</td>
<td>Results</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>-------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2010</td>
<td>Peplow et al</td>
<td><strong>review—human and animal cells in culture</strong></td>
<td>-- results consistently demonstrated the potential of laser irradiation to affect cellular proliferation in a wavelength-and dosage-dependent manner</td>
</tr>
<tr>
<td>2002</td>
<td>Pinheiro et al</td>
<td>Laryngeal carcinoma cells- H.Ep.2 cells</td>
<td>--670nm laser irradiation led to an increased cell proliferation when compared to both control and 635nm irradiated cells -- best cell proliferation was found with 670nm laser irradiated cultures exposed to doses of doses of 0.04 to 0.48 J/cm2 -- both dose and wavelength are factors that may affect cell proliferation of HEP2 cells</td>
</tr>
<tr>
<td>2005</td>
<td>Werneck et al</td>
<td>HEP2 cells</td>
<td>-- Time, treatment, and wavelength significantly influenced the proliferation process of HEP2 cells -- 685 and 830 nm</td>
</tr>
<tr>
<td>2007</td>
<td>Renno et al</td>
<td>normal primary osteoblast (MC3T3) and malignant osteosarcoma (MG63) cell lines</td>
<td>-- Osteoblast proliferation increased significantly after 830-nm laser irradiation (at 10 J/cm(2)) but decreased after 780-nm laser irradiation (at 1, 5, and 10 J/cm(2)) -- Alkaline phosphatase (ALP) activity in the osteoblast line was increased after 830-nm laser irradiation at 10 J/cm(2) -- each cell line responds differently to specific wavelength and dose combinations</td>
</tr>
<tr>
<td>2012</td>
<td>Rhee et al</td>
<td>Cochlear hair cells</td>
<td>-- LLLT may promote hair cell survival following gentamicin damage in the cochlea</td>
</tr>
<tr>
<td>2016</td>
<td>Saenko et al</td>
<td>HCT-116 and CHO-K cells (human colon carcinoma cell line)</td>
<td>-- Laser irradiation has induced a dose-dependent cell death via increasing intracellular reactive oxygen species (ROS) concentration, increase of DNA damage, decrease of mitochondrial potential, and reduced glutathione. -- the increase of the intracellular ROS concentration induced by mitochondrial damage contributes to the damaging effect of the laser irradiation at 1265 nm</td>
</tr>
<tr>
<td>Year</td>
<td>Authors</td>
<td>Species/Tissue</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>--------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2016</td>
<td>Bartos et al</td>
<td>HEI-OC1 auditory cells</td>
<td>Near infrared photobiomodulation therapy at 810 nm, 30 mW/cm², 100 seconds, 1.0 J, 3 J/cm² changed mitochondrial metabolism and oxidative stress response for up to 24 hours post treatment.</td>
</tr>
<tr>
<td>2015</td>
<td>Giuliani et al</td>
<td>Mouse Embryonic Fibroblast (MEF)</td>
<td>LLLT at 670 nm, when dispensed in pulsed mode, modifies mitochondria network dynamics, as well as expression level of mRNA encoding for selective matrix proteins in MEF.</td>
</tr>
<tr>
<td>2014</td>
<td>Agrawal et al</td>
<td>Multiple tissues</td>
<td>LLLT has been used for nearly 50 years to enhance tissue healing and to relieve pain, inflammation and swelling. The photons are absorbed in cytochrome(c) oxidase (unit four in the mitochondrial respiratory chain), and this enzyme activation increases electron transport, respiration, oxygen consumption and ATP production.</td>
</tr>
<tr>
<td>2016</td>
<td>Zhou et al</td>
<td>stress-induced premature senescence (SIPS) model</td>
<td>5-aminolevulinic acid photodynamic therapy (ALA-PDT) elicits oxidative damage and apoptosis in photoaged fibroblasts in vitro, which may be the basis for the rejuvenating effects on photoaged skin.</td>
</tr>
<tr>
<td>2014</td>
<td>Mocan et al</td>
<td>pancreatic cancer cells (PANC-1)</td>
<td>Multi-walled carbon nanotubes and polyethylene glycol (PEG) laser mediated treatment (808 nm, 2W) leads to mitochondrial membrane depolarization that activates the flux of free radicals within the cell and the oxidative state mediate cellular damage in PC cells via apoptotic pathway.</td>
</tr>
<tr>
<td>2013</td>
<td>Huang et al</td>
<td>Mouse cortical neuron</td>
<td>LLLT increases ROS in normal neurons, it reduces ROS in oxidatively-stressed neurons. In both cases mitochondrial membrane potential is increased.</td>
</tr>
</tbody>
</table>
Table 5: Studies on the Effect of EGCG

<table>
<thead>
<tr>
<th>Year</th>
<th>Author(s)</th>
<th>Cell Line/Type</th>
<th>Major Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>2015</td>
<td>Legeay et al</td>
<td><strong>Review</strong></td>
<td>-- EGCG modulates cellular and molecular mechanisms of various symptoms leading to metabolic syndrome</td>
</tr>
<tr>
<td>2012</td>
<td>Min et al</td>
<td><strong>non-specific</strong></td>
<td>-- Preferential death of cancer cells by EGCG could be caused by the cancer-specific induction of ROS and epigenetic modulation of expression of apoptosis-related genes, such as hTERT</td>
</tr>
<tr>
<td>2003</td>
<td>Gupta et al</td>
<td>human prostate carcinoma cells</td>
<td>-- EGCG causes an induction of G1 phase cks, which inhibits the cyclin-cdk complexes operative in the G0/G1 phase of the cell cycle--causes an arrest, leading to apoptotic cell death</td>
</tr>
<tr>
<td>2015</td>
<td>Flores-Perez et al</td>
<td>A549 lung cancer cells</td>
<td>-- Abrogation of HDGF by EGCG enhances cisplatin-induced apoptosis and sensitize A549 cells to chemotherapy -- Cell death was associated to increased apoptosis, disruption of the mitochondrial membrane potential, and activation of caspase-3 and caspase-9</td>
</tr>
<tr>
<td>2015</td>
<td>Irimie et al</td>
<td>SSC-4 human oral squamous cell carcinoma (OSCC)</td>
<td>-- EGCG treatment suppresses cell proliferation of SSC-4 human oral squamous cell carcinoma (OSCC), (dose- and time-dependent) -- Activation of apoptosis and autophagy in response to EGCG exposure in SSC-4 cells</td>
</tr>
<tr>
<td>2015</td>
<td>Lee et al</td>
<td>Oral squamous cell carcinoma (OSCC)</td>
<td>-- EGCG attenuates cell proliferation of OSCC cells by upregulating BTG2 expression via p38 and ERK pathways</td>
</tr>
<tr>
<td>2015</td>
<td>Liu et al</td>
<td>human esophageal squamous cell carcinoma cell lines, Eca-109 and Te-1</td>
<td>-- EGCG inhibits proliferation and induces apoptosis through ROS production, caspase-3 activation, and a decrease in VEGF expression in vitro and in vivo -- Tumor cells were arrested in the G1 phase</td>
</tr>
<tr>
<td>Year</td>
<td>Authors</td>
<td>Tissue/Cell Type</td>
<td>Effects and Findings</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>-----------------------------------</td>
<td>--------------------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| 2013 | Lee et al        | head and neck squamous carcinoma (HNSC) | -- EGCG inhibits the self-renewal capacity of HNSC CSCs by suppressing their sphere forming capacity, and attenuates the expression of stem cell markers, such as Oct4 and Sox2  
-- EGCG decreased the transcriptional level of Notch, resulting in the inhibition of Notch signalling |
| 2009 | Wang et al       | Laryngeal squamous cell carcinoma (LSCC) cell line Hep-2 | -- treatment of Hep-2 cells with EGCG decreased the cell viability, inhibited the growth and proliferation, induced apoptosis and increased the activity of caspase-3 in a dose-dependent manner  
-- EGCG-treatment repressed telomerase activity effectively in a concentration-dependent manner |
| 2010 | Lee et al        | human laryngeal epidermoid carcinoma of the larynx Hep2 cells | -- p53-mediated mitochondrial pathway and the nuclear translocation of AIF and EndoG play a crucial role in EGCG-induced apoptosis of human laryngeal epidermoid carcinoma Hep2 cells |
| 2011 | Muneyuki et al   | head and neck squamous cell carcinoma (HNSCC) | --EGCG has shown clinical efficacy and unique biological effects on lipid rafts that are an important platform of numerous biophysical functions including RTKs signalings. |
| 2011 | Joseph et al     | squamous cell carcinoma of the head and neck (SCCHN) | -- EGCG modulates several key molecular signaling pathways at multiple levels and has synergistic or additive effects when combined with many other natural or synthetic compounds (review) |
| 2009 | Chung et al      | **review**                         | --EGCG inhibit enzyme activities and signal transduction pathways, resulting in the suppression of cell proliferation and enhancement of apoptosis, as well as the inhibition of cell invasion, angiogenesis and metastasis |
Materials and Methods
**Cells and Culture:** The FaDu cell is a squamous cell carcinoma originating from a hypopharyngeal tumor removed from a patient in India (Wiley). For this experiment, the FaDu cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The FaDu cell line was grown in 90% Eagle’s Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS). FaDu cells were cultured and plated in either 24-well tissue culture plates at a density of 1.0 x 10^6 cells per well or in 6-well tissue culture plates at 5 x 10^6 for the laser experiments since the area of the well (9.5 cm^2) was comparable to the head of the laser. The cells were then incubated in a CO_2 (5% CO_2 and 95% air) at 37°C until treatment.

**Treatment of Cells:** For the first set of experiment in the 24 well plates, cells in the first row were left untreated and served as the control and cells in rows 2-4 were treated with IC_{50} doses of either Genistein (25 mM), TQ (16 µM) and 5-FU (16 µM). For experiments in 6 well plates involving LLLT, cells were treated with laser or IC_{50} doses of EGCG, TQ, and 5-FU, and placed in the incubator for 30 minutes prior to LLLT treatment and then incubated for 24, 48, and 72-hour intervals. For LLLT, cells were treated with 10 Joules (1.05 J/cm^2) at 830 nm wavelength. (Genistein was replaced with the IC_{50} dose of EGCG (3 µM)). Additional experiments were performed with daily treatments of LLLT and followed for 24, 72, and 96 hours (change in harvest time was used since the doubling time for these cells was found to be 48 hours) of treatment with the chemotherapeutic agent 5-FU for 30 minutes followed by daily treatments with LLLT. In the final experiments, the cells were treated and incubated for 30 minutes with the IC_{50} doses with TQ, 5-FU, EGCG, followed daily laser therapy. In the first phase of this set, the cells were treated for one day, then LLLT was performed for each day (24,
48, and 72 hours). In the second phase, the cells were treated each day, 30 minutes prior to LLLT being performed. Table 6 below summarizes the experimental design and treatments for each set and phase.

Table 6: Experimental Design

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Process</th>
<th>G</th>
<th>TQ</th>
<th>5-FU</th>
<th>EGCG</th>
<th>LLLT</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24, 48, 72 hours; continuous treatment</td>
<td>25 mM</td>
<td>16 µM</td>
<td>16 µM</td>
<td>X</td>
<td>X</td>
<td>Protein</td>
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<td></td>
<td></td>
<td></td>
<td>Glut</td>
</tr>
<tr>
<td>2</td>
<td>24, 72, 96 hours; continuous treatment</td>
<td>X</td>
<td>X</td>
<td>16 µM</td>
<td>X</td>
<td>830 nm 10 J</td>
<td>Protein Glut</td>
</tr>
<tr>
<td>3</td>
<td>24, 48, 72 hours. Phase 1: continuous treatment; LLLT on day 0 Phase 2: Continuous treatment with additional LLLT treatment each day</td>
<td>X</td>
<td>16 µM</td>
<td>16 µM</td>
<td>3 µM</td>
<td>830 nm 10 J</td>
<td>Protein Glut NO LDH</td>
</tr>
</tbody>
</table>
**Cell Collection:** After the allotted times (24, 48, 72, or 96-hour period after treatments), the supernatants from each group were collected and placed into appropriate labeled microcentrifuge tubes and were frozen. After the supernatant was collected, 1mL of PBS was added to the wells (6- and 24-well plates). All of the wells that contained PBS were scraped, and the cells were collected in microcentrifuge tubes. The microcentrifuge tubes containing the cells with PBS were placed into the centrifuge at 1500-2000 rpm for ten minutes. The PBS was then removed from the microcentrifuge tubes down to the 0.1 mark. The pellet was left, and another 1mL of PBS was added to the tubes and centrifuged again. The PBS was removed again down to the 0.1 mark, the 300µL of PBS was added to the tubes containing the pellet. The tubes were vortexed to resuspend the cells. The cells were frozen for at least one day prior to being assayed.

**Biochemical Analysis:** Colorimetric microtiter plate endpoint assays were performed to determine concentrations of protein, reduced glutathione, and nitric oxide. Lactate dehydrogenase activity was determined using spectrophotometric analysis of reduction of NADP. Cellular protein was determined to evaluate cell viability; intracellular glutathione was used to determine cellular stress and viability. Lactate dehydrogenase activity and nitric oxide levels were determined in the supernatants for cell toxicity and damage. The microtiter plates were analyzed using a Spectra plate reader to determine concentration using the Magellan curve fit software.

**Protein Assay:** The initial step in the BCA protein assay process involved making the standard solution. Eight microcentrifuge tubes were used for the standard. Albumin Standard Ampule (500 µl, 2mg/mL) was placed in the first tube, and then 500µL of PBS were placed in all eight tubes. A serial dilution was done, where 500µL were taken out
of each consecutive tube and placed into the next one, but not in tube eight. These standard solutions (25µL) were added into the first two columns of the 96-well plate followed by 25µL of cells into the remaining wells. The working reagent was determined by multiplying the number of wells with standards and cells by 0.2mL, which is equal to the total working reagent (WR). The equation, 1/50=X/total amount of WR gave the amount of reagent B when solved for X. By subtracting the amount of reagent B from the total WR, the amount of reagent A was calculated. After 200 µL of the WR was added to all the standards and cells, the cell plate was incubated (37˚C) for 30 minutes, covered. After the incubation period, the plate was read using the Magellan software. The parameters: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0 were used to compare the standards. Wavelength (540 nm) was used for protein assay analysis.

**Glutathione Assay:** The initial steps in the glutathione assay were to prepare fresh reagents. The chemicals used in the preparation included NADPH, DNTB, GSH 1, and 2, phosphate buffer, EDTA, and reductase. The standards are made by taking eight glass test tubes and placing 100µL of GSH 2 into tubes 1 and 2, then adding 100µL of distilled water into tubes 2-8, then doing a serial dilution, where 100µL was taken out of each consecutive tube starting from tube two and ending at tube seven. Each standard (50 µL) was added into the 96-well plate, then 50µL of the cells were also added into the plate. Depending on how many samples being tested, the mixed reagent that was prepared initially was added to the standard and cell samples (100µL). The ratio of the amount of chemicals to use to make the mixed reagent: 1 to 1 to 1.15 (DNTB, NADPH, EDTA + Buffer). After the mixed reagent was added, the plate was read using the Magellan
software. After this initial reading, and incubation period of 10 minutes is done, then the plate was read again using the Magellan software.

**Nitric Oxide Assay:** The initial step in the Nitric Oxide assay was to mix the necessary chemicals to make reagent 1 and 2. Reagent 1 is made by weighing out 0.1 g of N-(1-naphthyl) ethylenediamine dihydrochloride (NED) and dissolving in 100mL of distilled water. Reagent 2 was made weighing out 1.0g of sulfaniladmine and dissolving in 100mL of 5% phosphoric acid. Aluminum foil was placed around the flask containing Reagent 1 and 2 and placed in the refrigerator. The Standard solution is made by weighing out 0.069 g of sodium nitrite and dissolving in 500mL of distilled water. Of this solution, 1mL is then placed in 9mL of distilled water. Eight glass test tubes were used and 300µL of the standard solution were pipetted into tubes 1 and 2. Of distilled water, 300µL were added into tubes 2-8. A serial dilution was performed, starting with taking out 300µL from tube 2, and then stopping at tube 7. Of the standards, 100µL were pipetted into the 96-well plate, and 100µL of the supernatants were also added into the 96-well plate. A working reagent was then prepared using reagents 1 and 2 by mixing 1 part of Reagent 1 with one part of Reagent 2. The total amount of working reagent was calculated, and then divided by 2 to determine the quantity of Reagent 1 and 2. Of the working reagent, 100µL were added into the 96-well plate. The plate was incubated at room temperature for at least 5 minutes before analysis at 492nm and 540 nm. Both parameters use the concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, and 0 for the curve fit parameters.
**Lactate Dehydrogenase (LDH) Assay:** The initial step in the LDH assay was to prepare the working reagent (mix 5 parts Buffer (R1) and 1 part Co-enzyme (R2)). Of reagent, 1.0mL is pipetted into the appropriate cuvette tubes and pre-warmed in incubator (37°C) for 5 minutes. The spectrophotometer was calibrated using a blank tube with water only at 340nm. Supernatant samples (50µL) were added to the cuvettes containing the reagents, mixed, then were placed in incubator for 30 seconds. The first absorbance (A1) was read and recording using the spectrophotometer. The samples are then placed back in the incubator for 30 minutes, and then A2 is read and recorded. The change in absorbance ((A2-A1)/30mins) multiplied by the factor 3376 yielded results in IU/L.

**Statistics and Graphing:** Sigmaplot 12.0 software was used for the statistical analysis and one-way ANOVA data. The preferred comparison option for one-way ANOVA was Dunnett’s and Tukey’s, however if this option was not available, Dunn’s method was used to compare the variables and evaluate significant differences, etc. The SlideWrite software was use for the graph production. Descriptive statistics was expressed as mean +/- standard error of the mean (SEM). The data collected from the experiments was statistically analyzed using the SigmaSTAT software. Group mean comparisons were determined using one-way analysis of variance (ANOVA) to determine if any difference differences existed among the groups. ANOVA used an F test to compare the mean of multiple groups initially to determine if differences exist. Multiple comparison testing was performed when significant differences were detected using ANOVA. Several different types of multiple comparison procedures exist, including Dunnett, Dunn, and Tukey. The Tukey posttest was used to determine which differences among the group means were statistically significant.
Results
Protein Assay (Figures 3-5):

The effects of Genistein (G), Thymoquinone (TQ), and 5-Fluorouracil (5-FU) on overall protein levels in FaDu cells were measured as a correlate to overall cell number and cell viability (Figures 3-5). The IC$_{50}$ doses (concentration found to inhibit cellular replication by 50%) determined at the 72-hour time point of G, TQ, and 5-FU were 25 mM, 16 µM, and 16 µM respectively, informed by previous studies such as Williams et al 2015 and Lin et al 2001. Cells treated with TQ displayed the greatest decrease in protein levels as compared to the untreated control group at all time points (24-72 hours). In particular, protein levels were significantly decreased by 68%, 76%, and 76% at 24, 48, and 72 hours respectively as compared to untreated control. 5-FU significantly decreased protein levels, as compared to untreated control, at 48 and 72 hours by 51% and 96%, respectively. In contrast, G did not significantly affect protein levels, as compared to the untreated control, at any time point. As the concentrations of all drugs were chosen based on previously calculated IC$_{50}$ values, the lack of G effect was surprising, but consistently noted.
Figure 3: Average 24-hr cellular protein assay of FaDu cells treated with Genistein, Thymoquinone, and Fluorouracil. Bar graph representing amount of cellular protein after treated with 25mM Genistein, 16µM TQ, and 16µM 5-FU and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

**Normality Test (Shapiro-Wilk)** Passed (P = 0.091)
Figure 4: Average 48-hr cellular protein assay of FaDu cells treated with Genistein, Thymoquinone, and Fluorouracil. Bar graph representing amount of cellular protein after treated with 25mM Genistein, 16µM TQ, and 16µM 5-FU and control that contained cells only. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk.

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Kruskal-Wallis One Way Analysis of Variance on Ranks
Figure 5: Average 72-hr cellular protein assay of FaDu cells treated with Genistein, Thymoquinone, and Fluorouracil. Bar graph representing amount of cellular protein after treated with 25mM Genistein, 16µM TQ, and 16µM 5-FU and control that contained cells only. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

Normality Test (Shapiro-Wilk)  Failed (P < 0.050)
Kruskal-Wallis One Way Analysis of Variance on Ranks
Glut Assay (Figures 6-8):

The effects of G, TQ, and 5-FU on overall glutathione (glut) levels in FaDu cells were measured as a correlate to overall cellular damage. Figures 6-8 display glutathione levels of cells when treated with previously determined IC\textsubscript{50} doses of G, TQ, and 5-FU. G significantly decreased glutathione levels, as compared to the untreated control, at 24 and 48 hours by 33\% and 22\%, respectively. TQ significantly increased in glutathione levels at 48 hours by 200\%, which was reversed to normal by 72 hours. 5-FU did not significantly affect glutathione levels, as compared to the untreated control, at any time point.

** Although G displayed significant change in glutathione levels, G was removed as a treatment and replaced with EGCG (seen in the upcoming data) because EGCG was shown to have more overall significant effect.
Figure 6: Average 24-hr intracellular glutathione assay of FaDu cells treated with Genistein, Thymoquinone, and Fluorouracil. Bar graph representing oxidative damage/glut levels after treated with 25mM Genistein, 16µM TQ, and 16µM 5-FU and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

Normality Test (Shapiro-Wilk)  Passed (P = 0.147)
Figure 7: Average 48-hr intracellular glutathione assay of FaDu cells treated with Genistein, Thymoquinone, and Fluorouracil. Bar graph representing oxidative damage/glut levels after treated with 25mM Genistein, 16 µM TQ, and 16µM 5-FU and control that contained cells only. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

Normality Test (Shapiro-Wilk) Failed (P < 0.050)
Kruskal-Wallis One Way Analysis of Variance on Ranks
Figure 8: Average 72-hr intracellular glutathione assay of FaDu cells treated with Genistein, Thymoquinone, and Fluorouracil. Bar graph representing oxidative damage/glut levels after treated with 25mM Genistein, 16 µM TQ, and 16µM 5-FU and control that contained cells only. Any significant differences (p<0.05) multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.289)

Normality Test (Shapiro-Wilk) Failed (P < 0.050)
Protein Assay Phase 1 (Figures 9-11; 12):

The effects of TQ, EGCG, and 5-FU on overall protein levels in FaDu cells were measured again, as was 3 µM of EGCG, the IC$_{50}$ dose as reported in Williams et al 2015 (Figures 9-11). TQ, EGCG, and 5-FU, as compared to the untreated control, did not significantly affect protein levels at any time point. These results are not consistent with previous literature or with the results found in Figures 3-5, where TQ displayed significant decrease at all time points.

The effects of each treatment in combination with laser on overall protein levels in FaDu cells were also measured (Figures 9-11). The literature-based IC$_{50}$ concentrations for each treatment were used to treat the cells. LLLT alone significantly increased protein levels, as compared to the untreated control, at 48 hours by 74%. TQ + laser significantly increased protein levels, as compared to the untreated control, at 48 hours by 20%, and significantly decreased protein levels, as compared to the untreated control at 48 hours by 68%. TQ + laser also significantly decreased protein levels, as compared to LLLT, at 72 hours by 63%. All other treatments did not significantly affect protein levels when compared to the untreated control or LLLT alone.

An additional analysis evaluating the effects of 5-FU and 5-FU + laser on protein levels was performed to investigate what happens to protein levels at 96 hours (Figure 12). 5-FU + laser significantly decreased protein levels, as compared to LLLT alone, at 24 hours by 44%. No other significant differences were observed. The purpose of investigating just 5-FU at 96 hours is because we wanted to test the effects of this drug that is actually used in treating laryngeal and hypopharyngeal carcinomas and see the prolonged effects.
Figure 9: 24-hr Phase 1 cellular protein assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing amount of cellular protein after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Any significant differences (p<0.05) for multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.079).

Normality Test (Shapiro-Wilk) Passed (P = 0.826)
Figure 10: 48-Hr Phase 1 cellular protein assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing amount of cellular protein after treated with 10 joules (1.05J/cm²) of 830 nm laser 3µM EGCG, 16µM TQ, and16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Kruskal-Wallis One Way Analysis of Variance on Ranks
Figure 11: 72-Hr Phase 1 cellular protein assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing amount of cellular protein after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment grouped compared to Control + Laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Passed (P = 0.522)

Kruskal-Wallis One Way Analysis of Variance on Ranks
Figure 12: Average 24-72-96-hr cellular protein assay of FaDu cells treated with Laser, fluorouracil, and fluorouracil plus laser. Bar graph representing amount of cellular protein after treated with 10 joules (1.05J/cm²) of 830 nm laser, 16µM 5-FU, a combination of these two treatments, and control that contained cells only. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

72hrs/96hrs: The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.352), (P=.396) respectively

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Kruskal-Wallis One Way Analysis of Variance on Ranks
Glut Assay (Figures 13-15; 16):

The effects of TQ, EGCG, and 5-FU, at the same previously discussed concentrations, on overall glutathione levels in FaDu cells were measured as a correlate to overall cellular damage (Figures 13-15). 5-FU significantly increased glutathione levels, as compared to the untreated control, at 48 hours by 38%. The other treatments did not significantly affect glutathione levels at any time point.

The effects of each treatment in combination with laser on glutathione levels in FaDu cells were also measured (Figures 13-15). The IC$_{50}$ concentrations for each treatment were still used to treat cells. LLLT alone significantly increased glutathione levels, as compared to the untreated control, at 48 hours by 20%. TQ + laser significantly decreased glutathione levels, as compared to LLLT alone, at 24 hours by 27%. 5-FU + laser significantly increased glutathione levels, as compared to the untreated control, at 48 hours by 41%. The other treatments did not significantly affect glutathione levels at any time point.

An additional analysis evaluating the effects of 5-FU and 5-FU + laser on glutathione levels was performed to investigate what happens to protein levels at 96 hours in Figure 16. There are no significant differences in any time point.
Figure 13: 24-Hr Phase 1 intracellular glutathione assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing oxidative damage/glut levels after treated with 10 joules (1.05J/cm$^2$) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used to for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to Control + Laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Passed (P = 0.229)

Equal Variance Test: Passed (P = 0.910)
Figure 14: 48-Hr Phase 1 intracellular glutathione assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing oxidative damage/glut levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

Normality Test (Shapiro-Wilk) Passed (P = 0.543)
Figure 15: 72-Hr Phase 1 intracellular glutathione assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing oxidative damage/glut levels after treated with 10 joules (1.05J/cm$^2$) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunn’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

**Normality Test (Shapiro-Wilk) Passed (P = 0.624)**

**Equal Variance Test:** Failed (P < 0.050)
Figure 16: Average 24-72-96-hr intracellular glutathione assay of FaDu cells treated with Laser, fluorouracil, and fluorouracil plus laser. Bar graph representing oxidative damage/glut levels after treated with 10 joules (1.05J/cm$^2$) of 830 nm laser, 16µM 5-FU, a combination of these two treatments with laser, and control that contained cells only. Any significant differences (p<0.05) for multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

24,72hr,96 : The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.098), (P = 0.261), (P = 0.136).
NO Assay Phase 1 (Figures 17-19):

The effects of TQ, EGCG, and 5-FU on overall nitric oxide (NO) levels in FaDu cells were measured as a correlate to overall cellular stress (Figures 17-19). EGCG displayed the greatest increase in NO levels, as compared to the untreated control, at all time points (24-72 hours). In particular, NO levels were significantly increased by 180%, 186%, and 556% at 24, 48, and 72 hours, respectively as compared to the untreated control. The other treatments did not show any significant differences at any time interval.

The effects of each treatment in combination with laser on NO in FaDu cells were also measured (Figures 17-19). EGCG + laser significantly increased NO levels, as compared to the untreated control, at 48 and 72 hours by 223% and 620%, respectively. EGCG + laser also significantly increased NO levels, as compared to LLLT alone, at 48 and 72 hours by 600% and 603% respectively. TQ + laser significantly increased nitric oxide levels, as compared to the untreated control and LLLT alone, at 72 hours by 441% and 407%, respectively. The other treatments did not display any significant differences at any time intervals.
Figure 17: 24-Hr Phase 1 nitric oxide assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing Nitric oxide levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunn’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

Normality Test (Shapiro-Wilk) Failed (P < 0.050)
Figure 18: 48-Hr Phase 1 nitric oxide assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing Nitric oxide levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to Control + Laser is denoted with a percent sign (%).

**Normality Test (Shapiro-Wilk) Failed (P < 0.050)**
Figure 19: 72-Hr Phase 1 nitric oxide assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing Nitric oxide levels after treated with 10 joules (1.05J/cm$^2$) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunn’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). Treatment groups compared to control + laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Kruskal-Wallis One Way Analysis of Variance on Ranks
LDH Assay Phase 1 (Figures 20-22):

The effects of TQ, EGCG, and 5-FU on overall lactate dehydrogenase (LDH) levels in FaDu cells were measured as a correlate to overall cellular damage and toxicity (Figures 20-22). EGCG displayed the greatest increase in LDH levels, as compared to the untreated control, at all time points. Specifically, LDH levels significantly increased by 600%, 900%, and 950% at 24, 48, and 72 hours, respectively. 5-FU significantly increased LDH levels, as compared to the untreated control, at 48 and 72 hours by 150% and 350%, respectively.

The effects of each treatment in combination with laser on LDH levels in FaDu cells were also measured (Figures 20-22). EGCG + laser significantly increased LDH levels, as compared to the untreated control, at 24, 48 and 72 hours by 725% and 650%, and 262%, respectively. EGCG + Laser also significantly increased LDH levels, as compared to LLLT alone, at 24, 48 and 72 hours by 726% and 262%, and 775%, respectively. 5-FU + laser significantly increased LDH levels, as compared to the untreated control, at all time intervals. Specifically, LDH levels significantly increased by 325%, 200%, and 500% at 24, 48, and 72 hours, respectively. 5-FU + laser also significantly increased LDH levels, as compared to LLLT alone, at all time intervals. In particular, LDH levels significantly increased by 325%, 100%, and 500% at 24, 48, and 72 hours, respectively.
Figure 20: 24-Hr Phase 1 lactate dehydrogenase assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing Lactate Dehydrogenase levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to Control + Laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Passed (P = 0.993)
Figure 21: 48-Hr Phase 1 lactate dehydrogenase assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing Lactate Dehydrogenase levels after treated with 10 joules (1.05J/cm²) of 830 nm laser 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to Control + Laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Passed (P = 0.051)
Figure 22: 24-Hr Phase 1 lactate dehydrogenase assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing lactate dehydrogenase levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to Control + Laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Passed (P = 0.537)
Protein Assay Phase 2 (Figures 23-25):

The effects of TQ, EGCG, and 5-FU on overall protein levels in FaDu cells were measured as a correlate to cell number and cell viability (Figures 23-25). Phase 2 differs from phase 1 because additional laser treatment was added each day rather than continuous laser treatment on day 0 for phase 1. TQ, EGCG, and 5-FU did not display any significant difference at any time point.

The effects of each treatment in combination with laser on protein levels in FaDu cells were also measured in Figures 23-25. TQ + laser significantly decreased protein levels, compared to control and control + laser, at 72 hours by 60% and 66%, respectively. 5-FU + laser significantly decreased protein levels, as compared to control + laser, at 72 hours by 49%. The other treatments do not display any significant differences.
Figure 23: 24-hr Phase 2 cellular protein assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing amount of cellular protein after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Any significant differences (p<0.05) for multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.488)

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Kruskal-Wallis One Way Analysis of Variance on Ranks
Figure 24: 48-hr Phase 2 cellular protein assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing amount of cellular protein after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Any significant differences (p<0.05) for multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.021).

**Normality Test (Shapiro-Wilk)** Failed (P < 0.050)

**Kruskal-Wallis One Way Analysis of Variance on Ranks**
Figure 25: 72-Hr Phase 2 cellular protein assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing amount of cellular protein after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to Control + Laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Passed (P = 0.131)
Glut Assay Phase 2 (Figures 26-28):

The effects of TQ, EGCG, and 5-FU on overall glutathione levels in FaDu cells were again measured as a correlate to overall cellular damage (Figures 26-28). Phase 2 consisted of laser treatment each day rather than continuous later treatment on day 0. EGCG significantly decreases glutathione levels at 48 hours by 36%. The other treatments did not display any significant differences.

The effects of each treatment in combination with laser on glutathione levels in FaDu cells were also measured in Figures 26-28. The IC$_{50}$ concentrations for each treatment were still used to treat cells. There are no significant differences displayed at any time point.
Figure 26: 24-Hr Phase 2 intracellular glutathione assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing oxidative damage/glut levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

Normality Test (Shapiro-Wilk) Passed (P = 0.229)
Figure 27: 48-Hr Phase 1 intracellular glutathione assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing oxidative damage/glut levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

Normality Test (Shapiro-Wilk) Passed (P = 0.208)
Figure 28: 72-Hr Phase 2 intracellular glutathione assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing oxidative damage/glut levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunn’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk.

Normality Test (Shapiro-Wilk) Passed (P = 0.088)
NO Assay Phase (Figures 29-31):

The effects of TQ, EGCG, and 5-FU on overall NO levels in FaDu cells were measured as a correlate to overall cellular function (Figures 29-31). Phase 2 consisted of laser treatment each day rather than continuous later treatment on day 0. EGCG significantly increased NO levels, as compared to the untreated control, at 24, 48, and 72 hours by 690%, 300%, and 3067%, respectively. TQ significantly increased nitric oxide levels, as compared to the untreated control, at 48 hours by 125%. The other treatments did not display any significant differences.

The effects of each treatment in combination with laser on glutathione levels in FaDu cells were also measured in Figures 29-31. EGCG + laser significantly increase nitric oxide levels, as compared to the untreated control, at 24, 48, and 72 hours by 733%, 250%, and 2700%, respectively. EGCG + laser also significantly increase nitric levels, as compared to laser alone, at 24, 48, and 72 hours by 734%, 192%, and 2233%, respectively. The other treatments did not display any significant differences.
Figure 29: 24-Hr Phase 2 nitric oxide assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing Nitric oxide levels after treated with 10 joules (1.05J/cm$^2$) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to Control + Laser is denoted with a percent sign (%).

**Normality Test (Shapiro-Wilk) Failed (P < 0.050)**

**Kruskal-Wallis One Way Analysis of Variance on Ranks**
Figure 30: 48-Hr Phase 2 nitric oxide assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing Nitric oxide levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to Control + Laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Passed (P = 0.363)
Figure 31: 72-Hr Phase 2 nitric oxide assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing Nitric oxide levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunn’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). Treatment group compared to Control + laser denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Kruskal-Wallis One Way Analysis of Variance on Ranks
**LDH Assay Phase 2 (Figures 32-34):**

The effects of TQ, EGCG, and 5-FU on overall LDH levels in FaDu cells were measured as a correlate to overall cellular damage and toxicity (Figures 32-34). Phase 2 consisted of laser treatment each day rather than continuous later treatment on day 0. EGCG significantly increase LDH levels, as compared to the untreated control, at 48 and 72 hours by 320% and 483%, respectively. The other treatments did not display any significant differences.

The effects of each treatment in combination with laser on LDH levels in FaDu cells were also measured (Figures 32-34). EGCG + laser significantly increase LDH levels, as compared to untreated control, at 48 and 72 hours by 420% and 980%, respectively. EGCG + laser also significantly increased LDH levels, as compared to LLLT alone, at 48 and 72 hours by 150% and 210%, respectively. 5-FU + laser significantly increased LDH levels, as compared to untreated control, at 48 and 72 hours by 260% and 667%, respectively. 5-FU + laser also significantly increase LDH levels, as compared to LLLT alone, at 72 hours by 119%. The other treatments did not display any significant differences.
Figure 32: 24-Hr Phase 2 lactate dehydrogenase assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing lactate dehydrogenase levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunn’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunn’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*).

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.655)

Normality Test (Shapiro-Wilk) Passed (P = 0.704).
Figure 33: 48-Hr Phase 2 lactate dehydrogenase assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing lactate dehydrogenase levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to control + laser is denoted with a percent sign (%).
Figure 34: 72-Hr Phase 2 lactate dehydrogenase assay of FaDu cells treated with Thymoquinone, Thymoquinone + Laser, EGCG, EGCG + Laser, Fluorouracil, and Fluorouracil + Laser. Bar graph representing lactate dehydrogenase levels after treated with 10 joules (1.05J/cm²) of 830 nm laser, 3µM EGCG, 16µM TQ, and 16µM 5-FU, a combination of the treatments with laser, and control that contained cells only. Dunnett’s Method was used for multiple comparisons versus the control group. Any significant differences (p<0.05) for Dunnett’s multiple comparison procedure of treatment group compared to the control are denoted with an asterisk (*). An All Pairwise Multiple Comparison Procedure was done using a Tukey Test, and any significant differences (p<0.05) for Tukey multiple comparison procedure of treatment group compared to control + laser is denoted with a percent sign (%).

Normality Test (Shapiro-Wilk) Passed (P = 0.994)
Discussion
Based on the findings of the current study, FaDu cells treated with 3 µM EGCG showed the greatest overall anti-cancer effect, in terms of promoting damage to FaDu cells, especially notable with increased changes in NO and LDH, compared to the untreated control. In the first part of the experiment, TQ consistently showed a significant decrease in protein levels and glutathione levels; however, these effects were not consistent and are not displayed in the other protein analyses or any other analyses performed. This drastic change in the results could be due to the possibility that the TQ stock used during the phase 1 and 2 experiments could have been too old, and was not as effective as when it was during the first set of experiments. 5-FU + laser displayed significant effects similar to EGCG when observing the LDH levels as compared to the untreated control; however, the results were not as consistent or effective as EGCG. Overall, the laser seems to not have any significant effects by itself or when combined with the treatments.

Many chemotherapeutic drugs stop cells in cycling through the cell cycle. The cell cycle is divided into four parts: G1, S, G2, and M. Cancer cells treated with the nucleoside analog chemotherapeutic agent, 5-FU, stops division of cells in the S phase of the cell cycle by being incorporated into RNA and DNA and halting translation, transcription, and replication, respectively (Wilkes et al 2008). Limited and conflicting information exists regarding natural agents, genistein and TQ. Both treatments have been shown to stop cells in the G0-G1 phase of the cell cycle and induce apoptosis possibly by increasing the ratio of Bax/Bcl-2 protein expression and decreasing Bcl-xL protein, which are type of anti- or pro-apoptotic regulatory proteins depending on the amount present in the cell (Gali-Muhtasib et al 2004; Shoieb et al 2003). Because G did not have
a significant effect on the cells, this treatment was later exchanged for EGCG, which was found in our lab to be effective against cancer cells, particularly hypopharyngeal carcinoma (Williams et al 2015). EGCG also arrests cells in the G0-G1 phase of the cell cycle by inhibiting the cyclin-dependent kinase complexes that are involved in cell cycle control in those phases (Gupta et al 2003; Liu et al 2015). Most of these therapies and their effects on the cell cycle have been investigated with different types of cancer cells such as human esophageal/oral squamous cell carcinoma (Liu et al, 2015; Irimie et al, 2015), human prostate carcinoma (Gupta et al,2003), and lung cancer cells (Flores-Perez et al, 2015). Based on these previous studies, laryngeal and hypopharyngeal carcinomas seem like they would also be plausible cancer cell types that could be affected by the pro-apoptotic effects of these therapies by cell cycle arrest.

Throughout our study we noted that our findings were inconsistent, especially for TQ. Our initial results show TQ to be effective at decreasing cell proliferation and inducing cell damage; but significant TQ effects were not noted throughout the entirety of the study. Notably, the time when the first set of results was done several months earlier than the experiments including the laser was conducted. This could possibly hint at the idea that the stock of TQ used could have been old, and the effects were not presented accurately. Many studies have displayed TQ as being an effective, putative treatment against different cancer cells. For example, TQ induced p53- dependent apoptosis in human osteosarcoma cells (Roepke et al 2007), and increased apoptosis in ID8-NGL mouse ovarian cancer cells (Wilson et al 2015). TQ was also found to significantly inhibit proliferation in human breast adenocarcinoma cells (Dehghani et al 2015). TQ’s pro-apoptotic effects, however, are not clearly and consistently seen in this
study. TQ likely becomes an inducer of glutathione as the cells are trying to recover and protect cells from the protein loss and damage due to treatment. A change in glutathione levels is indicative of toxicological responses and oxidative stress, which can lead to apoptosis (Gokce et al 2016). Due to these inconsistencies in our results, it would be plausible to re-evaluate the effects on TQ on FaDu cells.

EGCG was a plausible treatment option for laryngeal or hypopharyngeal cancer in the present study. Previous studies have shown that EGCG increases apoptosis and decreases cell proliferation in cancer cells such as lung cancer cells, human oral squamous cell carcinoma cells, and hypopharyngeal carcinoma cells (Flores-Perez et al 2015; Lee et al 2015; Williams et al, 2015). In the current study, EGCG was found to significantly affect nitric oxide and LDH levels, but not overall protein or glutathione levels. The increased nitric oxide levels due to EGCG are consistent with the study by Harpole et al (2015). Nitric oxide has many versatile biological effects on the system, and increased nitric oxide levels could indicate either a positive or negative response on the system. For example, increased nitric oxide levels could lead to oncogene expression, oxidative DNA damage, angiogenesis, and inhibition of DNA repair enzymes and/or dysregulation of apoptosis; however, nitric oxide can also generate apoptosis/death. This uncertainty of the meaning of the increased nitric oxide levels due to EGCG is clarified when evaluating the LDH levels. LDH is used to evaluate the presence of damage to, and toxicity of, cells. The LDH levels were significantly increased by EGCG, indicating the FaDu cells were being damaged. These results align with studies showing that EGCG displays pro-apoptotic effects on cancer cells (Lee et al, 2015; Liu et al, 2015).
Low level light therapy (LLLT) has been controversial in the literature in regards to treating different types of cancer. LLLT induced cell death in a human colon carcinoma cell line (HCT 116) by increasing intracellular reactive oxygen species (ROS) concentrations, which were induced by mitochondrial damage (Saenko et al 2016). The photons in LLLT absorb cytochrome-C oxidase, which is part of the mitochondrial chain. This reaction leads to increased electron transport, respiration, oxygen consumption, and ATP production (Tanupriya et al 2014). Studies of LLLT by Kreisler et al (2002 and 2003) showed enhanced cell proliferation, including on human laryngeal carcinoma in vitro, from 1.96-7.84 J/cm² with LLLT of wavelength 809 nm. This is in contrast to some of the results of this study, where LLLT did not consistently show significant increase in overall protein levels, although in one set of cells from one duplicate experiment at 48 hours, the protein assay did show a significant increase in protein levels, which aligned with the studies by Kreisler et al. Pinheiro et al (2002) reported that LLLT with 635 nm wavelength did not significantly stimulate laryngeal carcinoma cells, whereas, the LLLT with a wavelength of 670 nm wavelength significantly stimulated these cancer cells compared to the control, with every irradiation being 0.04-0.48J/cm². Other researchers, such as Werneck et al (2005) who used LLLT with wavelengths of 685 nm and 830 nm significantly increased the proliferation of laryngeal carcinoma cells. Notably, the results of Renno et al (2007), reported an increase in normal osteoblast cells and reduction in osteosarcoma cells after treating with LLLT at 830 nm and a total energy impact of 10 J/cm². In our present study, we impacted a total energy of 10 J, which is equivalent to 1.05 J/cm² into each well using LLLT of 830 nm wavelength. The conflicting results one
may observe in the literature, and between previous report and the present study, may be due to time of exposure, treatment and wavelength or parameters used.

In the present study, we wanted to combine LLLT with the treatments, TQ, EGCG, and 5-FU, in order to possibly find a combination that would be most effective. Most combinations of the drug treatments with LLLT did not highlight a therapeutic benefit. However, TQ + laser showed significant decrease in protein levels from one duplicate experiment at 48 and 72 hours, but these results may not be enough to make any conclusive analysis about TQ + laser. Notably, 5-FU + laser significantly increased LDH levels, which is not seen in 5-FU alone, which could possibly mean that LLLT aids in cellular damage and toxicity. LLLT combined with EGCG did show a significant difference showing similar results with the treatment of EGCG alone, but the combination did not prove to be more effective than the treatments alone. In conclusion, our results consistently demonstrate reduction in hypopharyngeal tumor cells following treatment EGCG alone and when combined with laser therapy, but that there was no overall additive effect. TQ and 5-FU results were inconsistent, and this study requires further investigation in order to clarify consistent findings and further treatments to test in-vivo.

Currently, researchers are trying to find out if certain drugs, vitamins, or other supplements can help prevent precancerous lesions from developing into laryngeal and hypopharyngeal carcinomas. Surgery techniques are also being refined in order to effectively take out laryngeal and hypopharyngeal tumors. More chemotherapeutic drugs are also being tested for these types of cancer (American Cancer Society, 2016). In this present study, there were many inconsistent findings that were contrary to previous
studies. Although treatment with EGCG seems to be the most effective treatment compared to the others, there could be possible combinations of these drugs that could prove to be even more effective. For future studies, it would be plausible to investigate the effects of EGCG in combination with 5-FU and TQ, and also with different wavelengths of LLLT other than 830 nm.
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