CHAPTER-2

ELUCIDATING THE ANTI-CANCER POTENTIAL OF CINNAMON IN SiHa CELLS

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Abstract:

In the present study, we have reported the anti-neoplastic activity of cinnamon in cervical cancer cell line, SiHa. Cinnamon altered the growth kinetics of SiHa cells in a dose-dependent manner. Cells treated with ACE-c exhibited reduced number of colonies compared to the control cells. The treated cells exhibited reduced migration potential that could be explained due to downregulation of MMP-2 expression. Interestingly, the expression of HER-2 oncoprotein was significantly reduced in the presence of ACE-c. Cinnamon extract induced apoptosis in cervical cancer cells through increase in intracellular calcium signaling as well as loss of mitochondrial membrane potential. All these data suggest cinnamon could be explored as a potent chemopreventive drug in cervical cancer.
2.1. Introduction

Cinnamon is a small evergreen tree, approximately 10-15 m tall, originally found in Sri Lanka and Southern India. The bark of cinnamon (Figure 2.1) is a common spice used as a culinary all over the world (Das et al., 2013). The production of cinnamon is mostly limited to the wettest low land areas of South east Asia. Cinnamon is cultivated up to an altitude of 500 m above the sea level having the mean temperature of 27°C and an annual rainfall varying from 2000 to 2400 mm (Das et al., 2013). Cinnamon consists of a variety of resinous compounds, including cinnamaldehyde, cinnamate, cinnamic acid and numerous essential oils (Senanayake et al., 1978). The presence of a wide range of essential oils, such as trans-cinnamaldehyde, cinnamyl acetate, eugenol, L-borneol, caryophyllene oxide, b-caryophyllene, L-bornyl acetate, E-nerolidol, α-cubebe, α-terpineol, terpinolene and α-thujene, has been reported (Tung et al., 2008).

![Cinnamon Bark](image)

*Figure 2.1: A typical cinnamon bark material*

The therapeutic utilities of cinnamon bark have been indicated in traditional systems of medicine such as Ayurveda, Unani, Chinese and Japanese (Ravindran et al., 2003). Preparations containing the bark of cinnamon have been prescribed in native Ayurvedic system for flu, indigestion, mouth washes and gynecological ailments.
In traditional Chinese medicinal system, cinnamon has been prescribed for the treatment of cold, fever, diarrhea, pain, nephritis, purulent dermatitis, and hypertension as well as for improvement of an appetite depressed by influenza or the common cold (Cheng et al., 1983). In Arabian and Unani systems of medicine, cinnamon has been considered to be an aromatic, astringent and carminative. Local inhabitants use cinnamon bark powder to treat nausea, vomiting, flatulence, dyspepsia, abdominal colic and heart burn (Alqasoumi et al., 2012). Fresh plant materials, crude extracts and isolated components of cinnamon have been shown to possess a wide spectrum of pharmacological activities such as antifungal (Singh et al., 2007; Matan et al., 2006), anti-inflammatory (Lee et al., 2005), antidiabetic (Khan et al., 2003; Kim et al., 2006; Qin et al., 2003), antiulcer (Amar et al., 2010), antihypertensive (Preuss et al., 2006), antioxidant (Dragland et al., 2003), as well as lowering of cholesterol and lipid (Kim et al., 2006; Khan et al., 2003). Recently, the anti-tumor activity of cinnamon has been shown both in vitro (Schoene et al., 2005; Kamei et al., 2000; Singh et al., 2009) and in vivo (Kwon et al., 2009). Cinnamaldehyde, the bioactive component of cinnamon, has been shown to inhibit proliferation of several human cancer cell lines including breast, leukemia, ovarian, and lung tumor cells (Lee et al., 2009). Earlier studies from our lab for the first time showed a comparative analysis of cytotoxic effect of aqueous extract of cinnamon (ACE) from C. zeylanicum with that of commercial cinnamaldehyde on a variety of cell lines (Singh et al., 2009). ACE proved to be more cytotoxic compared to the commercial cinnamaldehyde owing to the presence of polyphenolic compounds, besides cinnamaldehyde, that may synergistically act to induce enhanced cytotoxicity.

In the present work, we report for the first time the antineoplastic activity of the aqueous extract of cinnamon bark from Cinnamomum cassia (ACE-c) L. family Lauraceae, in human cervical cancer cell line, SiHa. We observed that cinnamon altered...
the growth kinetics of cells in a dose-dependent manner. The colony formation and soft agar assays demonstrated that the number of colonies in cells treated with (ACE-c) was less compared to the untreated control cells. The ACE-c treated cells exhibited slow migration compared to the control cells that could be explained due to reduced MMP-2 expression in the former. Cinnamon extract induced apoptosis in cervical cancer cell line due to the loss of mitochondrial membrane potential (ΔΨm) through increase in mitochondrial calcium flux.

2.2. Materials and Methods

2.2.1. Reagents

Tissue culture plastic ware was purchased from BD Biosciences, CA, USA, Axygen Scientific Inc, CA, USA and Nunc, Roskilde, Denmark. Dulbecco’s Modified Eagles Medium (DMEM) was obtained from Himedia Corporation, Mumbai, India. Penicillin and streptomycin were obtained from Gibco BRL, CA, USA. Fetal bovine serum was purchased from Moregate Biotech, Australia, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), FCCP, JC-1 were purchased from Sigma-Aldrich (St. Louis, MO). HER-2 antibody was purchased from Santa Cruz Biotechnology, CA, USA, Donkey anti-Mouse IgG Cy-3-conjugate (Millipore, MA) and Annexin V-FITC apoptosis kit #3 from Invitrogen (CA, USA). All other common reagents were procured from Qualigens fine chemicals (Mumbai, India).

2.2.2. ACE-c preparation and characterization

The bark of Cinnamomum cassia was purchased from Shivam Ayurvedics Pune, Maharashtra, India. Voucher specimen number for Cinnamomum cassia bark was 104. Sample was authenticated from Regional Research Institute (AY) Kothrud, Pune (ref
no.1045). The bark was weighed, powdered and extracted in double distilled water (ratio of cinnamon: water used was 1:16) in a hot water extractor (The Ayurvedic Pharmacopoeia of India). The resulting extract was centrifuged at 13000 rpm for 15 min to remove the particulate matter. The supernatant was further filter-sterilized using swiney filter (pore size, 0.45 µm) and the resultant filtrate was stored in aliquots at -80°C until use. The bark identity was further confirmed by detecting the marker molecule cinnamaldehyde in ACE-c (17 mg/ml stock solution) by HPTLC analysis as described previously (Singh et al., 2009; Gopu et al., 2008). Further, the total polyphenolic content of ACE-c was measured by Folin-Ciocalteau method as described previously (Singh et al., 2009).

2.2.3. Cell culture

The human cervix carcinoma (SiHa) cell line used in the study was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2 mM L-glutamine and supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin. The cells were incubated in a humidified 5% CO₂ incubator at 37°C.

2.2.4. Cell viability

The anti proliferative activity was determined by MTT dye uptake method (Singh et al., 2009). Briefly, SiHa cells were seeded at 1 x 10⁵/ml density in 96-well plate. An untreated group was kept as a negative control. The aqueous cinnamon extract (ACE-c) was added at following concentrations: 10, 20, 40, 80, 160 and 320 µg/ml, in each well in triplicates. The MTT solution (5 mg/ml) was added to each well, and the cells were cultured for another 4 h at 37°C in 5% CO₂ incubator. The formazan crystals formed
were dissolved by addition of 90 µl of SDS-DMF (20% SDS in 50% DMF). After 15 min, the amount of colored formazan derivative was determined by measuring optical density (OD) with the ELISA microplate reader (Biorad, Hercules, CA) at 570 nm (OD$_{570-630}$). The percentage viability was calculated as: % Viability = (OD of treated cells / OD of control cells) x 100

2.2.5. Cell growth analysis

SiHa cells were seeded at a density of $1 \times 10^5$/ml in 24-well plates in triplicates. Next day, the cells were dosed with different concentrations of ACE-c (0, 10, 20, 40 and 80 µg/ml) and grown for 24, 48 and 72 h. The cells were harvested and counted for viability with trypan blue dye exclusion using a hemocytometer.

2.2.6. Colony formation assay

The cells were plated at a seeding density of $1 \times 10^3$ cells/ml in 6-well plates. After 24 h, the cells were exposed to various concentrations of ACE-c (0-80 µg/ml). Plates were incubated at 37°C in a 5% CO$_2$ incubator for one week. This was followed by fixing the colonies with 4% paraformaldehyde and staining with 0.5% crystal violet (Kaul et al., 2003). The colonies were photographed with Sony DSC-S75 cyber-shot camera.

2.2.7. Soft agar assay

Control SiHa cells ($5 \times 10^3$ cells/ml) as well as cells treated with different concentrations of ACE-c (0-80 µg/ml) were mixed at 40°C with 0.35% agarose (DNA grade, GIBCO BRL, CA, USA) in culture medium and gelled at room temperature for 20 min over a previously gelled layer of 0.5% agarose in culture medium in 6-well plates. After
incubation for 10 days, colonies were photographed and counted using an Axiovert 200M microscope (Carl Zeiss, Germany) (Kaul et al., 2003).

2.2.8. Wound healing assay

Cells were plated at a seeding density of 4 x 10^5/ml in 24-well plates and grown overnight at 37°C in 5% CO₂ incubator. An artificial wound was made with 10µl micropipette after 6 h serum starvation in control cells as well as cells treated with different concentrations of ACE-c (10-80 µg/ml). Time-lapse imaging of migrating cells in wound healing assay was performed on Nikon Eclipse TE2000-E microscope (Nikon, Tokyo, Japan) over 15 h in serum containing medium in a humidified chamber at 37°C and 5% CO₂ atmosphere. Images were obtained every 20 min using a 10X phase objective of NA 0.25 and analyzed by image analysis software Metamorph Universal Imaging, USA. The average migration rate in µm/h was calculated and graphs were plotted using Microsoft Excel and Sigma plot program.

2.2.9. RT-PCR analysis

Total cellular RNA from control as well as cells treated with different doses of ACE-c (10-80 µg/ml) was extracted by a 1-step acid guanidine isothiocyanate-phenol method using TRI reagent (Sigma, St. Louis, MO), precipitated with isopropanol and estimated by spectrophotometry. 10 µg total RNA was used for each RT-PCR reaction. 50 Units of Moloney murine leukemia virus reverse transcriptase (MMuLV) (Bangalore Genei, Bangalore, India) were added in a typical 50 µl reaction (10 µg RNA, 1X first-strand buffer, 1 mM DTT, 2.5 mM dNTPs, 50ng/µl random primers and 15U/µl RNase i) and incubated for 1 h at 42°C followed by incubation at 95°C for 5 min. The purified cDNA template was amplified using different sets of primers. The primers used were as
follows: β-actin-F: 5'-taccactggcactgtaggact-3'; β-actin-R: 5'-tttctgcatctgtaggaaat-3'; MMP-2-F: 5'-ggtggtcagtggc-3'; MMP-2-R: 5'-agatctttcttcaaggacggtt-3'. PCRs were performed in 25 µl volume in which 1X PCR buffer, 2.5 mM dNTPs, 1.5 mM MgCl₂, 1U of Taq polymerase and 100 ng of the specific primers were added. A brief initial denaturation at 95°C for 5 min was followed by 35 cycles with the following steps: 95°C for 1 min, annealing at 55-55.2 °C for 1 min and extension at 72°C for 1 min. RT-PCR products were then separated on a 1.2% agarose gel and visualized by staining with ethidium bromide. The intensities of the bands corresponding to the RT-PCR products were quantified using phosphorimager (Alpha Imager using Alpha Ease FC software, Alpha Innotech) and normalized with respect to the β-actin product.

2.2.10. Gelatin zymography

The Gelatin zymography was performed to detect the extracellular MMP-2 (Rangaswami et al., 2004). The conditioned medium of control cells as well as cells treated with 80 µg/ml ACE-c was collected and concentrated in Centricon YM-30 tubes (Millipore, MA). The control and treated samples containing an equal amount of total proteins were mixed with sample buffer (2% SDS, 25% glycerol, 0.1% bromophenol blue and 60 mM Tris- HCl pH 6.8) and loaded onto 7.5% SDS-polyacrylamide gel containing gelatin (0.5 mg/ml). The gel was washed with 0.25% Triton X-100 and incubated overnight in incubation buffer (150 mM NaCl, 100 mM CaCl₂, 50 mM Tris-HCl pH 7.5, 1% Triton X- 100, 0.02%NaN₃) at 37°C. The gel was stained with staining solution (0.1% Coomassie Brilliant blue R-250 in 40% isopropanol) and destained in 7% acetic acid. Gelatinolytic activity was detected as unstained bands on a blue background. The quantitation of bands in control and treated samples was performed by densitometric analysis on Alpha Imager using Alpha Ease FC software, Alpha Innotech.
2.2.11. Immunoblotting
Cell extracts were prepared from control and cells dosed with different concentrations of ACE-c. Briefly, the cell pellet was resuspended in 80 µl lysis buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 0.5% NP40, 50 mM NaF, 1 mM DTT, 0.1 mM PMSF, 0.5 µg/ml leupeptin (Pro-pure Amersco, Solon, USA), 1 µg/ml pepstatin (Amresco, Solon, USA), 150 mM NaCl, 0.5 µg/ml aprotinin (Amersco, Solon, USA) and protease inhibitor cocktail (Roche, Lewes, UK) and incubated on ice for 1 h with intermittent mixing. The extract was centrifuged for 20 min at 4°C at 12000 rpm. The protein was estimated using Bradford reagent (Biorad Laboratories Inc, CA, USA). Equal amount of protein was loaded on a 10% SDS-polyacrylamide gel and transferred electrophoretically to Amersham Hybond-P PVDF membrane (GE Healthcare, UK) in sodium phosphate buffer (pH 6.8). The membrane was blocked in 5% BSA in TST and incubated at room temperature for 1 h with mouse monoclonal antibody for HER-2 and tubulin (Santacruz, CA, USA) at a 1:1000 and 1:2500 dilution, respectively. The membrane was washed in TST and incubated with donkey anti mouse IgG HRP conjugate at 1:5000 (for HER-2) and 1:3000 (for tubulin) dilutions. Proteins were visualized with a chemiluminescence kit (Amersham ECL western blotting kit, GE Healthcare, UK) and densitometric analysis was performed on Alpha Imager using Alpha Ease FC software, Alpha Innotech.

2.2.12. Measurement of Apoptosis
The cells were plated at a seeding density of 5 x 10^5 per well and treated with different concentrations of ACE-c. After 48 h, the cells were harvested by trypsinization and washed with PBS twice. Cells were stained with Annexin V-FITC (for early apoptosis) and propidium iodide (PI) following the manufacturer’s instructions (Annexin V-FITC apoptosis kit #3, Invitrogen) and analyzed by FACS using CellQuest Software.
2.2.13. Intracellular calcium measurement

Intracellular Ca$^{2+}$ levels were analyzed in control cells as well as cells treated with different doses of ACE-c by flow cytometry (Yoon et al., 2006). Cells were loaded with 5 µM Fluo-3/AM (Sigma, St. Louis, MO) and 100 µg/ml of Pluronic F127 (Sigma, St. Louis, MO) in centrifuge tubes and incubated at 37°C, 5% CO$_2$ for 1 h in the dark. The cells were resuspended approximately every 20 min to ensure even dye loading. The cell pellet were washed twice with 0.9% saline, finally resuspended in 3 ml Hank’s Balance salt solution (HBSS) in FACS tubes. Fluorescence intensities were measured at 525nm in the resuspended cells by FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) to obtain baseline readings. Mean channel fluorescence intensities were calculated using CellQuest software.

2.2.14. Detection of Mitochondrial Membrane Potential (Δψm) using JC-1

Mitochondrial membrane potential was estimated using the fluorescent dye JC-1 either by confocal microscopy or by flow cytometry. For confocal studies, control as well as cells (1 x 10$^5$) treated with different concentrations of ACE-c (10-80 µg/ml) were seeded in 6-well plates. After 48 h, cells were incubated with culture medium containing JC-1 dye for 30 min at 37°C in the dark. Cells were washed with PBS twice and fixed with 2.5% paraformaldehyde made in 200 mM HEPES buffer for 15 min at room temperature followed by PBS wash. Slides were then mounted in antifade mounting medium (Ultracruz mounting medium, Santacruz) and analyzed with a Zeiss LSM510 META confocal laser scanning microscope (Zeiss, Thornwood, NY) using LSM Image Examine software. For detection by flow cytometry (Herold et al., 2002), control as well as cells (5 x 10$^5$) treated with different concentrations of ACE-c (10-80 µg/ml) were harvested by trypsinization and washed with PBS. The cells were incubated with culture medium
containing JC-1 for 30 min at 37°C in the dark. Cells were washed in PBS twice and analyzed for \( \Delta \psi_m \). The fluorescence intensities were measured at 527nm (green) and 590nm (red). Analysis was done by Cell Quest software.

2.2.15. **Immunofluorescence microscopy**

For immunostaining, SiHa cells were plated on coverslips in 6-well plates at a seeding density of \( 2 \times 10^5 \) cells/ml. After 24 h, the cells were dosed with different concentrations of ACE-c (0-80 µg/ml). Twenty four hours post-treatment, the cells were washed with PBS and fixed in 2.5% paraformaldehyde made in 200 mM HEPES buffer for 15 min at room temperature. Cells were washed for 5 min in PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked in 10% FBS in PBS for 1 h. For detection, cells were incubated with HER-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer at 1:100 dilution. After washing with PBS, the cells were incubated with the CY3-conjugated secondary antibody (Millipore) that was used at a dilution of 1:300. Slides were then mounted in antifade mounting medium (Ultracruz mounting medium, Santacruz) and analyzed with a Zeiss LSM510 META confocal laser scanning microscope (Zeiss, Thornwood, NY) using LSM Image Examine software.

2.2.16. **Statistical analysis**

All experiments were performed in triplicates and repeated at least five times and the data were presented as mean ± SD. Statistical analysis was conducted with the SigmaStat 3.5 program (Systat Software, Inc.) using 1-way ANOVA. The \( \alpha \) level used for comparisons was \( \alpha = 0.05 \).
2.3. Results

2.3.1 HPTLC analysis of aqueous cinnamon extract (ACE-c)

Aqueous extract of Cinnamon (ACE-c) prepared from C. cassia was analyzed for the presence of cinnamaldehyde as well as polyphenols to ensure the quality and purity of the preparation (Figure 2.2 A and B). The concentration of cinnamaldehyde (µM) present in the ACE-c was determined by HPTLC analysis. It was found that ACE-c contained 20 µM/mg of cinnamaldehyde. The presence of polyphenols was confirmed by Folin-Ciocalteau method. The quantity of polyphenols in ACE was found to be around 380.83 µg/ml (equivalent to 0.76% w/w).

![HPTLC chromatogram and calibration curve](image)

**Figure 2.2**: Detection and quantification of cinnamaldehyde in aqueous cinnamon extract from C. cassia (ACE-c). (A) The figure shows HPTLC chromatogram of standard mixture of piperine, cinnamaldehyde and eugenol (I) as well as ACE-c (II). (B) Calibration curve for quantification of total polyphenolic content in ACE-c by Folin-Ciocalteau method.
2.3.2. Effect of Aqueous extract of Cinnamon (ACE-c) on cell viability

We initially performed MTT assay to define the optimal concentration at which cinnamon was non-toxic to cells. SiHa cells were treated with ACE-c at various concentrations (0-320 µg/ml) for 24 h. Up till 320 µg/ml ACE-c concentration, the cells exhibited 100% survival (Figure 2.3). Based on it, we chose non-cytotoxic concentrations of ACE-c (0-80 µg/ml) in our assays.

![Graph showing the effect of ACE-c on cell viability](image)

**Figure 2.3: Cytotoxic effect of ACE-c on human cervical cancer cells.** SiHa cells were treated with different concentrations (0-320 µg/ml) of ACE-c for 24 h. The cell viability was measured by MTT assay.

2.3.3. Cinnamon treatment altered growth kinetics of SiHa cells

To test the effect of cinnamon on the growth kinetics, SiHa cells were treated with different concentrations of ACE-c: 0, 10, 20, 40, and 80 µg/ml and were grown for 24, 48 and 72 h. At the end of each treatment, the cells were stained with trypan blue, and the viable cells that excluded the dye were counted. It was observed that there was a dose-dependent decrease in the growth kinetics of ACE-c-treated cells compared to the untreated control cells (Figure 2.4 A). Moreover, it was found that at around 80 µg/ml concentration of ACE-c treatment, there was a significant decrease (~2-fold) in the
growth kinetics compared to that observed in the untreated control cells (p≤0.05 for 24 h; p≤0.001 for 48 h and 72 h).

**Figure 2.4: Cinnamon altered growth kinetics of cervical cancer cells.** (A) The growth kinetics has been presented in the figure taken at different time points. Data represent means ±SD of five different experiments. (B) The cells (1 x 10^3/ml) were grown in 6-well plates and treated with various concentrations (0-80 µg/ml) of ACE-c for one week. The cells were then stained with crystal violet and photographed. The experiments were repeated five times. (C) The cells (5 x 10^3) were treated with various concentrations (0-80 µg/ml) of ACE-c and grown in soft agar for 10 days, and the colonies were counted. Colonies were counted from at least 10 different areas and the average of each is plotted. The data represent means ±SD of five independent experiments.

This was further confirmed by colony forming assay wherein at a lower seeding density, cells were treated with different concentrations of ACE-c for one week. At 80 µg/ml concentration of ACE-c, the cells exhibited relatively lesser colonies compared to
the control cells (Figure 2.4 B). Consistent with the slow growth rate, it was observed that Cinnamon extract induced a dose-dependent decrease in the number of soft agar colonies. Interestingly, at 80 µg/mL ACE-c concentration, cells exhibited significant reduction (~3-fold) in the number of soft agar colonies compared to the untreated control cells (p≤0.001) (Figure 2.4 C).

All these data indicate that cinnamon alters the growth kinetics of SiHa cells in a significant manner that could be a positive indicator for testing its antineoplastic activity in cervical cancer cells.

2.3.4. Cinnamon induced apoptosis

To further elucidate the anti-cancer mechanism of cinnamon in cervical cancer cells, we performed apoptosis studies. After treating the cells with different doses of ACE-c, the percent apoptotic cells were assessed by Annexin V-FITC and propidium iodide staining, followed by flow cytometric analysis (Figure 2.5). It was found that at 80 µg/ml ACE-c concentration, there was ~2.6-fold (p≤0.001) increase in the population of cells undergoing apoptosis compared to the untreated control cells. Doses below 80 µg/ml concentration could not induce significant apoptosis in cells.

Figure 2.5: Cinnamon induced apoptosis. SiHa cells were treated with different concentrations of ACE-c (0-80 µg/ml) followed by Annexin V-FITC and PI staining to analyse the effect of cinnamon in apoptosis. This was determined by FACS analysis showing the percentage of early (lower right quadrant) and late (upper right quadrant) apoptotic cells.
2.3.5. **Cinnamon increased intracellular calcium**

Since intracellular Ca$^{2+}$ is a powerful activator of apoptosis, we studied the Ca$^{2+}$ signaling mechanism in cells treated with ACE-c to elucidate the cause of apoptosis (D'Herde et al., 1997; Kaddour-Djebbar et al., 2006). It was observed that after treatment of SiHa cells with various concentrations of ACE-c (0-80 µg/ml), there was a dose-dependent increase in the intracellular levels of calcium. It was noted that the calcium increase was maximal (~2.64; $p \leq 0.001$) at the concentration of 80 µg/ml (Figure 2.6) compared to the control cells. Ionomycin (30 µM) was used as a positive control in the experiment.

![Figure 2.6: Cinnamon increased intracellular calcium. Flow cytometric analysis of the rapid calcium release in SiHa cells after treatment with cinnamon. Cells (5 x 10$^3$ cells) were treated with different doses (0-80 µg/ml) of ACE-c for 24 h. This was followed by loading the cells with Fluo-3/AM for 1 h before analysing in calcium-free HBSS. Ionomycin was used as a positive control. Fluorescence intensities were measured with FACS Calibur flowcytometer. The data represents mean ±SD of five different experiments.](image-url)
2.3.6. **Cinnamon decreased mitochondrial membrane potential**

It is well known that increase in mitochondrial levels of calcium Ca\(^{2+}\) induces apoptosis through the loss of \(\Delta\Psi_{m}\) (Zoratti et al., 1995). To analyse whether the increased intracellular Ca\(^{2+}\) induced by ACE-c treatment resulted into mitochondrial dysfunction, we stained the cells with \(\Delta\Psi_{m}\) indicator, JC-1. By both confocal as well as flow cytometry assays, we observed that the cells exposed to ACE-c exhibited a dose-dependent decrease in JC-1 staining (Figure 2.7A and B, respectively). This indicates loss of mitochondrial membrane potential after treatment with cinnamon, which approaches the loss of potential observed after treatment with the positive control agent, FCCP (10 \(\mu\)M) (Figure 2.7 B).

As clearly observed from the figure, cinnamon induces significant depolarization at 80 \(\mu\)g/ml ACE-c concentration wherein there was ~5-fold reduction in the ratio of red-green fluorescence intensity (p<0.001). Taken together, all these results suggest that cinnamon extract exhibited a potent antineoplastic effect in cervical cancer cells through increase in calcium flux resulting into loss of mitochondrial membrane potential, ultimately leading to apoptosis.
Figure 2.7: Cinnamon dysregulated mitochondrial membrane potential. (A) Confocal images showing mitochondrial membrane depolarization induced by cinnamon. Control and cinnamon-treated SiHa cells were stained with JC-1 and the staining pattern was monitored by confocal laser scanning microscopy. For detection of J-aggregate form (red) (Panel II) and J-monomer alone (green) (Panel I), Argon-Krypton laser line was excited at 590 nm and 527 nm, respectively. Panel III represents the merge images. (B) Flow cytometric analysis with JC-1 dye showing decrease in red to green fluorescence ratio. Control (5 x 10^5) and cells treated with various concentrations (0-80 µg/ml) of ACE-c were stained with JC-1 dye for 30 min. Fluorescence intensities were measured with FACS Calibur flow cytometer. The data represents mean ±SD of five independent experiments.
2.3.7. Cinnamon decreased cell migration through reduction in MMP-2 expression

To examine the effect of ACE-c on cell migration, we performed wound healing assay on confluent monolayers of SiHa cells. After making the wound with a pipette tip, the cells were cultured in presence or absence of different concentrations of the aqueous cinnamon extract and imaged with real time-lapse video for a period of 15 h. It was observed that ACE-c effectively inhibited the migration of cells in a dose- and time-dependent manner compared to the untreated control cells (Figure 2.8 A and B). The latter filled-up the wound gap completely after 15 h whereas at higher concentrations, particularly at 80 µg/ml, ACE-c suppressed the migration capability of SiHa cells significantly (~1.5-fold; p≤0.001) thereby affecting the rate of migration.

Since MMP-2 is known to play a significant role in the invasive property of tumor cells, we investigated the mechanism behind delay in wound healing by ACE-c by testing the expression of MMP-2 in cells treated with/without cinnamon extract. It was observed that the expression of MMP-2 was significantly down-regulated both at mRNA (Figure 2.8 C) as well as protein level (Figure 2.8 D) in a dose-dependent manner compared to the untreated control cells. Interestingly, at 80 µg/ml concentration of ACE-c, there was a ~1.6 fold and ~4 fold (p≤0.001) down regulation in the expression of MMP-2 in transcript and translation levels, respectively. These data suggest that through down-regulation of MMP-2 expression, ACE-c could induce decrease in the migration of cervical cancer cells.
Figure 2.8: Cinnamon inhibited migration of SiHa cells. (A) Time-Lapse image at the end of 15 h in a wound-healing assay in cells treated with different concentrations (0-80 µg/ml) of ACE-c treatment. The upper panel of the image shows the wound made at 0 h. The lower panel shows cell movement corresponding to the distance travelled by the cells at 15 h of time-lapse imaging. (B) Rate of migration of cells during the wound healing assay analyzed by the time-lapse imaging of SiHa cells. Migration rate (µm/h) for each sample from five different fields was calculated. Error bars represent standard deviation and the data is representative of five independent experiments. (C) ACE-c treatment reduces the MMP-2 expression at mRNA level that has been shown by RT-PCR. β-actin was used as the loading control. Densitometric analysis of MMP-2 expression was performed using phosphorimager. The data represents mean ±SD of five independent experiments. (D) Gelatin zymography showing down regulation of MMP-2 expression in SiHa cells at 80 µg/ml ACE-c treatment compared to the untreated control cells. The bands were quantified by densitometry using phosphorimager and the data represents mean ±SD of five independent experiments.
2.3.8. Cinnamon treatment down regulates the expression of HER-2 oncoprotein

Various studies have shown that a variable proportion of cervical carcinoma tumors overexpress HER-2 oncoprotein (Chavez-Blanco et al., 2004). To examine the effect of cinnamon aqueous extract on HER-2 expression, SiHa cells were treated with different concentrations of ACE-c (0-80 µg/ml). Interestingly, the cinnamon extract could down regulate the expression of HER-2 protein in a dose-dependent manner compared to the control cells (Figure 2.9 A and B), the maximum reduction being at 80 µg/ml (~2.6 fold; p≤0.001).

![Figure 2.9: Cinnamon decreased the expression of HER-2 oncoprotein.](image)

(A) Western blot analysis shows the expression levels of HER-2. Tubulin was used as a loading control. (B) Densitometric analysis of the western blot showing fold change in HER-2 protein levels upon ACE-c treatment. The data represents mean ± SD of three independent experiments. (C)
Confocal images of the cells treated with indicated concentrations of ACE-c showing decrease in HER-2 expression. The cells were stained indirectly for HER-2 using Cy3 conjugated antibody (Panel II) and counterstained with DAPI (Panel I). Panel III represents the merge images.

This was further proved by confocal studies wherein at 80 µg/ml of ACE-c treatment, a significant reduction in the expression of HER-2 could be observed (Figure 2.9 C). These results further strengthen the potential antineoplastic role of cinnamon in cervical cancer through reduction of HER-2 expression, a critical marker of cervical cancer.

2.4. Discussion

In the present study, we have reported the anti-cancer potential of cinnamon extract in vitro in human cervical cancer cell line and have elucidated the possible underlying mechanism. The anti-tumor activity of Cinnamon has been reported in vitro (Schoene et al., 2005; Kamei et al., 2000; Singh et al., 2009) as well as in vivo (Kwon et al., 2009); however, its role in cervical cancer remained to be elucidated. We found that the aqueous cinnamon extract affected the growth rate of SiHa cells in a dose-dependent manner with a significant reduction in growth kinetics. This data was further supported by results from colony formation and soft agar assays, which demonstrated statistically significant reduction in the number of colonies in ACE-c treated cells compared to the untreated control cells. Thus Cinnamon could be proposed as a suitable candidate that could be used for restricting the growth of cervical cancer cells.

It is well known that metastasis, being one of the major causes of mortality in cancer, involves various steps such as cancer cell adhesion, invasion, and migration (Liotta et al., 1986). Thus to know the effect of Cinnamon extract on migration of SiHa cells, wound healing assays were performed on untreated control and ACE-c treated cells. Interestingly, Cinnamon inhibited the migration of cancer cells in a highly
significant manner (~1.5 fold), further strengthening its potential use as an anti-cancer drug in cervical cancer.

One of the key steps in the invasive progress of cancer cells is the degradation of extracellular matrix (ECM) proteins by a family of zinc-binding enzymes called as matrix metalloproteinases (Overall et al., 2002). To elucidate the reason behind the poor migration of ACE-c treated cells, we tested the expression of MMP-2 (gelatinase) in control as well as cinnamon extract treated cells. A significant decrease in the expression of MMP-2 (gelatinase) was observed at both mRNA as well as protein levels in ACE-c treated cells that resulted into their reduced migration compared to the control cells. Thus, inhibition of MMP-2 expression by Cinnamon could be regarded as a rational approach towards metastatic disease therapy in cervical cancer.

Apoptosis plays a key role in the regulation of normal tissue homeostasis and participates in the elimination of abnormal cells. Most of the antitumor drugs kill the cancer cells by stimulating the apoptotic pathway (Evan et al., 2001). It is known that mitochondria play an important role in the regulation of apoptosis. To test whether Cinnamon could induce apoptosis in cervical cancer cell line SiHa, we carried out apoptosis studies in control as well as cells exposed to ACE-c. At an effective concentration (80 µg/ml), a significantly higher population of cells was observed to undergo apoptosis compared to the control cells. To further elucidate the mechanism of apoptosis, we tested whether there is any disruption of calcium signaling mechanism as it is known to be one of the main causes of apoptosis.

Intracellular Ca$^{2+}$ trafficking is known to govern a number of vital cellular functions that affect cell survival. Cytosolic calcium, (Ca$^{2+}$)$_{c}$, is usually maintained at lower level (~100 nmol/L) compared to the extracellular concentration (~1 mmol/L). The cells regulate (Ca$^{2+}$)$_{c}$ primarily by regulating the Ca$^{2+}$ trafficking across the plasma
membrane and in and out of key organelles, such as the endoplasmic reticulum and the mitochondria (Berridge et al., 2000). The endoplasmic reticulum is the largest reservoir of Ca\(^{2+}\) in normal cells whereas mitochondrial levels of Ca\(^{2+}\) are quite low. But during apoptosis, mitochondria are known to accumulate Ca\(^{2+}\), especially when the (Ca\(^{2+}\))\(_{c}\) level is high (Kaddour-Djebbar et al., 2006). Increase in mitochondrial calcium, (Ca\(^{2+}\))\(_{m}\), induces apoptosis resulting into loss of \(\Delta \psi_m\), expansion of the matrix, and the rupture of the outer mitochondrial membrane (Ehrenberg et al., 1988). Interestingly, we found that at 80 µg/ml concentration of cinnamon extract there was a significant increase in the levels of intracellular calcium in SiHa cells that could result into their apoptosis.

Since cinnamon extract led to increase in calcium flux in cells, it was obvious that it would result into mitochondrial dysfunction. Thus, we tested the \(\Delta \psi_m\) in cinnamon treated cells by using the fluorescent dye, JC-1 that aggregates into healthy mitochondria and fluoresces red. When the mitochondria collapses in apoptotic cells, the JC-1 no longer accumulates and instead, it is distributed throughout the cell resulting into decrease in red fluorescence. In accordance with this, we found that ACE-c indeed disrupted the mitochondrial membrane potential as observed by decrease in the red fluorescence. FCCP, a drug known to disrupt the transmembrane potential of mitochondria (Prehn et al., 1994; Oubrahim et al., 2001), was used as a positive control. Conclusively, all these data strongly implicate cinnamon as a potent antineoplastic agent in cervical cancer cells wherein it could induce apoptosis in cells through increase in calcium flux leading to loss of \(\Delta \psi_m\) (Salido et al., 2007).

It is well-known that HER-2/Erb2, a transmembrane receptor protein with tyrosine kinase activity from EGF3-receptor family, is a critical marker of cervical and breast cancer. Moreover, HER-2 is known to be overexpressed in a number of tumors (Chavez-Blanco et al., 2004). Interestingly, we found for the first time that cinnamon
could effectively and significantly down-regulate the expression of HER-2 in SiHa cells. It has been shown that HER-2 overexpression is related with the invasion capacity of the tumor cells that is related partly with the up-regulation of MMP-2 and MMP-9 expression as well as proteolytic activity (Pellikainen et al., 2004). Thus, downregulation of MMP-2 expression by cinnamon could be linked with the reduction in the expression of HER-2 oncoprotein.

These leads could be explored in detail to further establish the antineoplastic activity of cinnamon in cervical cancer that would in turn emphasize the chemopreventive potential of natural products.