

# Lemongrass Extract Possesses Potent Anticancer Activity Against Human Colon Cancers, Inhibits Tumorigenesis, Enhances Efficacy of FOLFOX, and Reduces Its Adverse Effects

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## Abstract

Current chemotherapeutics for metastatic colorectal cancers have limited success and are extremely toxic due to nonselective targeting. Some natural extracts have been traditionally taken and have shown anticancer activity. These extracts have multiple phytochemicals that can target different pathways selectively in cancer cells. We have shown previously that lemongrass (*Cymbopogon citratus*) extract is effective at inducing cell death in human lymphomas. However, the efficacy of lemongrass extract on human colorectal cancer has not been investigated. Furthermore, its interactions with current chemotherapies for colon cancer is unknown. In this article, we report the anticancer effects of ethanolic lemongrass extract in colorectal cancer models, and importantly, its interactions with FOLFOX and Taxol. Lemongrass extract induced apoptosis in colon cancer cells in a time and dose-dependent manner without harming healthy cells in vitro. Oral administration of lemongrass extract was well tolerated and effective at inhibiting colon cancer xenograft growth in mice. It enhanced the anticancer efficacy of FOLFOX and, interestingly, inhibited FOLFOX-related weight loss in animals given the combination treatment. Furthermore, feeding lemongrass extract to APC<sup>min/+</sup> transgenic mice led to the reduction of intestinal tumors, indicating its preventative potential. Therefore, this natural extract has potential to be developed as a supplemental treatment for colorectal cancer.

## Keywords

lemongrass extract, colorectal cancer, natural health products, FOLFOX, Taxol, NHP drug interaction, cancer prevention

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## Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in Canada, comprising 13% of all cases in 2018. It is estimated that 1 in 13 men and 1 in 16 women in Canada will develop CRC during their lifetime, and of those, 28% and 19%, respectively, are expected to die.<sup>1</sup> Great progress has been made in early detection techniques that have increased the prognosis of diagnosed patients.<sup>2–4</sup> If detected early, CRC-related fatality risk can be minimized by surgical resection. However, advanced-stage metastatic CRCs are very difficult to treat, and there are limited options of potentially toxic chemotherapeutic treatments.<sup>5</sup> They often target characteristics that are not unique to cancerous

cells such as DNA damage/replication and tubulin, thus affecting healthy cells and resulting in serious side effects to patients.<sup>6</sup>

The most common chemotherapy treatment for CRC is a combination of folinic acid (leucovorin), 5-fluorouracil, and oxaliplatin, otherwise collectively known as FOLFOX. Although there is potential short-term success, this

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treatment has severe side effects including gastrointestinal and neurotoxicities, and cannot be used as a long-term treatment option.<sup>7</sup> As a result, there is an urgent need to develop treatments that can selectively target cancer cells and induce cell death.

Natural health products (NHPs) have been used traditionally for their medicinal properties and widespread applications. Although their usage has been recorded over thousands of years, they often lack scientific credibility and experimental validation and are thus overlooked as a valid treatment or supplemental option. However, many currently used anticancer compounds are derived from or are chemically analogous to compounds found in plants.<sup>8,9</sup> Due to their relatively inexpensive cost and abundance, researchers are now shifting their attention to developing NHPs as well-tolerated and effective treatments for cancer.

Health care professionals are often hesitant to allow patients undergoing cancer treatments to take NHPs because of their unknown interactions with chemotherapies. It has been reported that increases in antioxidant effects of many NHPs may interfere with chemotherapeutic efficacy to induce apoptosis in cancer cells.<sup>10</sup> However, previous studies have shown that NHPs with potent anticancer effects have shown pro-oxidant activity selectively in cancer cells.<sup>11-14</sup> It is important to scientifically evaluate the anticancer efficacy of NHPs on their own, their interactions with chemotherapeutic drugs, and mechanisms of cell death.

Recent reports of lemongrass (LG) extract's medicinal properties and anticancer activity, in addition to its extensive traditional use across many cultures, makes it an especially promising NHP to study as a potential treatment for colon cancer. We have previously investigated the ability of ethanolic LG to produce reactive oxygen species (ROS) and induce apoptosis in lymphoma and leukemia cell models.<sup>11</sup> However, the efficacy of LG has not been studied in human colon cancer cells. Phytochemical analyses of LG have identified various compounds including terpenes, alcohols, ketones, and aldehydes such as citral. Three unique compounds, namely, elemicin, methyl isoeugenol, and lonicerin, found in ethanolic LG were tested for their anticancer activity but showed poor induction of apoptosis at high doses compared with the extract as a whole.<sup>11</sup> These findings indicate that the anticancer efficacy of LG may be attributed to its many constituents working together, rather than one or several active compounds leading to the induction of apoptosis.

Since LG can be taken over a long period of time, it has the potential to prevent the relapse of cancer, which has yet to be investigated. In order to study the preventive effect of NHPs, one can use transgenic mice susceptible to developing cancers. APC<sup>min/+</sup> mice are commercially available transgenic mice with a knockdown of the APC tumor suppressor gene, resulting in the development of intestinal tumors within 3 months of age.<sup>15</sup> These mice can be used as

a model for evaluating the ability of CRC treatments to prevent the development of cancer.

In this report, we demonstrate the ability of LG to induce apoptosis in aggressive human CRC (both p53 positive and negative) cell lines. Importantly, oral administration of LG inhibited growth of human CRC xenografts in immunocompromised mice. We showed that LG did not affect the efficacy of FOLFOX *in vitro*. However, *in vivo*, LG enhanced the efficacy of FOLFOX in reducing tumor growth. FOLFOX treatment on its own, although effective, led to weight loss in animals. Interestingly, when given in combination with LG, FOLFOX-related weight loss was inhibited. Furthermore, oral administration of LG on APC<sup>min/+</sup> mice significantly inhibited the occurrence of intestinal tumors, indicating the preventive efficacy of LG. Dandelion root, which has previously been investigated,<sup>12</sup> was included as a comparison for the LG extract.

## Materials and Methods

### Preparation of Herbal Extracts

Lemongrass (*Cymbopogon citratus*) was obtained from Premier Herbal Inc (Toronto, Canada). The total aerial part of dried LG was ground using a coffee grinder into a fine powder. The powder was extracted in 100% anhydrous ethanol (1 g leaf powder to 10 mL anhydrous ethanol) at room temperature overnight. The extract was filtered via gravity filtration with a P8 coarse filter, followed by vacuum filtration with a 0.45- $\mu$ m filter. The extract was evaporated using a RotoVap at 40°C and reconstituted in ethanol to obtain a final stock concentration of 200 mg/mL. The ethanolic extract was then passed through an Acrodisc 0.2- $\mu$ m dimethyl sulfoxide (DMSO)-safe syringe filter in a biosafety cabinet. Water extract of dandelion root was prepared as described previously.<sup>12</sup> Briefly, dried dandelion root (Premier Herbal Inc; Lot No. 319408SRP) was immersed in liquid nitrogen for about 5 to 10 minutes, until thoroughly frozen. The frozen pieces were ground up in an impingement grinder to an average particle size of  $\leq 45$   $\mu$ m. Following grinding, dandelion root powder was extracted in boiling water on low heat for 3 hours. The total extracted material was filtered through a NITEX nylon mesh filter (LAB PAK; Sefar BDH Inc, Chicoutimi, Canada), and the filtrate was spun down at 800  $\times$  g for 5 minutes at room temperature. The supernatant was filtered through a 0.45- $\mu$ m filter, followed by lyophilization. The dried extracted material was reconstituted in water to give a final stock solution of 100 mg/mL and then passed through a 0.22- $\mu$ m filter, in a biological safety cabinet and stored at 4°C.

### Cell Culture

The colon cancer cell line HT-29 (ATCC HTB-38) was cultured in McCoy's 5A Medium (ATCC 30-2007) supplemented

with 10% (v/v) fetal bovine serum (FBS; Thermo Scientific, Waltham, MA; Cat. No. 12484-020) and 0.4% (v/v) gentamicin (Gibco BRL; VWR, Mississauga, Canada; Cat. No. 15710-064).

The colon cancer cell line HCT-116 (ATCC CCL-247) was cultured in McCoy's 5A Medium supplemented with 10% (v/v) FBS and 0.4% (v/v) gentamicin.

The normal colon mucosa cell line (ATCC CRL-1831) was cultured in Dulbecco's Modified Eagle's Medium (ATCC 30-2002) supplemented with 10% (v/v) FBS and 0.4% (v/v) gentamicin.

The normal colon mucosa cell line NCM-460 (ATCC CRL-1831) was cultured in Dulbecco's Modified Eagle's Medium (ATCC 30-2002) supplemented with 10% (v/v) FBS and 0.4% (v/v) gentamicin.

All cells were maintained in an incubator at 37°C with 5% CO<sub>2</sub> and 95% humidity. All cells were cultured for less than 6 months with regular passaging.

### **Analysis of Cell Death: Annexin V Binding Assay and Propidium Iodide**

Annexin V (AV) binding assay and propidium iodide (PI) staining were performed to, respectively, monitor early apoptosis and cell permeabilization, a marker of necrotic or late apoptotic cell death using previously published standard procedure.<sup>11-14</sup> Briefly, colon cancer cells were grown to 50% confluency and treated individually or in combination with chemotherapeutics FOLFOX and Taxol as indicated in the Results section. Cells were washed with phosphate-buffered saline (PBS) and suspended in AV binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>, pH 7.4) with green fluorescent AV AlexaFluor-488 (1:20; Life Technologies Inc, Burlington, Canada; Cat. No. A13201) and 0.01 mg/mL of red fluorescent PI (Life Technologies Inc; Cat. No. P3566) for 15 minutes at 37°C protected from light. Percentage of early (green), late apoptotic cells (green and red), and necrotic cells (red) were quantified with a Tali Image-Based Cytometer (Life Technologies Inc; Cat. No. T10796). Cells from at least 18 random fields were analyzed using both the green (excitation [ex] = 458 nm; emission [em] = 525/20 nm) and red (ex 530 nm; em 585 nm) channels. Fluorescent micrographs were taken at 400× magnification using LAS AF6000 software with a Leica DMI6000 fluorescent microscope (Wetzlar, Germany). Cells monitored with microscopy were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR) with a final concentration of 10 μM during the 15-minute incubation.

### **Reactive Oxygen Species Quantification**

Whole cell ROS generation was monitored with the small molecule 2',7'-dichlorofluorescein diacetate (H<sub>2</sub>DCFDA)

as described before.<sup>14</sup> H<sub>2</sub>DCFDA enters the cell and is deacetylated by esterases and oxidized by ROS to the highly fluorescent 2',7'-dichlorofluorescein (DCF; ex = 495 nm; em = 529 nm). Cells were pretreated with 20-μM H<sub>2</sub>DCFDA (Sigma-Aldrich, Mississauga, Canada; Cat. No. D6883) for 30 minutes at 37°C protected from light at 5% CO<sub>2</sub>. Cells were treated for the indicated durations, collected, centrifuged at 3500 × g for 5 minutes, and resuspended in PBS. Percentage of DCF-positive cells was quantified using the Tali Image-Based Cytometer (Life Technologies Inc; Cat. No. T10796) using 13 random fields per group with the green channel (ex = 458 nm; em = 525/20 nm). Cells were monitored with microscopy and counterstained with Hoechst 33342. Images were taken with a Leica DMI6000 fluorescent microscope (Wetzlar, Germany) at 400× magnification using LAS AF6000 software.

### **Mitochondrial Potential Monitoring**

Tetramethylrhodamine methyl ester (TMRM; Gibco BRL; VWR; Cat. No. 89139-392) was used for detecting mitochondrial membrane potential (MMP), an indicator of healthy intact mitochondria, as described before.<sup>16</sup> Following incubation with TMRM, cells were collected, washed with 1 × PBS, resuspended in PBS, and then analyzed using the Tali Image-Based Cytometer (Life Technologies Inc; Cat. No. T10796). Cells from 13 random fields were analyzed using the red (ex = 530 nm; em = 585 nm) channel. Cells were monitored with microscopy and counterstained with Hoechst 33342. Images were taken with a Leica DMI6000 fluorescent microscope (Wetzlar, Germany) at 400× magnification using LAS AF6000 software.

### **In Vivo Assessment of LG Extract Efficacy and Combinations With FOLFOX**

Immunocompromised CD1 nu/nu mice, aged 6 weeks, were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed, and the protocols were followed using relevant guidelines and regulations that were approved by the University of Windsor Animal Care Committee (AUPP #17-15) in accordance with the Canadian Animal Care committee in a laboratory setting with 12-hour light/dark cycles. Following an acclimatization period, mice were injected subcutaneously with colon cancer cells (HT-29, HCT-116) cell lines suspension in Matrigel at a concentration of 1.0 × 10<sup>6</sup> cells per mouse in the hind flanks. On tumor formation, mice were randomly separated into 4 groups (control [n = 3], LG drinking water [n = 4], FOLFOX [n = 3] intraperitoneal injection, and FOLFOX intraperitoneal injection [n = 4]). Control and chemotherapeutic mice were given normal water, while LG treatment groups received water supplemented with 0.1 mg/mL LG (approximately 16 mg/kg/day) for 8 weeks. At 4 weeks,

intraperitoneal injections of FOLFOX (90 mg/kg folinic acid/50 mg/kg 5-fluorouracil/6 mg/kg oxaliplatin) commenced once per week for 4 weeks. Mice were then sacrificed using CO<sub>2</sub> chamber followed by cervical dislocation, and tumors were harvested. Tumor volumes (using the formula  $\frac{1}{2} \times [L \times W^2]$  to calculate approximate volume) and body weights of each mouse were measured throughout the length of the study once per week.

### ***In Vivo Assessment of LG Ability to Prevent Tumor Formation***

APC<sup>min/+</sup> mice from the Jackson Laboratory (Cat. #002020) were bred to obtain the APC<sup>min/+</sup> genotype. Mice were housed, and the protocols were followed using relevant guidelines and regulations that were approved by the University of Windsor Animal Care Committee (AUPP #17-15) in accordance with the Canadian Animal Care committee in a laboratory setting with 12-hour light/dark cycles. Control group were given plain drinking water (n = 5), LG group received water supplemented with 0.1 mg/mL LG extract in drinking water (n = 3), and DRE group received 0.2 mg/mL dandelion root extract (DRE) in their drinking water (n = 5). At approximately 3 months of age, mice were sacrificed using a CO<sub>2</sub> chamber followed by cervical dislocation, and the intestine was removed from the start of the duodenum to the end of the ileum. Intestinal segments were washed using PBS solution, fixed for 2 hours in 4% paraformaldehyde solution, rinsed, and placed in 30% sucrose solution at 4°C. All intestinal sections were opened similarly to standard protocol<sup>17,18</sup> and analyzed using a Zeiss 2000 stereoscope to count for adenomas and tumor occurrence.

### ***Statistical Analysis***

All statistical analysis was done using the GraphPad 6.0 Prism software. To test for statistical significance, a 2-way analysis of variance was used. All trials were conducted at least 3 independent times.

## **Results**

### ***Lemongrass Extract Induces Apoptosis in Colon Cancer Cells and Shows No Significant Cell Death in Normal Colon Mucosal Cells***

We assessed the ability of LG extract to induce apoptosis in 2 colon cancer cell models at 48-hour posttreatment. Cells were treated with varying doses of LG and assessed for apoptotic activity using AV binding, which detects the externalization of phosphatidylserine. PI was simultaneously used to detect necrotic cells given its ability to intercalate DNA to indicate cell permeabilization and death. Cells

positive for AV only (green) were considered to be early apoptotic cells, while necrotic cells were detected as positive for PI only (red). Those positive for both AV and PI reflected late apoptotic cells (orange). Finally, those negative for both AV and PI were considered viable cells (Figure 1).

At a 48-hour treatment time point, LG was able to induce apoptosis in both CRC model cell lines (Figure 1A). It displayed dose-dependent anticancer activity in the less aggressive HCT-116 cell line but was still able to significantly reduce cell viability in p53(-/-) HT-29 cell line. Staurosporine was used as a positive control for its ability to induce apoptosis.<sup>19</sup>

We further assessed the ability of LG to selectively kill cells using a normal colon mucosal cell line (NCM-460). Cells were treated and analyzed as described above (Figure 1B). Forty-eight hours after treatment, LG displayed no significant apoptotic activity in NCM-460 cells compared with control.

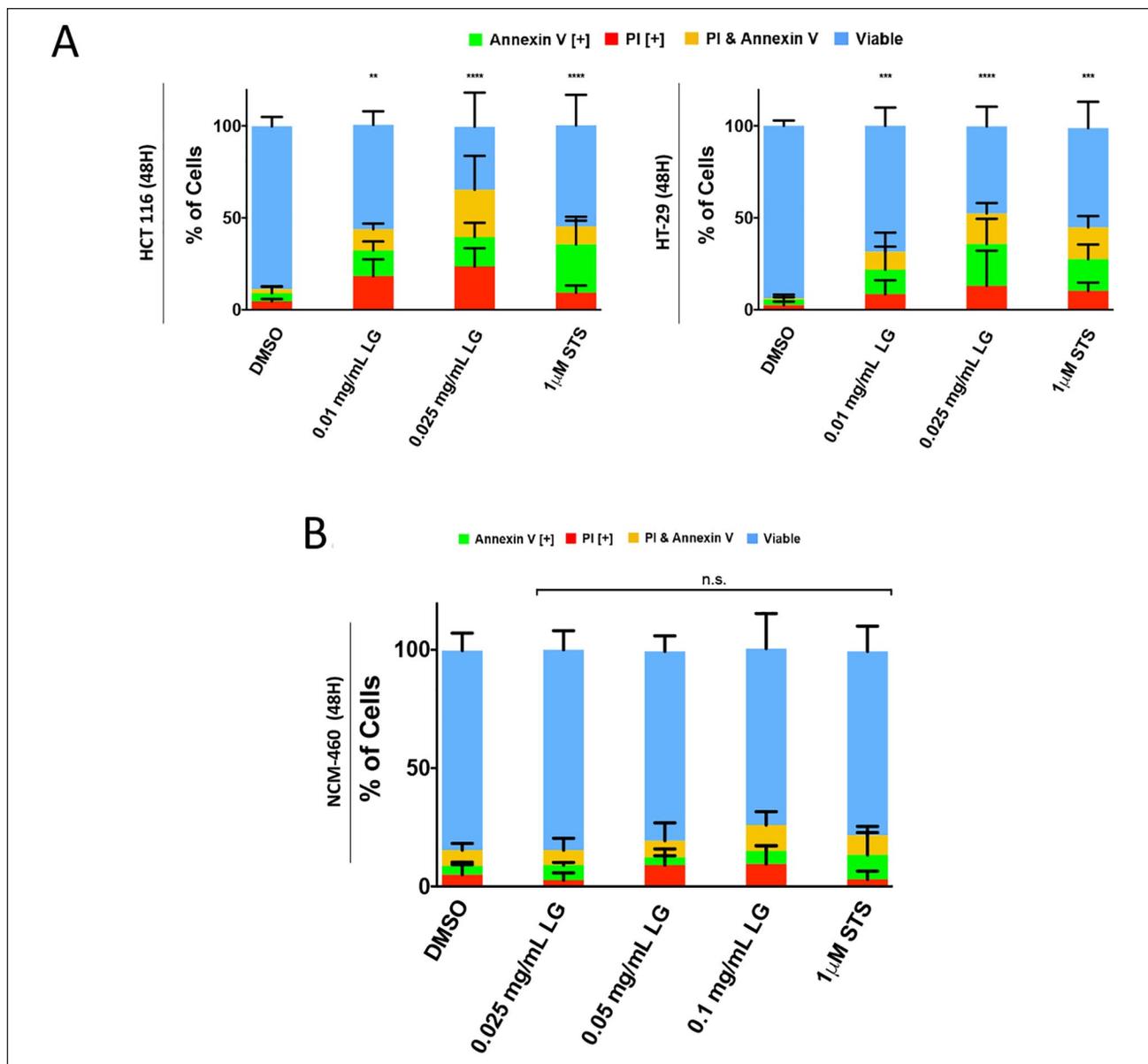
### ***Lemongrass Extract Does Not Inhibit Apoptosis Induced by FOLFOX and Taxol***

On its own, LG extract was able to induce apoptosis in colon cancer cell lines at very low doses. Next, we wanted to investigate whether treatment with LG in combination with synthetic chemotherapeutic drugs would have any effect on apoptosis of colon cancer cell lines.

To determine whether LG interacts with standard chemotherapeutic drugs to either enhance or reduce their efficacy, cells were treated with LG alone and in combination with either FOLFOX or Taxol and monitored for the induction of apoptosis using AV binding and PI staining after 48 and 96 hours with image-based cytometry (Figure 2). No significant changes were observed between the individual Taxol and combinatorial treatments involving LG extract. However, FOLFOX apoptotic induction was enhanced when used in combination with LG compared with FOLFOX treatment alone. In the more aggressive HT-29 cell line, a nonsignificant increase in cell apoptosis occurred when the treatments were used in combination.

### ***Changes in Morphology and Characterization of Apoptosis Following Treatment With LG Extract Alone and in Combination With Chemotherapeutics***

Morphological and qualitative analysis of HCT-116 and HT-29 cells was done to complement quantitative results of combination treatments. Cells were incubated with LG extract alone and in combination with standard chemotherapy drugs FOLFOX or Taxol for 48 hours then subjected to inverted fluorescent microscopy (Figure 3). Green (AV) and red (PI) staining were used as markers of apoptosis and were especially prominent in the treatment groups.



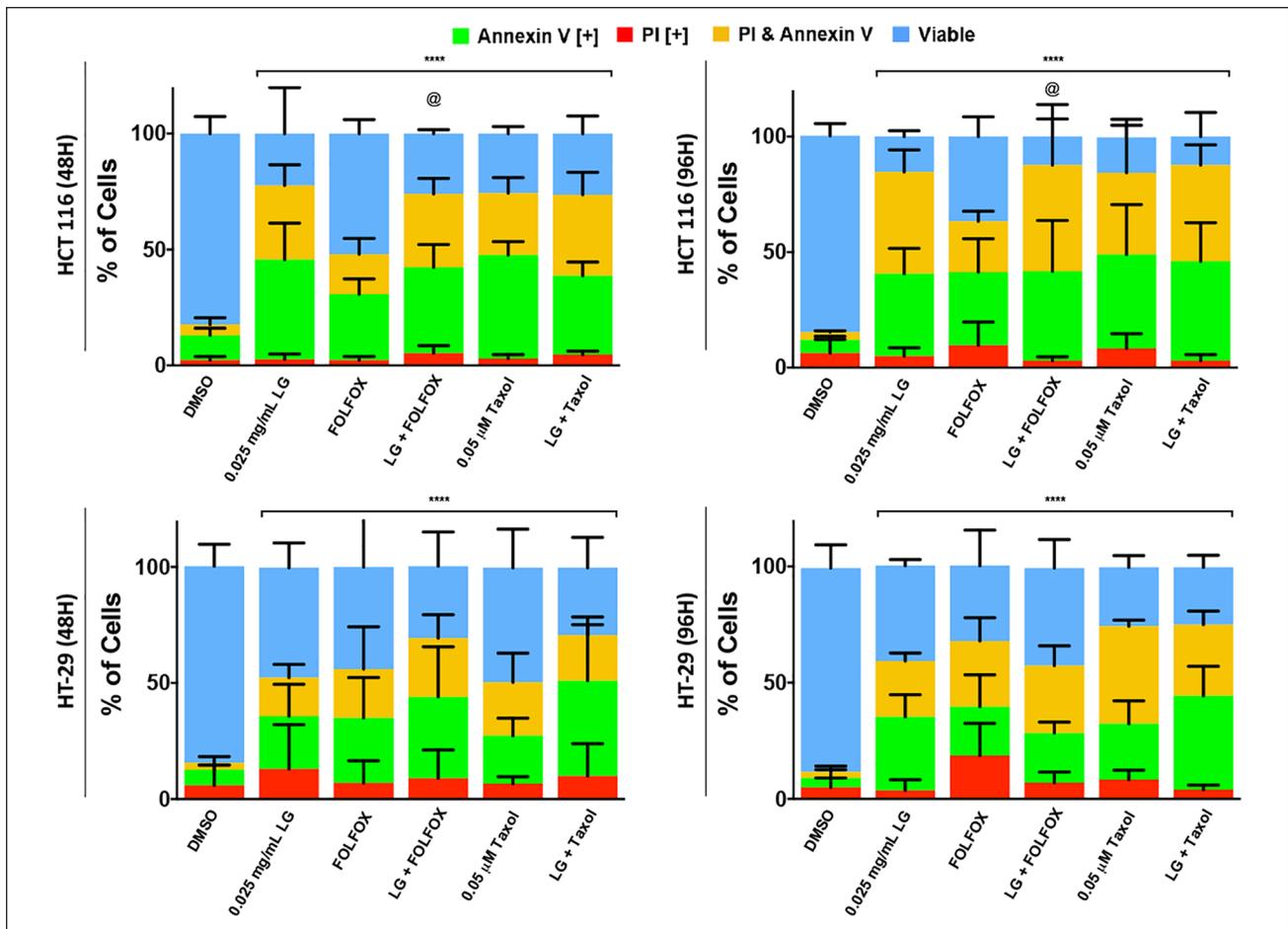
**Figure 1.** Lemongrass (LG) extract induces apoptosis in colorectal cancer cells but not healthy colon cells. (A) Colon cell lines HCT-116, HT-29, and (B) normal colon mucosa cell line NCM-460 were treated with various treatments of LG extract, and compared with a positive control of staurosporine (STS) at 48 hours. Results were obtained using image-based cytometry to assess the percentage of live cells compared with a vehicle control. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with annexin V (green), propidium iodide (PI, red), both (yellow), or negative for both annexin V and PI (blue). Values are expressed as mean  $\pm$  SD from 3 independent experiments. Statistical calculations were performed using 2-way analysis of variance multiple comparison. ns, not significant; \* $P < .05$  versus control; \*\* $P < .01$  versus control; \*\*\* $P < .001$  versus control.

Bright-field images also showed cell shrinkage and membrane blebbing as indicators of apoptosis.

### Lemongrass Extract Induces an Increase in ROS Production and Causes Depolarization of the Mitochondrial Membranes in CRC Cells

Lemongrass is an extract composed of many compounds that are able to interact in a complex manner. Determining

the method of apoptotic induction will allow for a greater understanding of how these complex extracts show the observed anticancer potency. In order to determine if LG is able to induce apoptosis in CRC through induction of oxidative stress,  $H_2DCFDA$  was used to monitor the generation of ROS in CRC cells treated with FOLFOX in the presence or absence of LG. Indeed, it was observed that LG treatment alone was able to induce significant ROS generation in treated cells (Figure 4A). The combination sample of



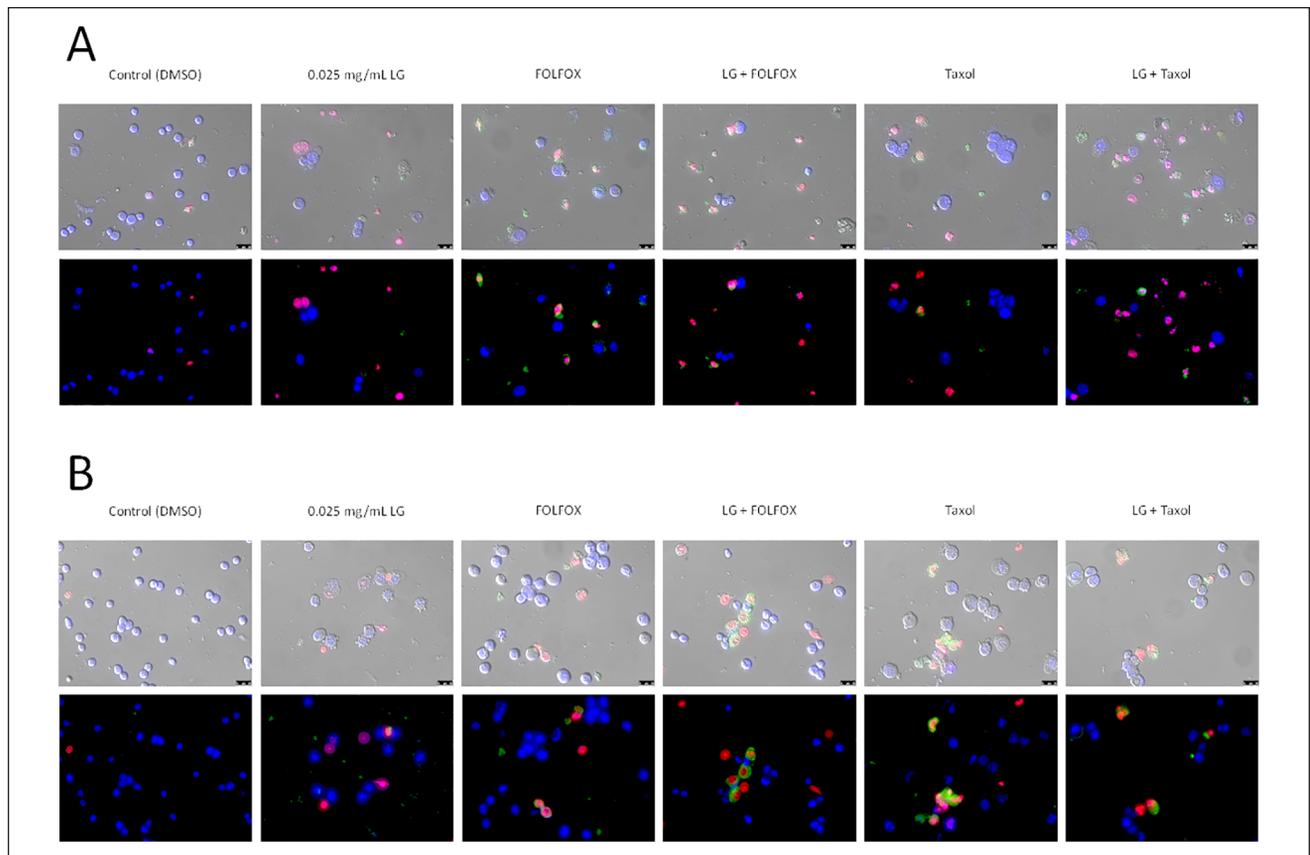
**Figure 2.** Lemongrass extracts do not inhibit chemotherapeutic efficacy when treated in combination on colorectal cancer cells. HCT-116 and HT-29 cells were treated with chemotherapeutics FOLFOX (10  $\mu$ M 5-fluorouracil/5  $\mu$ M folinic acid/1  $\mu$ M oxaliplatin) and Taxol (0.05  $\mu$ M) individually and in combination with 0.025 mg/mL lemongrass extract and assessed at 48 hours and 96 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with annexin V (green), PI (red), both (yellow), or negative for both annexin V and PI (blue). Values are expressed as mean  $\pm$  SD from 3 independent experiments. Statistical calculations were performed using 2-way analysis of variance multiple comparison. \* $P < .05$  versus control; \*\* $P < .01$  versus control; \*\*\* $P < .001$  versus control; @ $P < .05$  versus individual chemotherapy treatment.

FOLFOX and LG showed an increased capability to generate ROS compared with FOLFOX alone.

The mitochondria play a crucial role in apoptosis, and the collapse of MMP has been shown in cells undergoing apoptosis. The release of apoptogenic factors from the mitochondria will result in the induction of apoptosis. To monitor mitochondrial stability and depolarization, the fluorescent TMRM assay was used. TMRM molecules accumulate in mitochondria with an intact MMP. HCT-116 and HT-29 cells were treated with the respective treatment groups, incubated for 48 hours, and results were obtained using image-based cytometry. Interestingly, LG exhibited an even greater ability to disrupt MMP than the positive controls Taxol and FOLFOX, relative to the DMSO control (Figure 4B). These results indicate that LG was extremely effective in causing mitochondrial membrane collapse.

### Oral Administration of LG Extracts Reduces the Tumor Burden in Colon Cancer Xenograft Models in Immunocompromised Mice and Enhances the Efficacy of FOLFOX

Many anticancer agents that have shown to be effective in vitro have failed to show the same effect in an in vivo model due to poor bioavailability and stability. Therefore, it is crucial to assess the effect of LG in a more complex animal model. Human colon cancer HCT-116 and HT-29 cells were xenografted subcutaneously in immunocompromised mice. After tumor establishment, the mice were divided into 4 treatment groups as follows: a control group fed with regular drinking water, a LG treatment group fed with drinking water supplemented with LG, a FOLFOX group fed with regular water and injected intraperitoneally with a FOLFOX

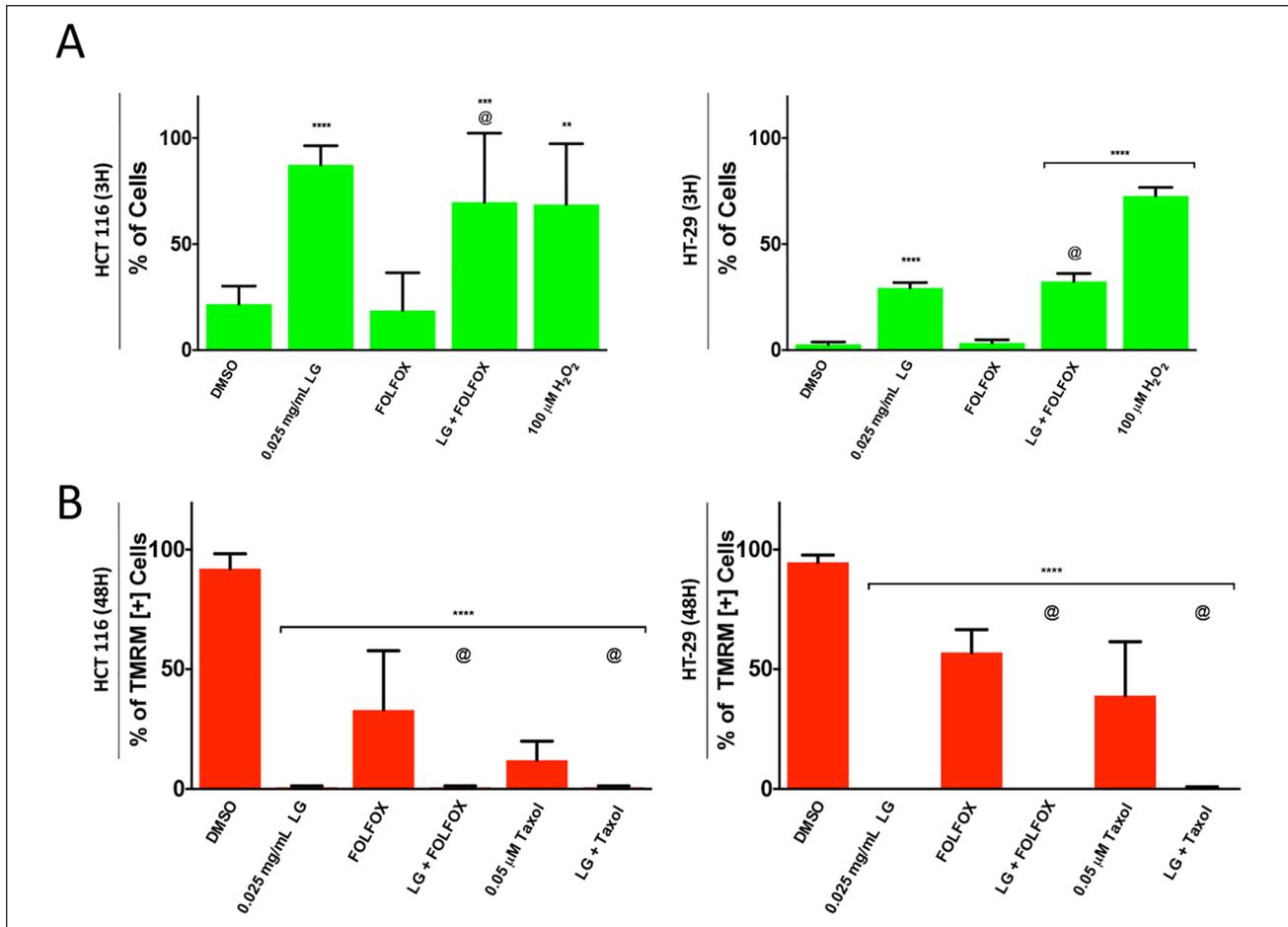


**Figure 3.** Lemongrass extracts induce a change in colorectal cell morphology. (A) HCT-116 and (B) HT-29 cells were treated with chemotherapeutics FOLFOX (10  $\mu$ M 5-fluorouracil/5  $\mu$ M folinic acid/1  $\mu$ M oxaliplatin) and Taxol (0.05  $\mu$ M) individually and in combination with 0.025 mg/mL lemongrass extract and assessed at 48 hours. Fluorescence microscopy images of individual hibiscus treatments. Top panels: Bright-field and fluorescent merged images at 400 $\times$  magnification. Bottom: Fluorescent images stained with annexin V (green), PI (red), and Hoechst (blue) at 400 $\times$  magnification. Scale bar is 50  $\mu$ m. Images are representative of 3 independent experiments.

preparation weekly, and a combinatorial (LG + FOLFOX) treatment group fed with LG-supplemented drinking water and injected with FOLFOX on a weekly basis. Indeed, LG was able to reduce the tumor burden of the xenografted mice as determined by tumor volume and weight compared with vehicle controls (Figure 5A and B). Interestingly, LG-supplemented FOLFOX groups showed the largest reduction in tumor burden compared with control, indicating that LG is able to enhance the effect of FOLFOX when taken orally (Figure 5A and B). After the second week of injections, animals in the FOLFOX treatment group began showing a decrease in weight gain relative to all other groups, indicating FOLFOX-related detrimental effects. However, when FOLFOX was administered in the mice drinking LG-supplemented water (LG + FOLFOX group), the weight gain was maintained similar to the control group (Figure 5C). This indicates that LG inhibited FOLFOX-related adverse effects in the mice.

### Oral Administration of LG Reduces Colorectal Tumor Formation in $APC^{min/+}$ Transgenic Mice

Due to extreme toxicity of chemotherapeutic drugs, they cannot be administered for extended periods. Thus, due to limited exposure to chemotherapy, there is a higher chance of relapse following the treatment period. The history of LG as a well-tolerated substance makes it safe for long-term usage.  $APC^{min/+}$  mice are susceptible to develop intestinal tumors and have been used as a model for tumorigenesis in the past. If LG has the ability to inhibit tumorigenesis, it can be easily tested in this model.  $APC^{min/+}$  mice were divided into 3 groups and were fed with water (control group), or water supplemented with LG (LG group) or DRE (DRE group) starting at the age of approximately 3 weeks (immediately after weaning) and continuing until the mouse reached 3 months of age. At 3 months, mice were sacrificed and tumors were counted as per the materials and methods.



**Figure 4.** Lemongrass extract induces oxidative stress and mitochondrial membrane dissipation on colorectal cancer and enhances FOLFOX effects. HCT-116 and HT-29 colorectal cancer cells were treated with FOLFOX (10  $\mu$ M 5-fluorouracil/5  $\mu$ M folic acid/1  $\mu$ M oxaliplatin) individually and in combination with 0.025 mg/mL lemongrass extract and assessed for (A) reactive oxygen species generation 3 hours posttreatment against a positive control of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and (B) mitochondrial membrane potential at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with the generation of reactive oxygen species (H<sub>2</sub>DCFDA, fluorescent green) and fluorescence associated with mitochondrial membrane potential (TMRM, fluorescent red). Values are expressed as mean  $\pm$  SD from 3 independent experiments. Statistical calculations were performed using 2-way analysis of variance multiple comparison. \* $P$  < .05 versus control; \*\* $P$  < .01 versus control; \*\*\* $P$  < .001 versus control; @ $P$  < .05 versus individual chemotherapy treatment.

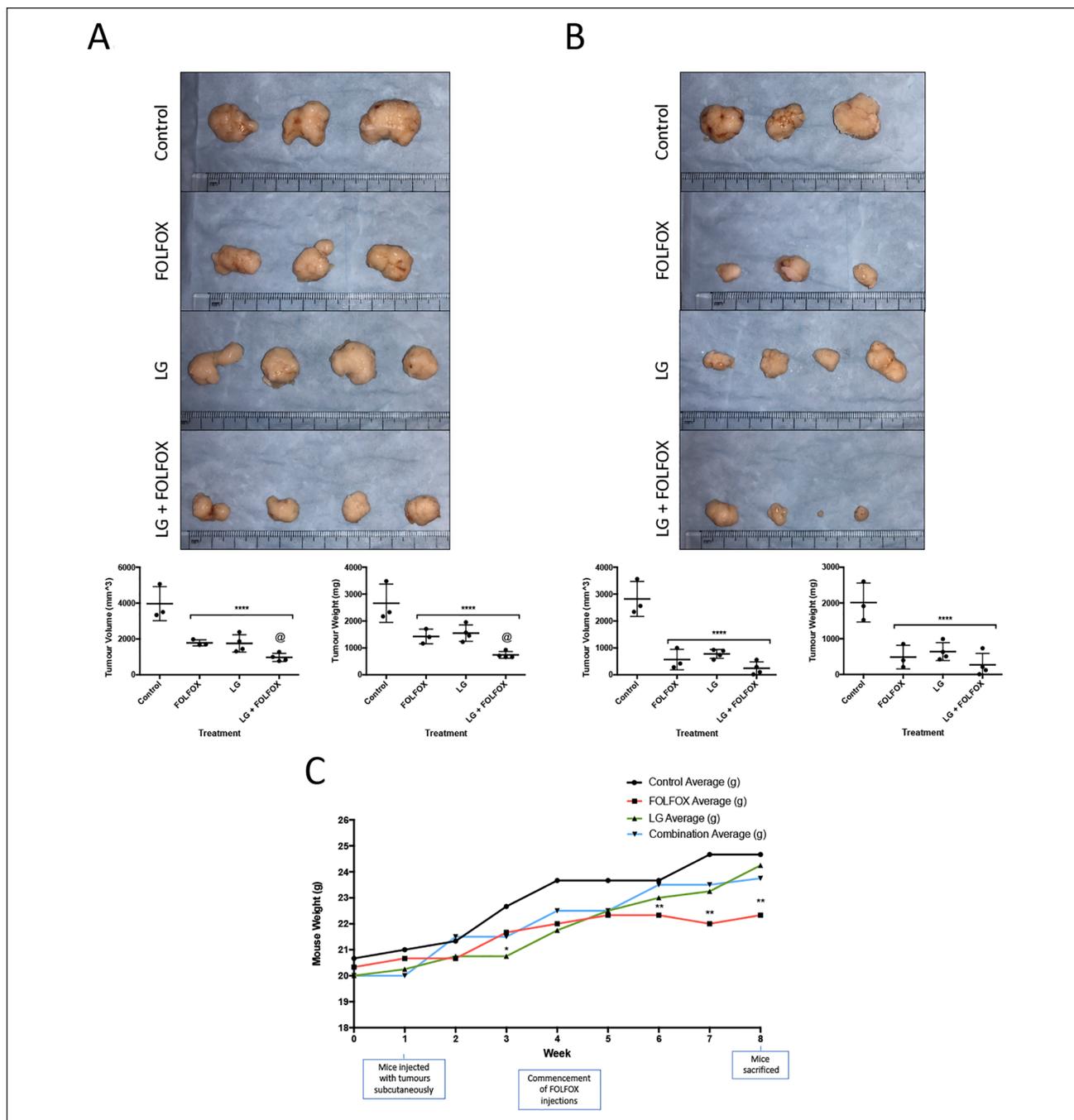
Indeed, the LG group showed a significant reduction in the occurrence of tumors compared with the control (Figure 6). DRE is another NHP extract that has been shown to be effective against CRC.<sup>12</sup> We also evaluated its preventative effects in APC<sup>min/+</sup> mice (DRE group). The results indicated that LG was more effective than DRE in reducing tumor occurrence.

## Discussion

In this report, we have demonstrated the efficacy of ethanolic LG as a potent anticancer agent against both p53-positive and p53-negative human colon cancer cells. We have shown that LG induces apoptosis selectively in cancer cells.

We observed that LG caused an increase in ROS production and depolarization of MMP in both cancer cell lines. Most important, when administered orally, the extract was able to inhibit the growth of human colon cancer xenografts in mice. Moreover, for the first time, we have shown that orally administered LG not only enhanced the efficacy of FOLFOX in inhibiting growth of colon cancer xenografts but also reduced the toxic effects of FOLFOX in mice. In addition to these important findings, we have also demonstrated that LG has the capability of preventing tumorigenesis in transgenic APC<sup>min/+</sup> mice that are susceptible to developing colon cancer.

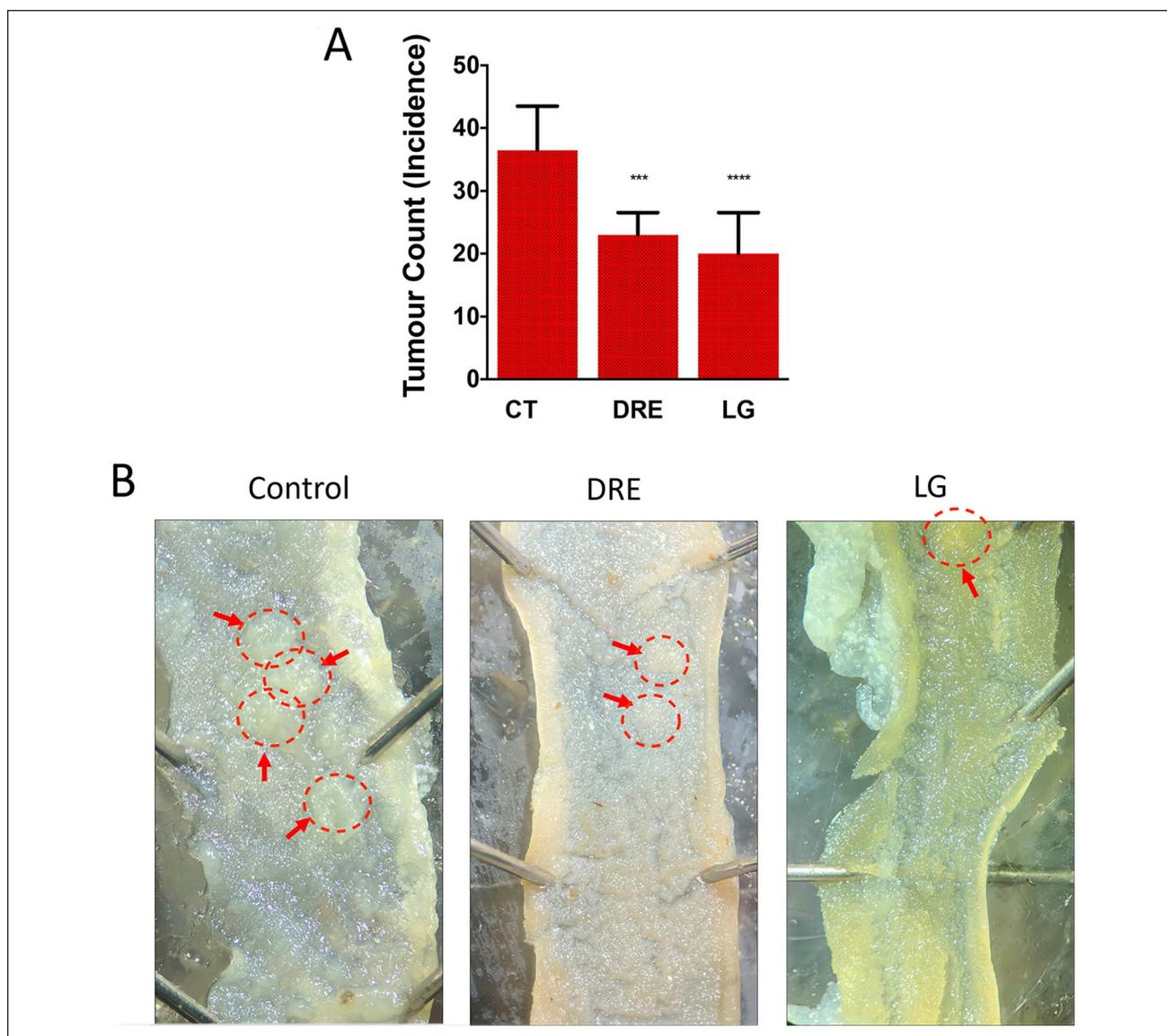
Since the discovery of apoptotic pathways, many efforts in cancer treatment development focus on targeting these



**Figure 5.** Lemongrass extract administered orally reduces the tumor burden on colorectal cancer xenografted mice and enhances FOLFOX efficacy. HCT-116 (A) and HT-29 (B) colorectal cancer cells were xenografted onto immunocompromised mice hind flanks subcutaneously. After tumor formation, these mice were orally administered lemongrass extract (16 mg/kg/day) for 8 weeks. After 4 weeks, FOLFOX (90 mg/kg folic acid/50 mg/kg 5-fluorouracil/6 mg/kg oxaliplatin) was administered intraperitoneal once a week. After mice were sacrificed, tumors were excised and measured for volume (using the formula  $\frac{1}{2} \times [L \times W^2]$  to calculate approximate volume). (C) Mouse body mass was measured once a week and averaged to compare between experimental groups.

pathways to induce apoptosis in cancer cells. Many therapeutics developed thus far have been effective in inducing apoptosis but not in a selective manner. LG was previously shown to be effective in inducing apoptosis in lymphomas.<sup>11</sup> Malignant colon cancers are difficult to treat and leave

patients with a poor prognosis. We evaluated the efficacy of LG on CRC. Our results indicate that in the HCT-116 and HT-29 cell lines, apoptosis and necrosis induction were observed at doses as low as 0.01 mg/mL LG in a dose-dependent manner. It is important to note that LG was more



**Figure 6.** Lemongrass extract prevents the formation of tumors in transgenic mice.  $APC^{min/+}$  mice were orally administered dandelion root extract (DRE; 40 mg/kg/day) and lemongrass (LG) extract (16 mg/kg/day) for a period of 3 months. Mice were sacrificed, and colon samples were obtained. (A) Tumor incidence in the colon of each mouse was counted, and (B) images were obtained digitally through a stereoscope. Red arrows indicate tumor incidence. Values are expressed as mean  $\pm$  SD from 3 different mice. Statistical calculations were performed using 2-way analysis of variance multiple comparison. \*\*\* $P < .005$  versus control; \*\*\*\* $P < .001$  versus control.

effective than FOLFOX, a cocktail of the most potent CRC chemotherapeutics (Figure 2). Additionally, its effects were comparable to those of positive control 1- $\mu$ M staurosporine, an agent known to induce apoptosis at low doses (Figure 1A). Agents that can trigger apoptosis in cancer cells must be tested in normal noncancerous cells for whether the treatment is selective to cancer and able to avoid having detrimental effects on healthy cells. We demonstrated that LG did not cause significant apoptotic induction in normal colorectal mucosal cells (NCM-460) even at higher doses

than those lethal to cancer cells. These cells are noncancerous proliferating colon mucosal cells that provide a reasonable normal healthy counterpart of colon cancer cells. The selectivity of LG as demonstrated by these results supports previous findings, wherein the same extract did not cause a decrease in cell viability of normal human fibroblast cells and peripheral blood mononuclear cells.<sup>11</sup> Therefore, these results indicate that LG extract induces apoptosis selectively in CRC cells with no significant effect on normal healthy cells.

It is unlikely that NHPs will eventually be prescribed and used as monotherapies in cancer therapy. Currently, cancer treatment strategies rely on the use of multiple therapeutics to target multiple vulnerabilities of cancer cells.<sup>20</sup> As mentioned, oncologists are hesitant to promote the use of NHPs alongside chemotherapeutics due to the risk of negative interactions. Thus, it is critical to examine whether LG, if taken as an adjuvant to chemotherapeutic drugs, will enhance or inhibit the anticancer effects of these drugs. FOLFOX and Taxol are the most common chemotherapeutic regimens used for treatment of aggressive colon cancers. When colon cancer cells were treated with FOLFOX and Taxol alone or in combination with LG, we did not observe any negative interaction. If at all, in HCT-116 cells, LG in combination with FOLFOX resulted in statistically significant enhancement of anticancer efficacy. Both cell lines showed increased cell death at the 96-hour time point, indicating that LG may act in a time-dependent manner (Figure 2). These results were further confirmed by morphological changes such as membrane blebbing and cell shrinkage in addition to AV and PI staining in treatment groups (Figure 3). In general, combination treatments involving LG with FOLFOX and Taxol did not indicate negative interactions. Although enhancement of FOLFOX anticancer activity was only observed in HCT-116 cells but not observed with Taxol, this may be attributed to the high potency of apoptotic induction by LG. Nonetheless, it is most important to note that LG did not inhibit the abilities of FOLFOX and Taxol to induce apoptosis.

The induction of apoptosis by LG in CRC may involve a complex cascade of events due to the many compounds present in the extract. The induction of apoptosis by LG may be dependent on its ability to induce oxidative stress. Indeed, LG treatment led to a significant increase in the generation of ROS in both CRC cell lines and enhanced the ability of FOLFOX to induce oxidative stress (Figure 4A). However, the difference in ROS generation could be primarily due to the action of LG, indicating the need for further analysis to conclusively analyze the true cause of this enhancement. Oxidative stress may lead to the destabilization of the mitochondria, leading to leakage of proapoptotic factors like cytochrome c and apoptosis-inducing factor, causing caspase-dependent and caspase-independent apoptosis.<sup>21</sup> Indeed, we did observe mitochondrial membrane collapse in HCT-116 and HT-29 cells following treatments with LG alone and in combination with FOLFOX and Taxol. LG treatments alone led to complete depolarization on the MMP, and the addition of FOLFOX and Taxol did not affect its ability to destabilize cancer cell mitochondria (Figure 4B). This kind of targeting could be selective to cancer cells due to metabolic and oxidative vulnerabilities of cancer cells.<sup>22-25</sup> There was an increase in production of ROS in cancer cells treated with LG. Wu et al have shown that high doses of substances known to have antioxidant

property tend to have pro-oxidant activities.<sup>26</sup> In order to further investigate the apoptotic pathways LG may utilize to induce apoptosis, we conducted experiments to see whether inhibition of certain pathways would reduce cell death with the respective treatments. Interestingly, the addition of n-acetyl cysteine (NAC; an antioxidant agent) and Z-VAD-FMK (an irreversible pan-caspase inhibitor) did not inhibit apoptosis in LG-treated cells (data not shown). Blocking ROS by NAC did not result in a decrease of apoptosis induction, indicating that compounds present in LG might directly target mitochondria. Furthermore, treatment with Z-VAD-FMK also did not inhibit apoptotic induction, indicating that caspase-independent apoptosis through factors such as apoptosis-inducing factor could be accomplished by LG treatment. The mechanistic studies with complex mixture of compounds like LG are difficult, and it is hard to pin point what exact target is being engaged. Although we find increase in oxidative stress by LG in cancer cells, quenching ROS by NAC could not inhibit apoptosis of cancer cells. There might be multiple pathways including mitochondrial destabilization that could be playing critical roles in apoptosis.

It is important to investigate the anticancer efficacy of LG in a more complex *in vivo* mouse model. When immunocompromised mice xenografted with human CRC cells were treated with LG (orally) or with FOLFOX (intraperitoneal injections), there was inhibition of tumor growth. Interestingly, LG was able to reduce the tumor burden on all mice at a comparable level to FOLFOX injections (Figure 5A and B). One of the most important observations was that there was an even greater reduction of tumor growth in combination treatments (LG + FOLFOX) when compared with LG or FOLFOX treatments alone. This may indicate that multiple components of LG or their metabolite must be absorbed and transported to the tumor sites in order to inhibit tumor growth, suggesting good bioavailability and stability of the compound or their metabolites in physiological conditions. Furthermore, these compounds clearly synergize with FOLFOX to reduce growth of xenografted tumors. In addition, LG treatment groups showed similar weight gain profiles with control mice, indicating that the treatment was generally well tolerated (Figure 5C). However, FOLFOX-injected mice stopped gaining weight after the second injection, indicating that mice suffered adverse effects of FOLFOX treatments such as loss of appetite. However, oral administration of LG was able to mitigate this effect in FOLFOX-injected mice, as shown by similar weight gain profiles of these mice compared with the control group. These results indicate that the compounds in the LG were absorbed and bioactive in physiological conditions, leading to inhibition of tumor growth of the subcutaneous tumor xenografts. Most important, LG is not only well tolerated by animals but also able to mitigate the toxic effects caused by FOLFOX treatments. There should

be detailed analysis of toxicology using histopathological parameters to confirm these results in future.

We further assessed the efficacy of LG against CRC to determine if it is able to prevent tumorigenesis. If effective, LG might contribute to the long-term maintenance of patients in remission to prevent the relapse of CRC. Interestingly, oral administration of LG in APC<sup>min/+</sup> mice was able to significantly reduce the occurrence of intestinal tumors when compared with mice fed with normal drinking water (Figure 6). For comparative purposes, we used another NHP, DRE, that has been shown to be effective against CRC in our preventative study.<sup>12</sup> DRE also demonstrated reduction in tumor occurrence in APC<sup>min/+</sup> mice. However, the preventative efficacy of LG was slightly better compared with DRE. This indicates that LG is a potent therapeutic that has not only been shown to reduce growth of CRC xenografts but is also able to prevent tumorigenesis in transgenic mice susceptible to developing CRC. Therefore, LG has potential activities as both a cancer therapeutic agent as well as a preventative agent for cancer relapse. The observation that the treatment groups still have tumor occurrences, although lower compared with control, can be explained by the fact that the LG treatment was administered only after 3 weeks of age. In this period, it is possible that some tumors would have already developed and that LG halted the formation of new tumors. Thus, these results indicate that LG is a safe and potent anticancer agent that can be given alongside FOLFOX and enhance its efficacy. Interestingly, it has the potential to be taken as a long-term supplement to prevent relapse of cancer.

## Conclusion

Lemongrass extract shows great potential as an anticancer agent in CRC models. When used alone or in combination with conventional chemotherapeutics, low dosages of LG selectively induce apoptosis in CRC and do not inhibit cytotoxic effects of the other drugs. LG was able to enhance the ability to generate oxidative stress and dissipate MMP when used in combination with FOLFOX and Taxol; however, these results require further testing to identify whether or not this happens in healthy cell lines. In xenografted mice models of colon cancer, LG was able to significantly reduce the tumor burden in mice, enhance the efficacy of FOLFOX, and reduce drug-related side effects. Thus, these results indicate that LG should be investigated further for its potential contributions to cancer treatment, prevention, and prevention of relapse.

## Authors' Note

This work is dedicated to the memory of Robin Palmer, a loving husband, father, poppa, teacher, mentor, and friend, who is greatly missed by many. May his memory continue to make a difference, continue to enthuse, and to inspire, as did his life.

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## Author Contributions

IR, CN, and SP were heavily involved in the design, execution, analysis, and manuscript preparation of this project. BS was involved in the execution and analysis of this project. CV helped in the experimental protocols including cell culture, image-based cytometry, and fluorescent imaging. OZ and KB helped in experimental protocols including cell culture, image-based cytometry, and mouse studies. AM helped in cell culturing and image-based cytometry. MN helped direct and facilitate transgenic mouse sacrificing and colon harvesting. SP is the principal investigator of this project.

## Declaration of Conflicting Interests

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