COVER SHEET

TITLE: Steam distilled extract of ginger is a potent mediator of apoptosis in endometrial cancer cells via the modulation of the p53 and Bcl-2 pathways

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ABSTRACT Form

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Development for new therapeutic and preventative agents in the treatment of gynecological cancers is in constant need. In the current study, we demonstrate that the steam distilled extracts of ginger are potent mediators of apoptosis in endometrial cancer cells. The treatment of endometrial cancer cells with the steam distilled extract of ginger results in significant decrease in the mitochondrial membrane potential, increase in the expression of cleaved caspase-3, phosphorylation of P53, and a significant decrease in the expression of Bcl-2. These results exhibit the potential of ginger extract to be used in the treatment and/or prevention of endometrial cancer.

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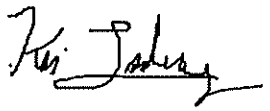

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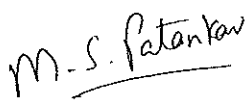

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Steam distilled extract of ginger is a potent mediator of apoptosis in endometrial cancer cells via the modulation of the p53 and Bcl-2 pathways

By

Kai Ludwig

under the direction of

Dr. Manish Patankar in Dept. of Obstetrics and Gynecology

Senior Honors Thesis

May 1st, 2012

I have supervised this work, read this thesis and certify that it has my approval.

5/1/12 — Date

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Abstract

Development for new therapeutic and preventative agents in the treatment of gynecological cancers is in constant need. In the current study, we demonstrate that the steam distilled extracts of ginger are potent mediators of apoptosis in endometrial cancer cells. The treatment of endometrial cancer cells with the steam distilled extract of ginger results in significant decrease in the mitochondrial membrane potential, increase in the expression of cleaved caspase-3, phosphorylation of P53, and a significant decrease in the expression of Bcl-2. These results exhibit the potential of ginger extract to be used in the treatment and/or prevention of endometrial cancer

Introduction

Endometrial cancer, also known as uterine cancer, consists of several malignancies in the endometrium of the uterine. The estimated diagnosis rate of 2012 in the US is 47,130 new cases while the estimated mortality rate is 8,010 deaths.[1] Five-year survival rates for stage I-IV are 70, 45, 30, and 15% respectively.[2] Even with advances in radiotherapy, surgery, and chemotherapy there is a desperate need for the development of novel and effective chemopreventive and chemotherapeutic agents for endometrial cancer.

Dietary supplements have long been used as traditional medicines and provide an important source of bioactive compounds that can serve as both chemopreventive as well as chemotherapeutic agents against endometrial and other types of cancers.[3] We are currently investigating the anti-cancer properties of compounds present in the rhizomes of ginger (*Ginger officinale*). Ginger has also been used for many centuries and

compounds found in ginger have been shown to have tumor-preventative properties.[4, 5, 6] Our studies on ginger are supported by previous studies undertaken showing ginger inducing apoptosis in pancreatic carcinoma cells through p53 signaling pathway.[7] Ginger treatment on cultured ovarian cancer cells induced growth inhibition and ginger treatment resulted in inhibition of NF-κB activation as well as diminished secretion of VEGF and IL-8.[8] In this study, we investigate the effect of ginger extract and its potential use in the treatment and prevention of endometrial cancer.

Materials and Methods

Reagents

DMEM (Dulbecco's Modification of Eagle's Medium) and RPMI-1640 was purchased from Cellgro (Manassas, VA). SuperSignal West Dura Extended Duration Substrate was purchased from Thermo Scientific (Waltham, MA). Primary caspase-3 Rabbit antibody, Bcl-2 Rabbit antibody, Bax Rabbit antibody, Phospho P53 Mouse antibody and β-actin Mouse antibody were purchased from Cell Signaling Technology (Beverly, MA). Peroxidase- conjugated AffiniPure Goat Anti-Rabbit IgG antibody and Peroxidase- conjugated AffiniPure Goat Anti-Mouse IgG antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC Annexin V Apoptosis Detection kit was purchased from BD Pharmingen (San Diego, CA). Pierce RIPA Buffer and Protease Inhibitor cocktail were purchased from Thermo Scientific.

Cell culture

Ishikawa and ECC1 cells were obtained from ATCC. Ishikawa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and ECC-1 cells were grown in RPMI-

1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics in a 5% CO_2 incubator.

Steam distillation of ginger rhizomes.

Ginger rhizomes were obtained from local vendors, cleaned with distilled water and cut into 0.5 cm pieces. Approximately 250-300g of the cut ginger pieces were transferred to a 1000 mL round bottom flask and submerged in 500 mL of deionized water. Steam distillation was carried out for 4-6 hours by heating the flask. The oil in the Clevenger apparatus, being less dense than water, was separated by periodically draining the liquid accumulating in the separation tube of the unit. The oil was immediately aliquoted into microfuge tubes and frozen until used in assays. The density of the oil was calculated to be 0.87 g/mL and this measurement was used to calculate the concentration of the extract used to conduct the biological assays.

Cell proliferation assay

Effect of steam distilled ginger extracts on the proliferation of the cancer cell lines was determined by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) uptake method.[9] Cancer cells were plated in a 96-well plate at a density of 5000 cells/well in their respective medium. The cells were then treated with various concentrations of ginger extract and incubated at 37°C in a 5% CO_2 environment for 24, 48 and 72 hours. After the designated time period, 20 mL of MTT was added to each well and the plates were incubated at 37°C for three hours. The formazan crystals formed in the wells were dissolved in 100 µL of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a Spectra MAX 190 (Molecular Devices, Sunnyvale, CA).

Mitochondrial membrane potential assay

The endometrial cancer cells, Ishikawa or ECC1, were grown in T-25 tissue culture flasks. Log-phase cells were treated with 250ng/mL or 2.5μg/mL of ginger extract for 24 hours. The cells were then washed with PBS twice and harvested using trypsin. $1x10^6$ cells were added to each flow tube from untreated, 250ng/mL, and 2.5μg/mL ginger extract treated cells. The cells were treated for 30 minutes with 40nM of $DiOC_6$ at 37°C. Cells were then washed and re-suspended in 400μL of PBS containing 2% FBS. The cells were then analyzed by FACSCalibur flow cytometer to assess the mitochondrial membrane potential. The data was analyzed using FlowJo software.

Measurement of Apoptosis by flow cytometry

Apoptosis was measured using the FITC Annexin V Apoptosis Detection kit. Briefly, 2×10^6 cells were treated with 2.5μg/mL ginger extract and incubated at 37°C for 24 hours. The cells were then washed twice with cold PBS and re-suspend in 1X Binding at a concentration of 1×10^6 cells/mL. The cells in 100 mL, the cells were transferred to 5 mL tubes and stained with 5 μL of FITC Annexin V and 5 μL of propidium iodide (PI). The cells were gently vortexed and incubated at room temperature for 15 minutes. After washing the cells with 1X binding buffer to remove the excess Annexin V and PI, the cells were analyzed on a FACSCalibur flow cytometer. The data was analyzed using FlowJo software.

Western blot analysis

After treatment of endometrial cancer cells with the steam distilled extracts of ginger, cells were washed with ice cold phosphate buffered saline (PBS) and lysed with radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail. The total amount of protein in the lysate was determined by using the bicinchonic acid

(BCA) assay[10]. Cell lysates were loaded with 25 mg of protein per well and separated via electrophoresis using a 7.5% resolving gel. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% milk in Tris buffered saline buffer, and probed with the appropriate primary antibodies. Horseradish peroxidase conjugated secondary antibodies and SuperSignal West Dura Extended Duration Substrate were used for detection of the proteins on the blots. The films were scanned using FLOURCHEM8900 and Image J software was used to quantify the intensities of the bands.

Results

Ginger oil used in all bioassays was isolated by steam distillation of rhizomes of ginger. In a modified Clevenger apparatus (Figure 1), ginger rhizomes were submerged in de-ionized water. Using steam distillation, the water immiscible oils from ginger roots separated as the low-density fraction in the collecting tube of the Clevenger apparatus. We have been able to isolate approximately 300mg of oils from 250g of ginger roots obtained from local commercial vendors.

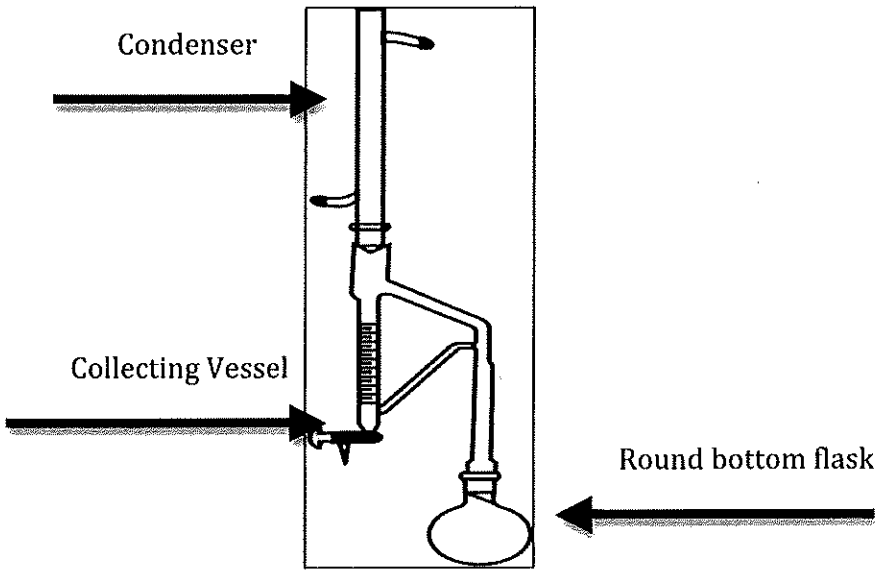

Figure 1: A Clevenger apparatus with a 1000 mL round bottom flask containing cut-up ginger rhizomes in de-ionized water. The heater is not shown but sits underneath the round bottom flask. Image taken from: http://medilabexports.com/clevenger--apparatus-165.html

The oil from the ginger was tested for its effect on the proliferation of one ovarian cancer (SKOV-3) and two endometrial cancer (ECC-1 and Ishikawa) cell lines. The three cell lines were treated with 25ng, 250ng, 2.5µg, 25µg, 250µg/ml concentrations of the steam distilled extracts of ginger for 24, 48, and 72 hours. Controls were not exposed to any ginger extract. Proliferation of the ginger oil-treated and control cells was measured by the MTT assay.[9] The proliferation of the ECC-1 and the Ishikawa cells was inhibited at a concentration of 2.5µg/mL and greater. The data shown in Fig. 2 shows proliferation curves for Ishikawa cells and Fig. 3 shows proliferation curves for ECC-1 cells. No control over proliferation was seen in SKOV-3 (data not shown) when compared to control. Thirteen replicates were used for each condition. Only minor variations in the measurements are seen as calculated by the standard deviation and shown as error bars.

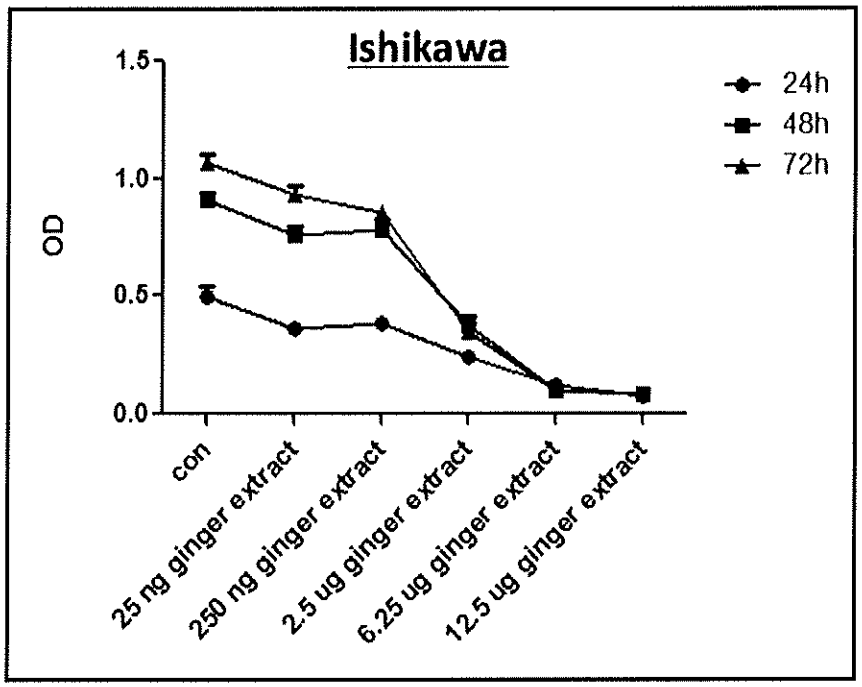

Figure 2: MTT assay performed on the endometrial cancer cell line Ishikawa after treatment with specific treatment of oil from ginger extract. 5000 cells were plated per well and subjected to MTT after allotted time. Optical Density (OD) measured at an absorbance at 570 nm. Control (con) was not treated with any ginger extract. Error bars are included as standard deviation of trials.

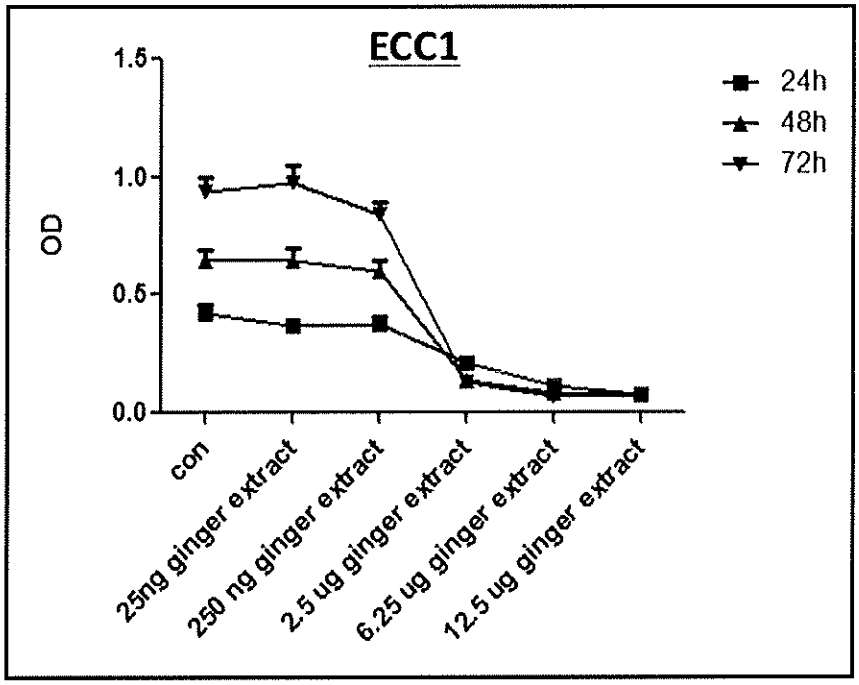

Figure 3: MTT assay performed on the endometrial cancer cell line ECC-1 after treatment with specific treatment of oil from ginger extract. 5000 cells were plated per well and subjected to MTT after allotted time. Optical Density (OD) measured at an absorbance at 570 nm. Control (con) was not treated with any ginger extract. Error bars are included as standard deviation of trials.

We next investigated the molecular mechanisms altered by ginger extracts that caused the decreased proliferation in the ECC-1 and Ishikawa cells. First, we tested if ginger extract treatment was affecting the mitochondrial membrane potential, an indicator for apoptotic cell death. During cellular damage, the mitochondrial permeability increases leading to a depolarization of the mitochondrial.[11] We studied the change in the membrane potential of the mitochondria by monitoring the uptake of the dye $DiOC_6$. The fluorescent dye accumulates in the mitochondria primary in healthy cells. The stain intensity decreases when there is a disruption in mitochondrial membrane potential. After treating Ishikawa cells with a ginger extract concentration of 250ng or 2.5µg, cells were subjected to 40nM of $DiOC_6$ for 30 minutes. The cells were then analyzed with a flow cytometer to determine the median fluorescence intensity (MFI). Figure 4 shows the results of the ginger-treated Ishikawa cells compared to two controls: one with no ginger treatment and the other with no ginger treatment and no $DiOC_6$. There was a significant decrease in mitochondrial membrane potential following ginger extract treatment in a dose-dependent fashion.

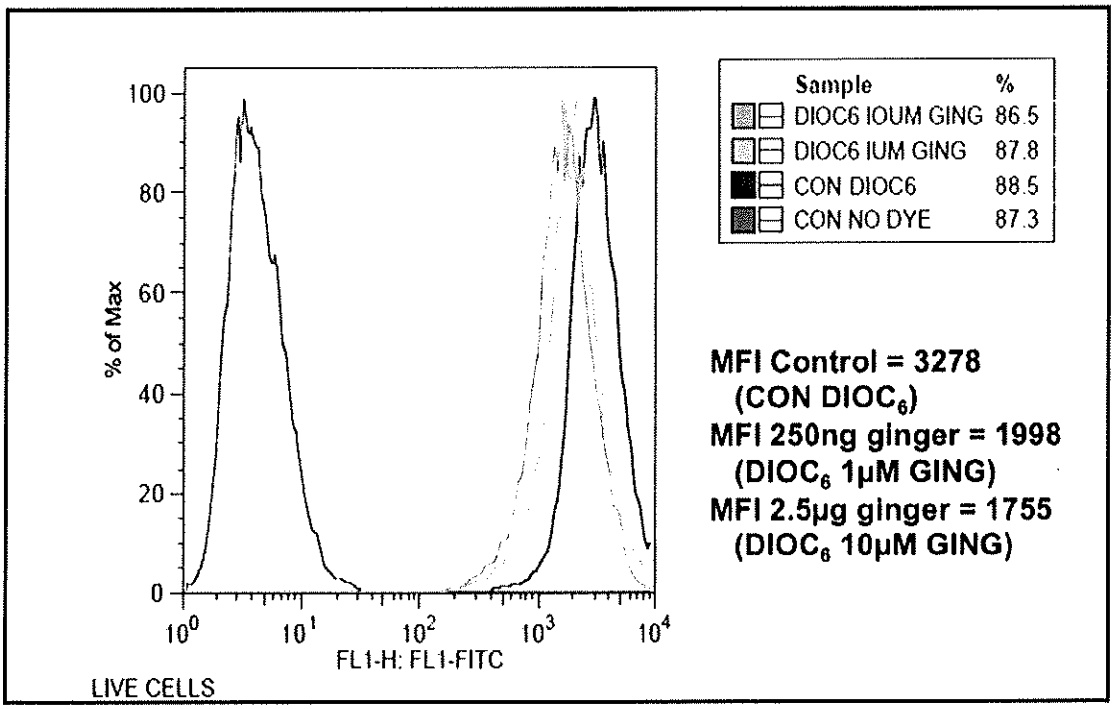

Figure 4: Mitochondrial membrane potential assay in Ishikawa cells treated with 250ng or 2.5µg ginger extract. Median fluorescence intensities (MFI) measured shows decreased membrane potential with increased concentration of ginger treatment.

From the MTT assay, cell death was occurring from ginger treatment. To confirm whether cell death was occurring we performed an Annexin V apoptosis assay. After treatment with ginger, cells were stained using Annexin V, which binds to phosphatidylserine (PS) in the cell membrane, and propidium iodide (PI), which is a DNA-binding dye.[12] Dead cells have an altered phospholipid bilayer, allowing Annexin to bind to PS and also enabling PI to enter the cell and bind to DNA. Figure 5 shows the flow cytometer data for both ginger-treated and control Ishikawa cells. Our data indicates a significant increase in the number of dead cells as a direct result of ginger extract treatment.

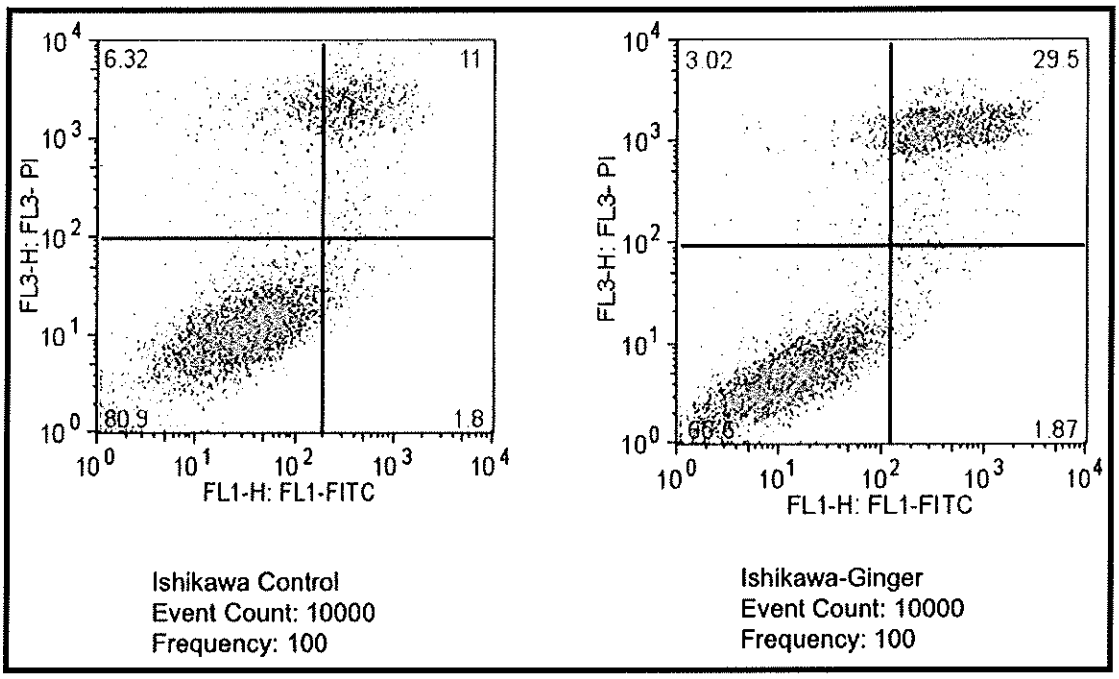

Figure 5: Flow cytometer data from Annexin V apoptosis assay on 8.5µg/mL ginger treatment on Ishikawa cells; figure on right. Figure on left is control with no ginger treatment. Bottom-left quadrant of both graphs represents cells with low PI staining and low Annexin V staining indicating viable cells. Top-right quadrant indicates high PI staining and high Annexin V staining indicating dead cells.

Cell death could be occurring by means of necrosis or apoptosis, programmed cell death. Caspase-3 was utilized as a marker to evaluate if apoptosis was occurring in endometrial cancer cells after ginger extract treatment. After 24, 48, and 72 hour incubation periods on the ECC-1 cells with 8.5µg/mL of ginger extract, cleaved caspase-3 levels were analyzed by western blot. Our data, in Fig. 5, shows that treatment with ginger extract results in a significant increase in cleaved caspase-3, the activated caspase that can lead to degradation of cellular proteins causing cell death. ß-actin was used as a loading control and cleaved caspase-3 levels were normalized to ß-actin bands.

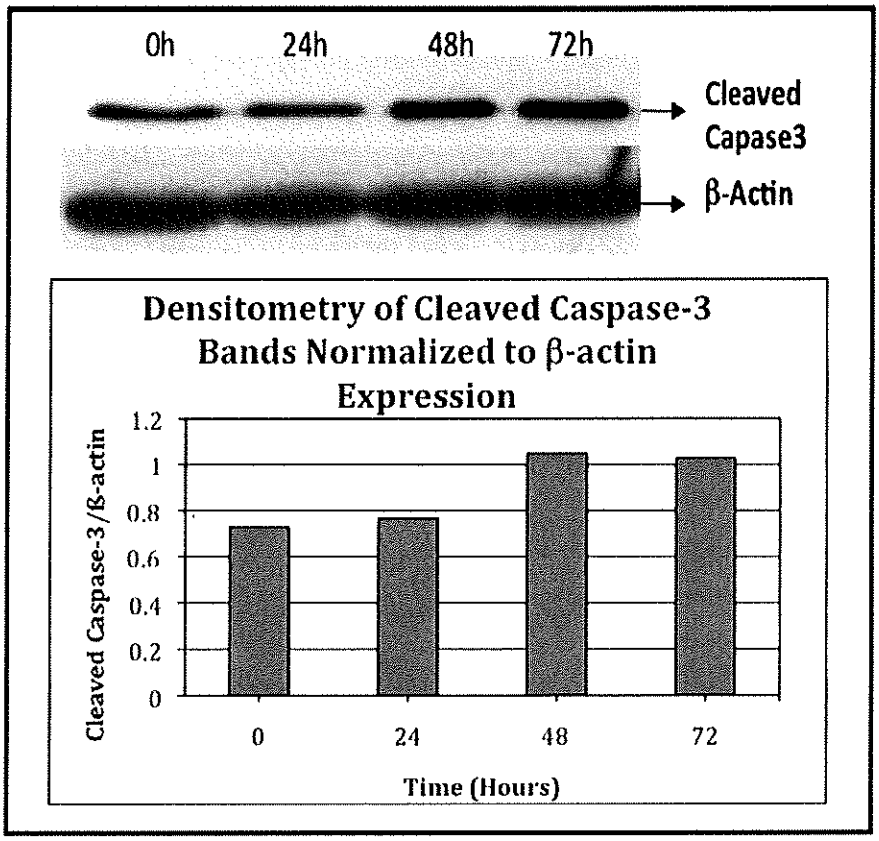

Figure 5: Treatment with ginger extract results in apoptosis of endometrial cancer cells. ECC-1 cells were treated for designated time points with 8.5µg/ml ginger extract and cleaved caspase-3 was monitored by western blotting. Bar graph shows densitometry of cleaved caspase-3 bands normalized to ß-actin expression.

The increase in cleaved Caspase-3, Annexin, and PI staining in the flow cytometry experiments coupled with decrease in mitochondrial membrane polarization led us to investigate if ginger extract was causing a change in the expression of the pro- and anti-apoptotic mitochondrial membrane-associated proteins Bax and Bcl-2, respectively. After 24, 48, and 72 hour incubation periods on the Ishikawa cells with 8.5µg/mL of ginger, there was slight increase in the expression of Bax. However, there was an almost complete loss of Bcl-2 expression in the endometrial cancer cells under these conditions leading to a significant increase in BAX/Bcl2 ratio. Western blotting

data is shown in Figure 6 along with ß-actin expression used as a loading control.

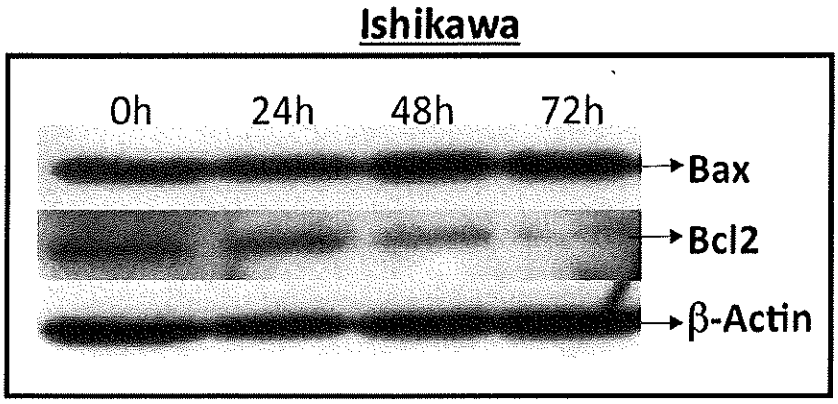

Figure 6: Western blot analysis showing expression of apoptosis signaling proteins Bax and Bcl2. Ishikawa cells subjected to 8.5µg/mL of ginger extract treatment for designated amount of time. Bax expression remains constant but Bcl2 expression decreases as a result of ginger extract treatment. Overall, ratio of Bax:Bcl2 increased suggesting increased apoptosis signaling. ß-actin used as loading control.

The tumor suppressor, p53, is a transcriptional factor that plays a key role in regulating various processes in the cell. The cellular processes regulated by P53 include regulation of cell cycle, apoptosis, and DNA repair.[13] Since SDGE induced apoptosis in endometrial cancer cells, we therefore decided to examine potential changes in the p53 pathway. Our data, shown in Figure 7, indicates a rapid rise in the phosphorylation of p53 protein on Ser-15. This data indicates an activation of the p53 pathway as shown by western blotting.

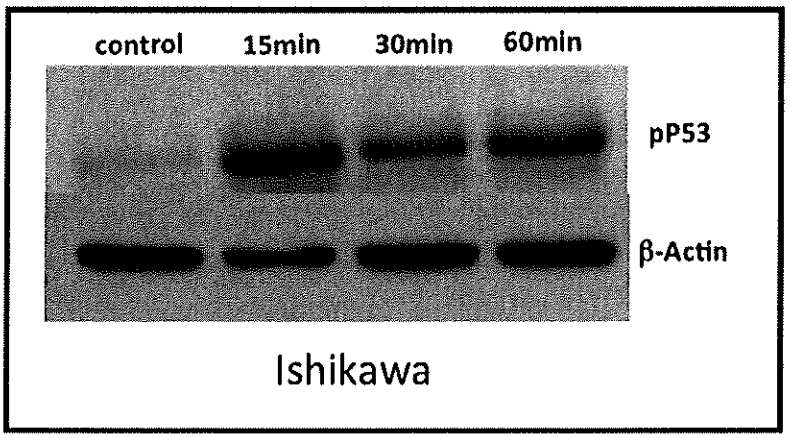

Figure 7: Treatment with ginger extract increases P53 phosphorylation. Ishikawa cells were treated with 8.5 μg/ml of ginger extract for designated time points and phospho-P53 levels were monitored by western blotting. The control represents no treatment with ginger extract. ß-actin used as loading control.

Discussion and Conclusions

Steam distilled ginger extract inhibits endometrial cancer cell growth in a dose and time dependent manner as seen from our proliferation assays. This effect was seen in both of the endometrial cancer cell lines, Ishikawa and ECC-1, in a 24 hours time period with a 2.5μg ginger extract treatment. The potent cytotoxic effect of ginger extract on endometrial cancer cells prompted us to do further research into the mechanism involved in growth inhibition.

To investigate the involvement of mitochondria in the cytotoxic effect caused by ginger extract treatment on endometrial cancer cells, we studied changes in the membrane potential of mitochondrial membrane by monitoring the uptake of the dye $DiOC_6$. There was a significant decrease in mitochondrial membrane potential following ginger extract treatment. Attenuation of mitochondrial membrane potential corresponds to the opening of the inner mitochondrial membrane permeability transition pore. The opening of this pore induces depolarization of the membrane potential, release of

apoptogenic factors, and loss of oxidative phosphorylation. The loss of mitochondrial membrane potential seen here may be either an early event in apoptosis or may be a consequence of the apoptotic-signaling pathway.

We found when endometrial cancer cell lines, Ishikawa and ECC1, were treated with ginger extract, the ratio of Bax and Bcl-2 was changed as shown in our protein blots. There was a no increase in the pro-apoptotic, Bax, a target protein of the transcriptional factor p53, whereas the expresstion of the anti-apoptotic protein family member, Bcl-2, was almost completely abrogated. The dramatic increase of the Bax/Bcl-2 ratio provides evidence of signaling for apoptosis, in addition to activated caspase cascade. Caspase-3 is a downstream target of Bax. Caspase-3 is the effector protein that can lead to degradation of the cellular proteins and cause cell death. As shown by western blotting, a significant increase in cleaved caspase-3 expression was observed providing evidence for apoptosis signaling through Bax and caspase-3.

Further, the P53 tumor suppressor protein is known to be involved in apoptosis. It transcriptionally regulates several apoptosis related genes. One way P53 can lead to apoptosis is by trans-activation of Bax. Translocation of Bax from cytosol to membranes followed by release of cytochrome c from mitochondria and activation of caspases leads to apoptotic cell death. Since Bax is the target protein for P53, we looked at the phosphorylation of P53 at serine-15 residue. P53 is phophoylated at serine-15 and becomes activated causing apoptosis. We observed there was a significant increase in the phosphorylation of P53 after 15 minutes of treatment with ginger extract. P53 plays a crucial role in the pathway for apoptosis in endometrial cancer cells when treated with steam distilled ginger extract.

Natural products of botanical origin are intensely investigated for their anti-cancer properties. They are one of the best reservoirs for new molecules with potential for use in treatments of a variety of cancers. We here examined ginger extract as a potential therapeutic in the treatment of endometrial cancer. It was shown that ginger extract treatment inhibits proliferation by inducing apoptosis via activating P53 and decreasing the Bcl2 expression in endometrial cancer cell. In conclusion, ginger extract could be developed as an agent used in the treatment and/or prevention of Endometrial cancer.

References

[1]American Cancer Society. *Cancer Facts & Figures 2012. (*2012). Atlanta: American Cancer Society

[2]American Cancer Society. *Endometrial (Uterine) Cancer: Survival by Stage.* Atlanta: American Cancer Society; Last Reviewed: 03/13/2012

[3]B.B. Aggarwal, S. Shishodia. "Molecular target of dietary agents for prevention and therapy of cancer." (2006). *Biochemical Pharmacology,* 71 pp. 1397–1421

[4]E.J. Park, J.M. Pizzuto. "Botanicals in cancer chemoprevention." (2002). *Cancer Metastasis Review*, 21 pp. 231–255

[5] Y.J. Surh, E. Lee, J.M. Lee. "Chemoprotective properties of some pungent ingredients present in red pepper and ginger." (1998). *Mutation Research,* 402 pp. 259–267

[6] Y.J. Surh, K.K. Park, K.S. Chun, L.J. Lee, E. Lee, S.S. Lee. "Anti-tumor-promoting activities of selected pungent phenolic substances present in ginger." (1999). *Journal of Environmental Pathology, Toxicology and Oncology*, 18 pp. 131–139

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[7] Zhang S, Liu Q, Liu Y, Qiao H, Liu Y. "Zerumbone, a Southeast Asian Ginger Sesquiterpene, Induced Apoptosis of Pancreatic Carcinoma Cells through p53 Signaling Pathway." (2012). *Evid Based Complement Alternat Med.* PMID: 22454691

[8] Jennifer Rhode, Sarah Fogoros, Suzanna Zick, Heather Wahl, Kent A Griffith, Jennifer Huang, and J Rebecca Liu. "Ginger inhibits cell growth and modulates angiogenic factors in ovarian cancer cells." (2007). *BMC Complement Altern Med*, 7: 44.

[9] Mosmann T. "Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays." (1983). *Journal of Immunological Methods*, 65 (1-2), pp. 55-63.

[10] Smith, P.K., *et al.* "Measurement of protein using bicinchoninic acid." (1985). *Anal. Biochem.* 150 (1): 76–85. doi:10.1016/0003-2697(85)90442-7. PMID 3843705

[11] Shapiro, H.M., Natale, P.F., and Kamentsky, L.A. "Estimation of membrane potentials of individual lymphocytes by flow cytometry." (1979). PNAS USA 76(11), pp. 5728-5730.

[12] Istvan Vermes, Clemens Haanen, Helga Steffens-Nakken, and Chris Reutelinsperger. "A novel assay for apoptosis Flow cytometric detection of phophatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V." (1995). *Journal of Immunological Methods* 184: 39-51.

[13] May P, May E. "Twenty years of p53 research: structural and functional aspects of the p53 protein". (1999). *Oncogene* 18 (53): 7621–36.

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