ANALYZING THE CHEMOPREVENTIVE POTENTIAL OF MEDICINAL PLANTS IN CERVICAL CANCER AND STUDYING THE UNDERLYING MOLECULAR MECHANISMS GOVERNING THEIR ANTICANCER ACTIVITY

A thesis submitted to

Bharati Vidyapeeth Deemed University, Pune

for the degree of

DOCTOR OF PHILOSOPHY

In Biotechnology

By

Mr. Amit Subhash Choudhari (M.Sc)

Under the guidance of

Dr. Ruchika Kaul-Ghanekar

Bharati Vidyapeeth Deemed University,
Interactive Research School for Health Affairs (IRSHA),
Pune-Satara Road, Pune - 411043
Maharashtra, India

September 2014

Certificate

This is to certify that the work incorporated in the thesis entitled "**Analyzing the Chemopreventive Potential of Medicinal Plants in Cervical Cancer and Studying the Underlying Molecular Mechanisms Governing their Anticancer Activity**" for the degree of **Doctor of Philosophy (Ph.D)** in the subject of Biotechnology under the faculty of Life Sciences has been carried out by **Mr. Amit Subhash Choudhari** in Cell and Translational Research Laboratory, Interactive Research School for Health Affairs, Bharati Vidyapeeth Deemed University, Pune under the guidance of Dr. Ruchika Kaul-Ghanekar.

Date :

Place :

Prof.Prabhakar Ranjekar

(Director)

Certificate of Guide

This is to certify that the work incorporated in the thesis entitled **"Analyzing the Chemopreventive Potential of Medicinal Plants in Cervical Cancer and Studying the Underlying Molecular Mechanisms Governing their Anticancer Activity"** submitted by **Mr. Amit Subhash Choudhari** for the degree of **Doctor of Philosophy (Ph.D)** in the subject of Biotechnology under the faculty of Life Sciences has been carried out in Cell and Translational Research Laboratory, Interactive Research School for Health Affairs, Bharati Vidyapeeth Deemed University, Pune under my direct supervision/ guidance.

Date :

Place :

Dr. Ruchika Kaul-Ghanekar

(Supervisor/Research Guide)

Declaration by the Candidate

I declare that the thesis entitled "**Analyzing the Chemopreventive Potential of Medicinal Plants in Cervical Cancer and Studying the Underlying Molecular Mechanisms Governing their Anticancer Activity**" submitted by me for the degree of **Doctor of Philosophy (Ph.D)** in Biotechnology to Bharati Vidyapeeth Deemed University is the record of the work carried out by me under the guidance of **Dr. Ruchika Kaul-Ghanekar** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institution of Higher learning.

I further declare that the materials obtained from other sources have been duly acknowledged in the thesis.

Date :

Place :

Mr. Amit Subhash Choudhari

Ph.D candidate

Acknowledgements

It is a great pleasure for me to acknowledge all those, who helped me in their own way, to make it possible for me to complete this work. I would like to take this opportunity to thank each and everyone, who have contributed in this effort to a successful completion.

First and foremost, I would like to express my deep sense of gratitude and sincere thanks to my research supervisor, Dr. Ruchika Kaul Ghanekar for her whole hearted encouragement, valuable suggestions and evolving me scientifically. It was she who nursed me to flourish in research and science with positive spirit. Thank you Madam for helping me at crucial times and providing me a great learning experience. It is also an honor for me to be her first Ph.D. student.

It is difficult to overstate my gratitude to Dr. Prabhakar Ranjekar, Director IRSHA, BVDU Pune, who provided me constant guidance, unflinching encouragement and support in various ways. I am grateful to him for keeping an eye on the progress of my work. I have been fortunate to benefit from his supervision and will remain in debt to him.

The progress of this research project has been positively gained due to valuable and critical inputs from Prof. Sahebrao. P. Mahadik, Professor Emeritus, Medical College of Georgia, Augusta, USA. I would like to thank him for the numerous discussions that helped me improve my knowledge in this area.

I would like to thank Dr GC Mishra, Director, NCCS, for allowing me to use FACS and Confocal facilities without which completion of this work was highly impossible. In this regard, I would like to thank Dr Limaye, Dr Patole, Ms Hemangini Shikhara, Mr Swapnil Walke, Mrs Pratibha and Mrs Ashwini Atre for technical assistance at NCCS.

Some people have a way of making the world a better place. I am lucky enough to have such personalities with me. It is hard to forget the company of Ms. Snehal for her continuous help and humour.

My special thanks to Ms. Savita, Mrs. Soumya and Ms. Prerna for their kind help in my experiments. I owe a big thank to my colleague Mr. Prakash, Ms. Rashmi,

Ms. Kirti and Mrs. Kavita for making lab atmosphere delightful. I will cherish the moments spent with you guys for all my life.

Members of administrative department, Mr. Gavade, Mrs. Kadam, Mr. Patil, Mr. Mote are acknowledged for their timely help. I am thankful to the support staff at IRSHA, Tushar, Ajay, Deshmukh, and Maushi for all their help which undoubtedly facilated the day-to-day laboratory work.

I am also thankful to Council of Scientific and Industrial Research (CSIR), India for providing me fellowship and financial assistance to conduct the research for my Doctoral study.

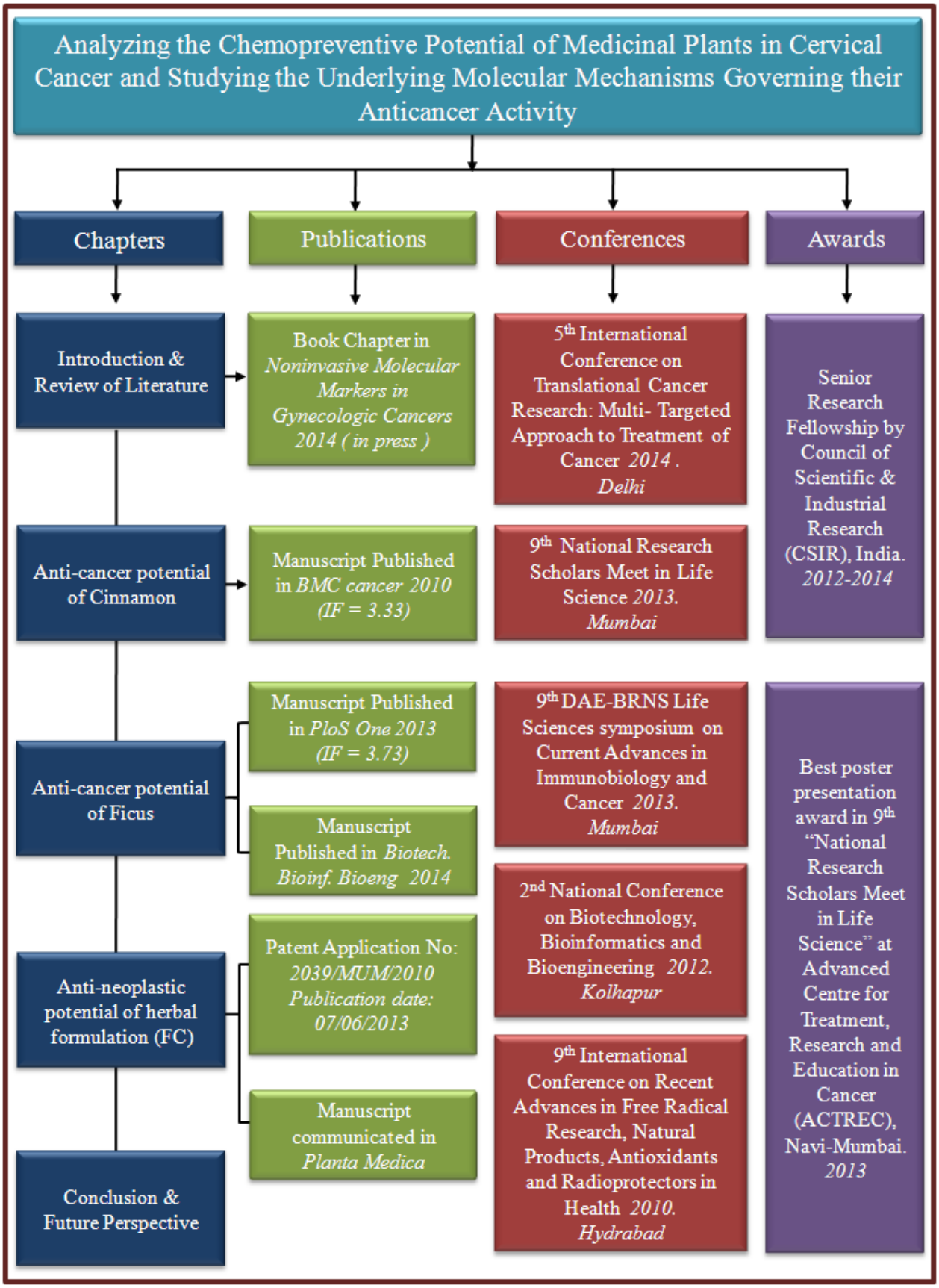
A whole bunch of my dear friends have supported and made this journey jovial. I would like to specially thank Vaibhav, Vitthal, Manmohan and Sadashiv who always were with me all the time during this period. It would not have been so easy without their support. I will forever be indebted to my several friends for their help and presence.

Last but not least I would like to thank my parents and family members for their unconditional support. Aai, Baba and Dada: You have been a constant source of encouragement and motivation to move ahead throughout this period. It would have been really difficult to achieve this goal of Ph.D without your love, affection and care. Words would be less to express my gratitude to God Almighty for giving me all the strength and such a nice bunch of people around me.

Amit Subhash Choudhari

To my Parents

Thesis at a Glance



Abbreviations and Symbols

$(Ca^{2+})_c$	Cytosolic Calcium
$(Ca^{2+})_m$	Mitochondrial Calcium
$\Delta\psi_m$	Mitochondrial Membrane Potential
AAPH	2,2'-azobis-2-methyl-propanimidamide
ACE	Aqueous Cinnamon Extract
ACE-*c*	Aqueous Cinnamon Extract from *C. cassia*
ACTREC	Advanced Centre for Treatment, Research and Education in Cancer
ADR	Adriamycin
AGUS	Atypical Glandular cells of Undetermined Significance
AIF	Apoptosis Inducing Factor
ALP	Anti-Lipid Peroxidation
ASC-US	Atypical Squamous Cells of Undetermined Significance
ASR	Age-Standardized incidence Rate
AUC	Area Under Curve
BAX	BCL2 Associated X protein
BCL-2	B-Cell Lymphoma 2
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAM	Complementary and Alternative Medicine
CIN	Cervical Intrapithelial Neoplasia
CIS	Carcinoma in-situ
CT	Chemotherapy
Cy 3	Cyanine 3
DAF-FM	4-Amino-5 Methylamino-2,7 difluorofluorescein
DAPI	4,6-diamidino-2-phenylindole
DCF-DA	2,7-dichlorofluorescin diacetate
DMEM	Dulbecco's Modified Eagles Medium
DMF	Dimethylformamide
dNTP	Deoxynucleotide triphophates
DTT	Dithiothreitol

ECM	Extracellular Matrix
EGF 3	Epidermal Growth Factor-3
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FC	*Ficus religiosa* and *Cinnamomum Zeylanicum* (2:1) formulation
FCCP	Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone
FIGO	International Federation of Gynecology and Obstetrics
FITC	Fluorescein isothiocyanate
FLUO3/AM	Fluo-3-acetoxymethyl
FR_{aq}	Aqueous extract of *Ficus religiosa*
HBSS	Hank's Balance Salt Solution
HEPES	Hydroxyethyl piperazinethanesulfonic acid
HER-2	Human Epithelial Receptor-2
HM	Herbal Medicine
HPTLC	High Performance Thin Layer Chromatography
HPV	Human Papilloma Virus
HR-HPV	High Risk Human Papilloma Virus
HRP	Horse Raddish Peroxidase
HSIL	High-grade Intraepithelial Lesion
HSV	Herpes Simplex Virus
IARC	International Agency for Research on Cancer
IFN-γ	Interferon-gamma
IL	Interleukin
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl enzamidazolocarbocyanin iodide
LR-HPV	Low Risk Human Papilloma Virus
LSIL	Low-grade Squamous Intraepithelial Lesion
MMP-2	Matrix Metalloproteinase-2
MMuLV	Moloney Murine Leukemia Virus
MPCC	Medicinal Plants Conservation Center
MTT	4,5-dimethylthiazol-2-yl-2,5-diphenylthiazolium bromide
NCCS	National Centre for Cell Science
NF-kB	Nuclear Factor kappa-B

NK	Natural Killer
NO	Nitric Oxide
OD	Optical Density
ORAC	Oxygen Radical Absorbance Capacity
PARP	Poly ADP-Ribose Polymerase
PBMC	Peripheral Blood Monocyte Cells
PBS	Phosphate Buffered Saline
PCNA	Poliferating Cell Nuclear Antigen
PMSF	Phenylmethanesulfonyl fluoride
RNOS	Reactive Nitrogen and Oxygen Species
ROS	Reactive Oxygen Species
RT	Radiotherapy
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
TBARS	Thiobarbituric Acid Reactive Substances
TIMP	Tissue Inhibitor of Metalloproteinase

Index		
Sr. No	**Particular**	**Page**
Chapter 1	**Introduction and Review of literature**	
Section 1	**Cervical Cancer- An Overview**	**1**
1.1	Cervical cancer	2
1.1.1	Cervical cancer facts and figures	2
1.1.1.1	Global incidence of cervical cancer	2
1.1.1.2	Cervical cancer burden in India	5
1.1.2	Etiology of cervical cancer	7
1.1.2.1	Human Papilloma viruses (HPV) infection	7
1.1.2.1.1	Molecular Pathophysiology of HPV infection- Role of E6 and E7	8
1.1.2.2	Other Risk Factors	10
1.1.2.2.1	Smoking	10
1.1.2.2.2	Parity	11
1.1.2.2.3	Sexually transmitted infections	11
1.1.2.2.4	Diet	11
1.1.2.2.5	Oral contraceptives	12
1.1.3	Development of Cervical cancer	12
1.1.4	Current therapies for cervical cancer	16
1.1.4.1	Surgery	16
1.1.4.2	Radiotherapy	16
1.1.4.3	Chemotherapy	17
1.1.4.4	Combination therapy	17
Section 2	**Medicinal Plants in Cervical Cancer- An Overview**	**19**

List of Tables

List of Figures

CHAPTER-1

INTRODUCTION AND REVIEW OF LITERATURE

The overview given in the chapter has been published in a book

Noninvasive Molecular Markers in Gynecologic Cancers (2014)

SECTION 1

Cervical Cancer- An Overview

1.1 Cervical cancer

Cervical cancer is a malignant neoplasm arising from the cells in the cervix. It has a major impact on the lives of women worldwide, particularly those in developing countries (Mukakalisa et al., 2014). The incidence of cervical cancer begins to rise at ages 30–39 and peaks in the fifth or sixth decade of life (Foley et al., 2011). The effective cervical cancer screening programs, which can detect and treat the precancerous lesions at an early stage, has dramatically reduced the incidence as well as death (Vicus et al., 2014). However, prognosis for the advanced cancer remains poor despite several efforts to improve treatment outcomes.

1.1.1 Cervical cancer facts and figures

1.1.1.1 Global incidence of cervical cancer

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females worldwide (Jemal et al., 2011). According to the Human Papillomavirus and Related Diseases Report 2014, globally estimations for cervical cancer have estimated 527,624 new cases and 265,653 deaths in 2008 year (Bruni et al., 2014a). The age-standardized incidence rates (ASR) of cervical cancer differs significantly between countries from high-risk regions i.e, Eastern Middle and Southern Africa (ASR greater then 30 per 100,000), Western Africa (29.3 per 100,000), Southern Asia (12.7 per 100,000), South and Central America (20.3 and 23.5 per 100,000 respectively) to low-risk regions i.e, Western Asia, Northern America and Australia/New Zealand (ASRs less than 7 per 100,000) (Bruni et al., 2014a) (Figure 1.1). Table 1.1 describes the key cervical cancer statistics in the world.

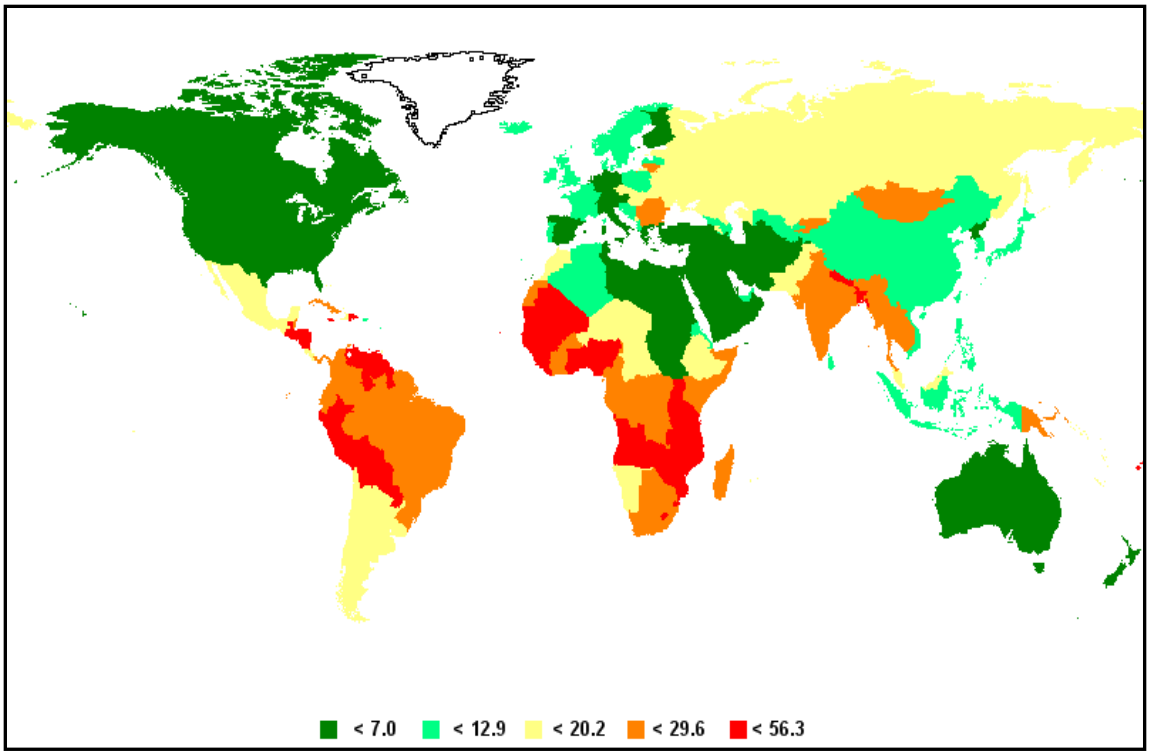


Figure 1.1: Cervical cancer age standardized incidence rates per 100,000 women *(Bruni et al., 2014a). Figure shows the age-standardized incidence rates of cervical cancer in the year 2008. The highest (>30 per 100,000) and lowest incidence rates (< 6 per 100,000) was found in Africa and Australia/New Zealand, respectively.*

Table 1.1: Key cervical cancer statistics worldwide (Bruni et al., 2014a).

Population	World	Developing regions	Developed regions
Number of women at risk for cervical cancer (Female population aged ≥15 yrs) (in millions)	2540.900	2001.450	539.440
Burden of cervical cancer			
New cases recorded per year	527,624	444,546	83,078
Cervical cancer deaths per year	265,653	230,158	35,495
Number of new cervical cancer cases anticipated in 2025*	720,060	668,875	81,868
Number of cervical cancer deaths anticipated in 2025*	395,095	380,653	38,291
Burden of cervical HPV infection			
Incidence (%) of HPV in the general population (women with normal cytology)	14	15.7	9.9
Incidence (%) of HPV 16/18 among women with-			
I. Normal cytology	5.4	5.5	5.9
II. Low-grade cervical lesions (LSIL/CIN-1)	25.8	24.6	26.1
III. High-grade cervical lesions (HSIL/ CIN-2 / CIN-3 / CIS)	52.2	47.6	53.8
IV. Cervical cancer	72.6	74.0	71.5

LSIL- low-grade intraepithelial lesions; HSIL- high-grade intraepithelial lesions; CIN-2/3, cervical intraepithelial neoplasia grade 2 or 3; CIS, carcinoma in-situ.

*Projected burden in 2025 has been estimated by applying current population forecasts for the country and assuming that the current incidence/mortality rates of cervical cancer are constant overtime.

1.1.1.2 Cervical cancer burden in India

Cervical cancer is ranked as the second most frequent cancer in women in India (Bruni et al., 2014b). According to the Human Papillomavirus and Related Diseases Report 2014, around 432.20 million women (above 15 years of age) are at a risk of developing cervical cancer (Bruni et al., 2014b). The current estimates indicate approximately 122,844 new diagnosed cases and 67,477 deaths annually in India, accounting for nearly one-third of the global cervical cancer deaths. Indian women face a 2.4% cumulative lifetime risk and 1.4% cumulative death risk from cervical cancer (Bruni et al., 2014b). The mortality from cervical cancer in India relative to the world, and the South Asia region is considerably at a higher side (Bruni et al., 2014b). In India, approximately 84.1% of cervical cancer is due to the most common HPV types 16 and 18 (Bruni et al., 2014b). In year 2010, approximately 33,400 deaths were accounted due to cancer of uterine cervix with a highest mortality rate in the state of Tamil Nadu and lowest in Jammu and Kashmir (Table 1.2). By 2025, the mortality due to cervical cancer has been projected to increase from 74,118 to 132,745 deaths in 2002 (GLOBOCAN, 2008) The cervical cancer death rate of 16 per 100 000 suggests 0.7% risk of dying from cervical cancer for a 30-year old woman before she can attain 70 years of age (Dikshit et al., 2012). By contrast, the risk of dying during pregnancy for Indian women aged between 15-49 years is about 0.6% (Meeta et al., 2013).

Table 1.2: Incidence of cervical cancer by state and regions in India (Dikshit et al., 2012)

States and regions	Estimated cancer deaths in 2010	Age standardized cancer mortality rate per 100 000
Urban	**8000**	**14·7**
Rural	**25400**	**16·6**
Northeast states†	400	15·2
Himachal Pradesh	300	19·7
Assam	200	3·5
Punjab	800	16·7
Jammu and Kashmir	40	2·3
Karnataka	1800	16·5
West Bengal	2700	15·5
Uttar Pradesh	4000	13·6
Andhra Pradesh	3000	17·6
Uttarakhand	..	..
Tamil Nadu	5400	35·7
Kerala	900	11·1
Gujarat	2000	16·8
Delhi	300	9·4
Madhya Pradesh	1200	10·1
Maharashtra	3800	19·0
Other states‡	200	17·5
Chhattisgarh	900	24·6
Rajasthan	1000	10·3
Haryana	400	11·4
Bihar	2000	12·9
Odissa	900	10·9
Jharkhand	1100	19·5
Poorest states§	**11400**	**12·5**
Richest states¶	**22000**	**18·7**
Total	**33400**	**16·0**

Rates have been standardized to the world population. †Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Tripura, and Sikkim. ‡Andaman and Nicobar Islands, Chandigarh, Dadra and Nagar Haveli, Daman and Diu, Goa, Lakshadweep, and Pondicherry. §The poorest states are the Empowered Action Group of States (Bihar, Chhattisgarh, Jharkhand, Madhya Pradesh, Orissa, Rajasthan, Uttarakhand, and Uttar Pradesh) plus Assam. ¶Remaining 26 states or union territories.

1.1.2 Etiology of cervical cancer

Cervical cancer develops from well-defined precursor lesions referred to as either cervical intra-epithelial neoplasia (CIN) or squamous intra-epithelial lesions. The infection by high risk human papillomaviruses (HR-HPV) is the principal etiologic agent for cervical cancer. Besides HPV, the risk for cervical cancer is also influenced by other endogenous and exogenous cofactors such as long-term use of oral contraceptives, high parity, smoking, diet and sexually transmitted infections such as those caused by human immunodeficiency virus, chlamydia and herpes simplex virus Type 2.

1.1.2.1 Human Papilloma Viruses (HPVs)

The association between HPV infection and carcinoma of the cervix has its origin in the works of Prof. Zur Hausen in the early 1980s (Zur Hausen et al., 1983). Since then, several studies have established that persistent infection with HR-HPV is the major risk factor for the development of high-grade pre-cancerous and cervical carcinoma. Out of 100 HPV types identified, approximately 40 can infect the cervix (Munoz et al., 2003) HPVs have been classified as "low-risk" (wart-causing) or "high-risk" with regard to their clinical 'oncogenic potential' (De Villiers et al., 2004). Low-risk (LR) HPV types include 6, 11, 42, 43, and 44 and high-risk (HR) HPV types include 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70. Of the HR-HPV types, HPV 16 causes approximately 55% of cervical cancers worldwide, and types 16 and 18 together account for about 70% of cervical cancers (Smith et al., 2007).

Human Papillomaviruses are ~7900-base pair, double-stranded, circular DNA viruses that can cause warty changes in epithelia of many host species. The HPV

genome comprises of early genes and late genes. Early genes (E1, E2, E4, E5, E6 and E7) encode for non-structural proteins that participate in cell transformation, DNA replication, transcriptional regulation as well as virus assembly and release. The late genes, L1 (major) and L2 (minor), encode for viral capsid proteins (Zheng et al., 2006) (Figure 1.2).

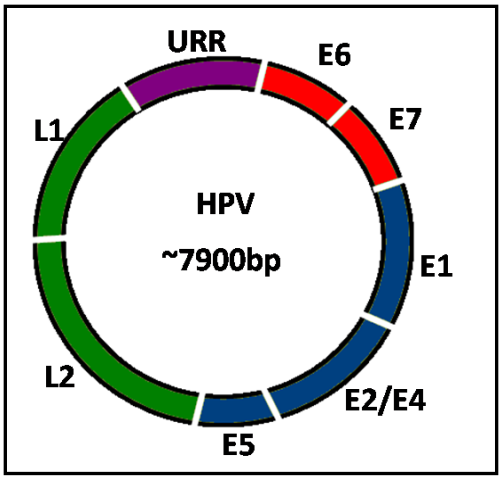


Figure 1.2: HPV genome showing early, late genes and URR (upstream regulatory region).

Based upon differences in their nucleotide sequences, HPVs are classified phylogenetically within the alpha, beta, gamma, delta and mu genera. 'Alpha' genus HPV types infect the genital and oropharnygeal mucosa exclusively and include the oncogenic HPV types associated with cervical cancer (De Villiers et al., 2004). Those HPVs that belong to the beta, gamma, and mu and nu groups are primarily related to cutaneous infections (De Villiers et al., 2004).

1.1.2.1.1 Molecular pathophysiology of HPV infection- Role of E6 and E7 oncoproteins

Cervical cancer is one of the best understood examples of how viral infection can lead to malignancy (Zur Hausen et al., 1983). The molecular mechanisms of oncogenic HPV infection are presented in Figure 1.3. p53 tumor suppressor protein,

restricts the growth of cells damage in their DNA; it either induces apoptosis or arrests the cell growth (Levine et al., 1994). Similar to p53, the tumor suppressor pRb also regulates the growth of the cells having damaged DNA by either inducing G_1 phase arrest or cell death (Genovese et al., 2006). E6 and E7 are the two viral oncoproteins known to interfere with the normal cell cycle machinery through biochemical interactions with several important cellular molecules (Lee et al., 2010; Huh et al., 2007). Prolonged cervical infection with HR-HPV genotypes is associated with viral DNA integration, which is a major factor responsible for the development of the pre-cancerous and cancerous lesions (Bosch et al., 2002). Integration of HPV-DNA disrupts or deletes the E2 region, which results into loss of its expression and an increase in E6 and E7 gene expression (Yoshinouchi et al., 1999).

The expression of E6 oncoprotein leads to degradation and inactivation of p53 protein through its association with the cellular E6-associated protein via the ubiquitin proteolytic pathway (Lee et al., 2010). In absence of p53, epithelial cells fail to recognize senescence or apoptosis signals and continue to divide throughout the stratified squamous epithelial layers. E7 has been reported to function in tandem with E6 to drive continuous division of differentiated epithelial cells. The HPV E7 inactivates pRb by blocking pRb-E2F binding, releasing the transcription factor E2F that controls numerous cellular processes (Huh et al., 2007).The inactivation of p53 and pRb proteins can give rise to an increased proliferation rate and genomic instability. As a consequence, the host cell accumulates more damaged DNA that cannot be repaired, leading to the transformed cells (Park et al., 1995).

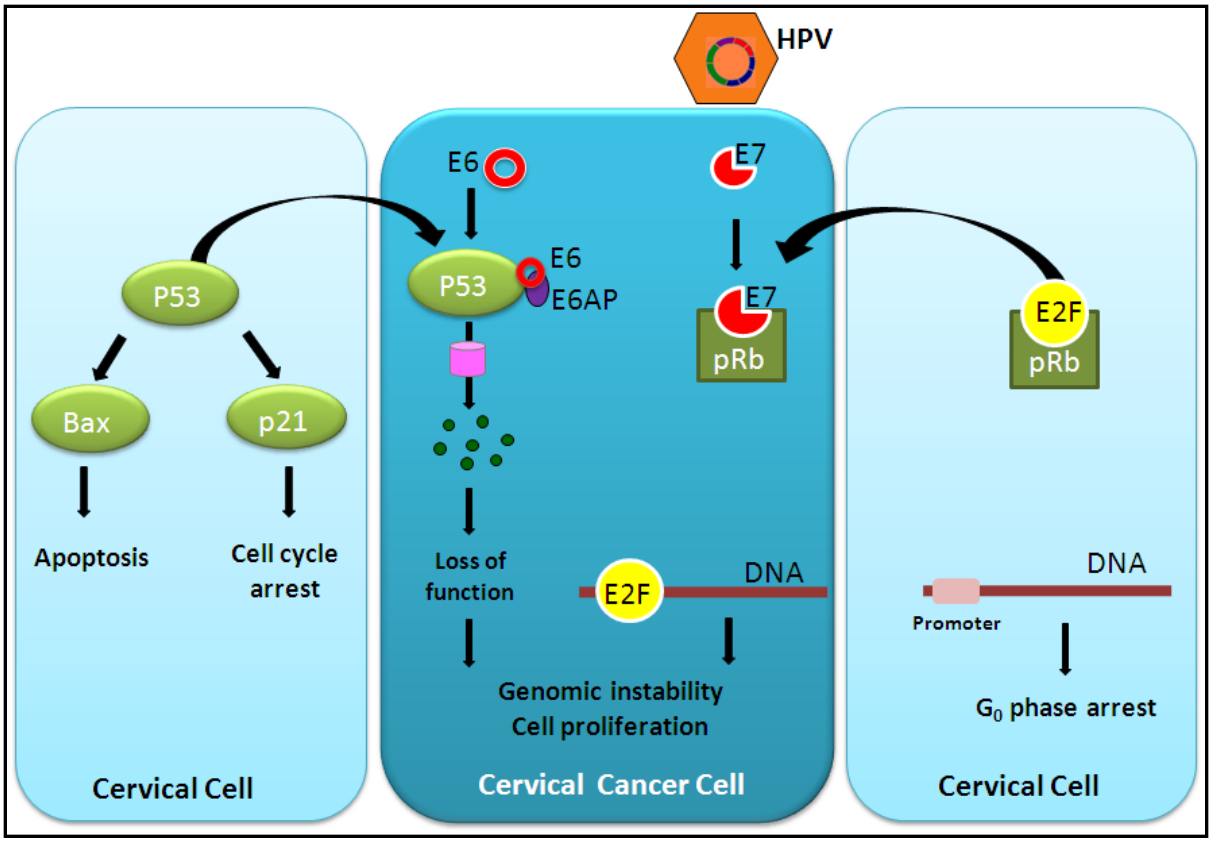


Figure 1.3: Molecular mechanisms of oncogenic HPV infection.

In addition to viral oncogenes (E6 and E7), other potential mechanisms contributing to the transformation in cervix cells include methylation of viral and cellular DNA, telomerase activation, and hormonal and immunogenetic factors (Burd et al., 2003).

1.1.2.2 Other risk factors

1.1.2.2.1 Smoking

The effect of smoking has been well studied and shows a strong association with cervical carcinogenesis (Sikström et al., 1995; Sasson et al., 1985; Hellberg et al., 1988; Yang et al., 1996; Vaccarella et al., 2008). The risk of cervical cancer is twice among women who smoke as compared to non-smokers. The strong beneficial effect of quitting smoking has been determined in cervical cancer patients (Vaccarella

et al., 2008). The possible reason might be tobacco by-products, such as cotinine and nicotine that may exert mitogenic effect by activating carcinogenic nitrosamines resulting into DNA damage and local immune suppression in the cervical epithelium.

1.1.2.2.2 Parity

High parity is also an important risk factor risk of squamous-cell carcinoma of the cervix among HPV-positive patients (Vaccarella et al., 2006). Women who had 3 or more full-term pregnancies have an increased risk of this cancer (Paramita et al., 2010). The possible reasons proposed are trauma to the cervix or hormonal effects (Paramita et al., 2010).

1.1.2.2.3 Sexually transmitted infections

Several clinical case control studies have shown that co-infection with sexually transmitted agents such as Chlamydia trachomatis (Lehtinen et al., 2011), Human immunodeficiency virus (HIV) (Ramogola-Masire et al., 2011; Zhao et al 2012) and herpes simplex virus type 2 (HSV) (Smith et al., 2002; Atalah et al., 2001) could support the persistence of HPV infection, and increase the risk of developing cervical cancer.

1.1.2.2.4 Diet

There is no evidence of diet from prospective studies on risk of cervical cancer, but there is a suggestion of a protective effect of some fruits and vegetables against HPV persistence. Some case-control studies suggest intake of fresh fruits (Atalah et al., 2001; Hirose et al., 1998) or citrus fruits (Rajkumar et al., 2003), total vegetables (Atalah et al., 2001), carrots (Atalah et al., 2001; Hirose et al., 1998)

cruciferous (Atalah et al., 2001; Rajkumar et al., 2003), garlic (Hernandez et al., 2004) and leafy vegetables (Brock et al., 1988). The possible explanation is that the dietary factors might be regulating carcinogenesis by enhancing mucosal immune response to infection or could act as efficient scavengers of free radicals and oxidants.

1.1.2.2.5 Oral contraceptives

Large-scale epidemiological evidence has shown that long term use of oral contraceptives significantly increases the risk of cervical carcinoma by up to four-fold in women who are positive for cervical HPV DNA (Appleby et al., 2007). However, the risk decreases significantly with decrease use of oral and/or injectable contraceptives (Appleby et al., 2007).

1.1.3 Development of cervical cancer

Cervical cancer begins in the lining of the cervix upon HPV infection and is associated with cellular transformation from the normal to pre-cancerous and cancerous phenotype (Figure 1.4). In a large number of cases, these changes in cell structure are not harmful and go away over time (Lehn et al., 1988). During the normal virus life cycle, HPV genomes are found as episomes in the nucleus of infected cells of the normal cervix. However, in some low-grade and in most of the high-grade lesions, HPV genomes are integrated into the host cell genome (Bosch et al., 2002).The integration of HR-HPV DNA leads to the development of invasive cervical cancers (Yoshinouchi et al., 1999).

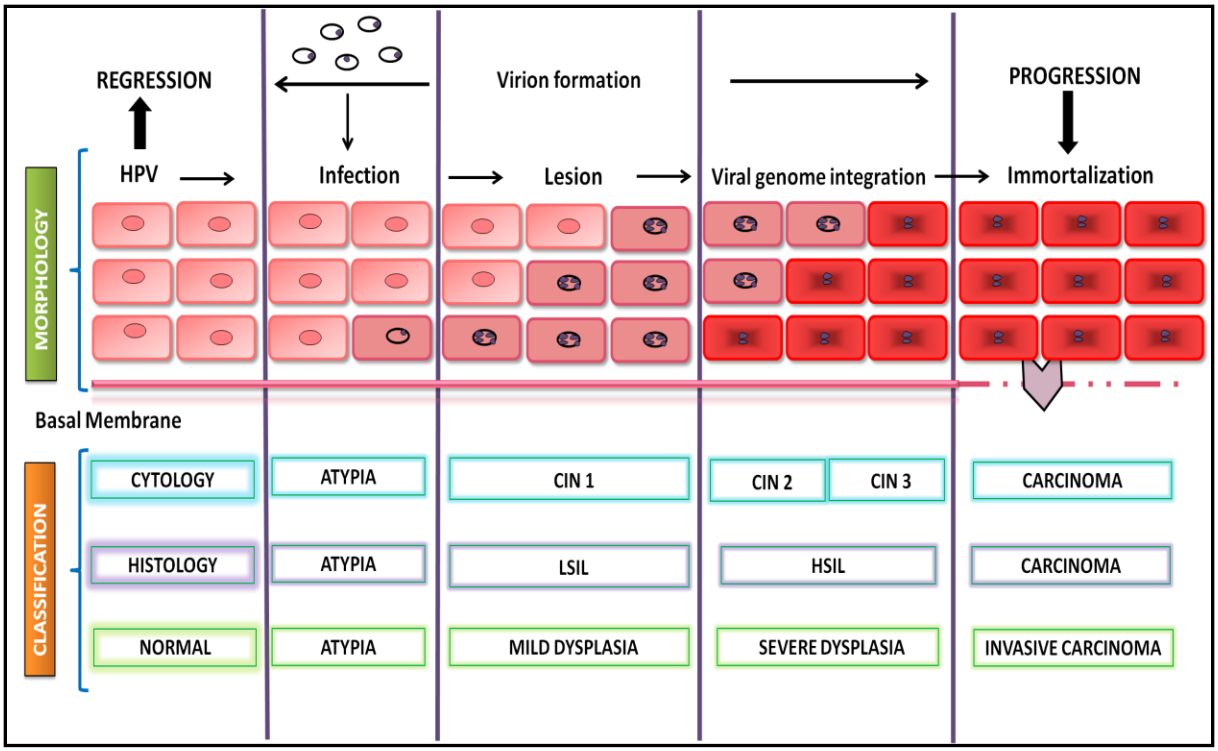


Figure 1.4: Model of cervical carcinogenesis. *Differences in epithelium morphology during the process of cervical carcinogenesis induced by HR-HPV infection with histological and cytological classification.*

These cellular changes and processes are classified by two systems:

The Cervical Intrapithelial Neoplasia (CIN) system (Buckley et al., 1982), grades the changes based on spread of atypical cellular changes in the basal epithelium. It histologically grades cervical intraepithelial neoplasia (CIN) as **(a)** mild dysplasia (CIN 1), wherein one-third of the epithelium is affected; **(b)** moderate dysplasia (CIN 2), involving two-thirds of the epithelium; and **(c)** severe dysplasia (CIN 3), involving more than two-thirds of the epithelium (Buckley et al., 1982).

The Bethesda System (TBS) (Solomon et al., 2004), based on the cytology grades the changes in the cervical cells into several different categories: **(a)** ASC-US (atypical squamous cells of undetermined significance), **(b)** AGUS (atypical glandular cells of undetermined significance), **(c)** LSIL (low-grade squamous intraepithelial

lesion) and **(d)** HSIL (High-Grade intraepithelial lesion). ASC-US is characterized by mild changes in the cell cytology, the cause of which is unknown. AGUS refers to glandular cells that may originate in the cervical canal or uterus. LSIL is a mild dysplasia wherein HPV infection may get cleared up on its own within two years (Solomon et al., 2004). HSIL is an immediate pre-cancerous lesion carrying a high risk of progression to invasive disease, if left untreated.

The invasive form of carcinoma is further classified into various stages, as per the International Federation of Gynecology and Obstetrics (FIGO) (Pecorelli et al., 2009), which is usually used to decide the treatment methods (Table 1.3).

Table 1.3: FIGO staging of cervical cancer (Pecorelli et al., 2009).

Stage	Sub Stages	Description
Early Stage	IA1	Carcinoma confined to the cervix, diagnosed only by microscopy. Measured invasion of stroma <3 mm in depth and lateral spread <7 mm
	IA2	Carcinoma confined to the cervix, diagnosed with microscopy. Measured invasion of stroma >3 mm and < 5 mm with lateral spread <7mm
	IB1	Clinically visible lesion or greater than A2, <4 cm in greatest dimension
	IB2	Clinically visible lesion, >4 cm in greatest dimension
	IIA1	Involvement of the upper two-thirds of the vagina, without parametrial invasion, <4 cm in greatest dimension
	IIA2	Involvement of the upper two-thirds of the vagina, without parametrial invasion, >4 cm in greatest dimension
Late Stage	IIB	Involvement of the upper two-thirds of the vagina, with parametrial invasion, >4 cm in greatest dimension
	IIIA	Cancer spread to lower third of vagina, but not to the pelvic wall
	IIIB	Cancer extends to pelvic wall and/or blocks urine flow to bladder
	IVA	Cancer spreads to bladder or rectum
	IVB	Cancer spreads to distant organs, e.g. lungs

1.1.4 Current therapy for cervical cancer

Cervical cancer is curable if detected and treated at an early stage; about 80% of those detected at the early stage are cured with suitable treatment methods. The choice of treatment modality depends upon the size and location of the tumor, stage of the disease as well as age and general health of the patient. The various treatment options include surgery, radiotherapy, chemotherapy and combination therapy.

1.1.4.1 Surgery

Surgery is recommended for small tumor in the cervix especially when the patient has no sign of metastasis in the lymph node (Landoni et al., 1997). Surgery is usually associated with several side-effects such as cramping, pain, bleeding, and difficulty in emptying bladder and so on.

1.1.4.2 Radiotherapy

Radiation therapy uses high-energy photons (X-rays and gamma-rays) or particle (electrons, protons, neutrons, carbon ions, alpha particles, and beta particles) radiation to destroy or injure cancer cells in the body (Halperin et al., 2006). The advanced stage of cervical cancer is usually treated with radiation therapy (Halperin et al., 2006). In some cases where the tumor is very large or has spread into the tissues around the cervix, radiotherapy is performed along with surgery or in combination with chemotherapy (Frumovitz et al., 2014). Most women (approximately 70-80%) experience diarrhea and dysuria as a common side effect during radiation treatment (Vaz et al., 2008). Radiotherapy also result in local side effects such as shortening, drying and adhesions of the vagina, symptoms of menopause, vaginal spotting and may even cause infertility (Rubin et al., 2014).

1.1.4.3 Chemotherapy

Chemotherapy includes use of cytotoxic drugs to kill or slow the growth of cancer cells. Cisplastin is the commonly used chemotherapic drug to treat cervical cancer (Cornelio et al., 2009). Other chemotherapic drug such as carboplatin, nedaplatin, paclitaxel, gemcitabine, capecitabine, vinorelbine, ifosfamide and topotecan are also used either alone or in combination (Cornelio et al., 2009). Chemotherapy is frequently associated with side effects such as vomiting, nausea, myelosuppression, alopecia and gastrointestinal symptoms (Marchiole et al., 2011). Chemotherapy may also cause periods to stop, either temporarily or permanently, causing premature menopause (Sklar et al., 2005).

1.1.4.4 Combination therapy

Randomized trials have suggested use of chemotherapy prior to radical hysterectomy and radiation therapy (Marchiole et al., 2011). However, in locally advanced disease, radiotherapy alone fails to control progression of cervical cancer. So, adjuvant cisplatin-based chemotherapy for patients undergoing radiotherapy is commonly practiced (Sklar et al., 2005). Table 1.4 summarizes the current therapeutic options and the new perspectives for cervical cancer treatment according to FIGO stage. Combined radiotherapy and chemotherapy can cause more side effects than radiotherapy alone. The side effects include a reduced number of white blood cells (leucopoenia), nausea and vomiting.

Table 1.4: Current therapeutic options and new perspectives for cervical cancer treatment (Cornelio et al., 2009).

FIGO Stage	Current Therapeutic Options	New Perspectives
IA - IB1	S or RT	------
IIA IB2	S + adjuvant RT	Neoadjuvant CT + S + RT
IIA	CT (with cisplatin) + RT + adjuvant S	Combination CT + RT Other CT agents* Biologic agents**
IIB - IVA	CT (cisplatin) + RT	Neoadjuvant CT Combination CT + RT Other CT agents* Biologic agents**
IVB	Palliative CT (cisplatin)	Combination CT Other CT agents* Biologic agents**

S: Surgery; RT: Radiotherapy; CT: Chemotherapy.

*including carboplatin, nedaplatin, paclitaxel, gemcitabine, capecitabine, vinorelbine, ifosfamide and topotecan.

** including cetuximab, bevacizumab, erlotinib, gefitinib, lapatinib, sorafenib and celecoxib.

SECTION 2

Medicinal Plants in Cervical Cancer- An Overview

1.2. Complementary and Alternative Medicine (CAM)

Complementary and Alternative Medicine (CAM) is **"a group of diverse medical health care systems, practices, and products"** that are not presently considered to be a part of conventional medicine; these can be used together with or in place of conventional medicine (NCCAM 2009a). The National Center for Complementary and Alternative Medicine has classified CAM therapies into five categories (NCCAM 2009b):

1. Alternative medical systems, such as traditional Chinese medicine or Ayurveda;
2. Mind–body interventions, such as meditation, prayer, healing or support groups;
3. Biologically based therapies, such as herbs, dietary supplements or vitamins;
4. Manipulation and body-based methods, such as massage, chiropractic or osteopathy; and
5. Energy therapies (i.e. biofield therapies such as Qi Gong and Reiki or bioelectromagnetic-based therapies such as magnetic fields).

Different CAM approaches are being used by a large percentage of people for various ailments. Well-known CAM modalities include acupuncture, aromatherapy, osteopathy, yoga, and herbal supplements (NCCAM 2009b). The National Health Interview Survey (2007) reported the use of natural products to be the most commonly used CAM treatments (Barnes et al., 2008)(Figure 1.5).

1.2.1. Prevalence of Complementary and Alternative Medicine

Over the last decade, there has been a steady and substantial increase in the use of complementary and alternative medicine. There seem to be differences in CAM usage, based on age, sex, income level, and educational status. The prevalence of CAM use vary widely in the literature from 6 to 84% (Boon et al., 2007; Samdup et

al., 2006; Foltz et al., 2005; Park et al., 2000; Harris et al., 2000; Roth et al., 2008; Quan et al., 2008; Millar et al., 2001), and broadly show that women (Park et al., 2000; Roth et al., 2008; Millar et al., 2001; Wiles et al., 2001; McFarland et al., 2002), those with high education levels (Park et al., 2000; Roth et al., 2008; Millar et al., 2001; Wiles et al., 2001; McFarland et al., 2002), those with high incomes (Foltz et al., 2005; Park et al., 2000; Wiles et al., 2001), certain ethnic groups (Roth et al., 2008; Quan et al., 2008) and those with a chronic condition (Park et al., 2000; Quan et al., 2008; Millar et al., 2001) are more likely to use CAM than the general population.

In cancer patients, the prevalence of CAM has been reported to vary from 40 to 83% (Arthur et al., 2012; Mao et al., 2007; Henderson et al., 2003) Supplement of vitamins and herbal remedies are the most frequently used, followed by mind-body medicine (Owens et al., 2009; Helyer et al., 2006; Montazeri et al., 2003) and alternative medical systems (Cui et al., 2004). Energy therapies (e.g., Qi gong, Reiki, therapeutic touch) have been ranked among the least used (Wanchai et al., 2004).

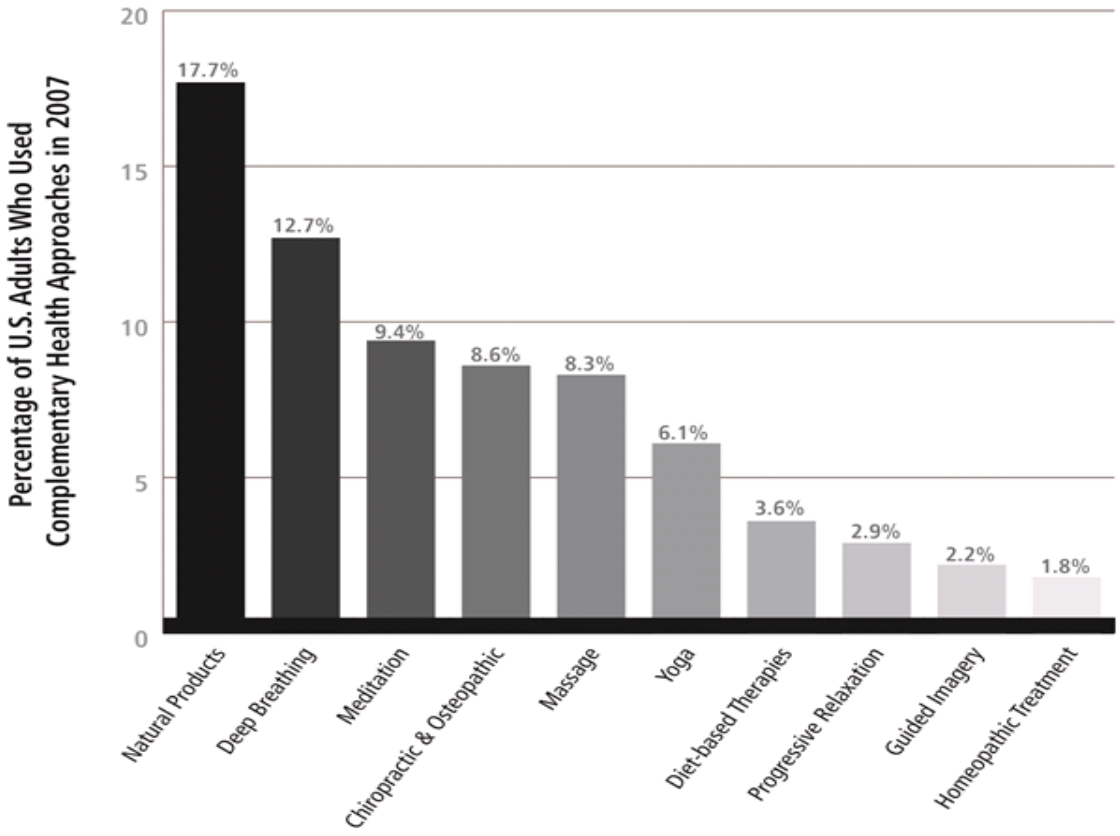


Figure 1.5: Common Complementary and Alternative Medicine among adults-2007 (Barnes et al., 2008).

1.2.2. Reason for use of CAM by cancer patients

CAM therapies have been found to help in recovering, healing, and improving health (Gulluoglu et al., 2009; Helyer et al., 2006; Navo et al., 2004), boosting the immune system (Rakovitch et al., 2004; Henderson et al., 2003), reducing the side effects of conventional therapies (Chen et al., 2008; Kremser et al., 2008; Molassiotis et al., 2006) improving emotional health of the patient (Lengacher et al., 2006; Van der Weg et al., 2003), reducing physical and psychological distress (Henderson et al., 2003; Lengacher et al., 2006), curing cancer (Cui et al., 2004, Rakovitch et al., 2004), and as an adjunct to conventional treatment (Rakovitch et al., 2004; Abdullah et al., 2003).

1.2.3. Potential of Herbal Medicines in Cancer

In recent age of targeted cancer therapy, despite the massive drug-development efforts, the numbers of successful drugs have not significantly increased (Giodano et al., 2008). The main reason has been its focus on target selection rather than clinical safety and efficacy. As cancer is a multistep process, involving alteration in number of genes, it raises an important question on the effectiveness of these targeted therapies. Thus, it has become necessary to rethink drug development strategies and emphasize upon natural and herbal medicines that possess multi-target capability.

Among various CAM therapies used, herbal remedies with either single herb or polyherbal formulations are playing prime role in the healthcare system (Moschèn et al., 2006; Lengacher et al., 2006; Van der Weg et al., 2003). In contrast to western medicine, herbal medicine (HM) does not focus on a single target but on multiple targets involved in a particular disease condition. The major reason for lack of enthusiasm of western academia towards HM is availability of insufficient data on clinical efficacy studies (Bent et al., 2008). However, the scenario is changing rapidly, and a number of in vitro, *in vivo* and clinical studies are being conducted with HM that provide their potential for prevention and treatment of cancer (Fujiki et al., 2003; Kumar et al., 2013; Li et al., 2011). The protective effects of HM have been attributed to the presence of phytochemicals and bioactive non-nutrient plant compounds present in them. These components have complementary and overlapping mechanisms of action, including free radical scavenging (Chaudhary et al., 2014), antimutagenesis (Sarac et al., 2014) induction of apoptosis (Li-Weber et al., 2013) and cell cycle arrest (Cao et al., 2013) in cancer cells. However, herbal remedies are yet to be integrated into main stream medicine due to a number of challenges, including

standardization, quality control issues, safety and toxicity concerns, interactions with existing therapeutic modalities, lack of proven clinical efficacy and mechanistic details (Sen et al., 2011). The recent clinical trials with HM focus on three major aspects of cancer research: (1) improving the response of cancer cells towards standard chemo- and radiotherapy, (2) reducing the severe adverse effects of standard cancer therapy, and (3) looking for unwanted interactions of standard therapy with herbal medicines. In addition, appropriate quality assurance and sustainable production methods are other important pre-conditions for the acceptance of HM in cancer therapy.

Various studies have tested the efficacy of medicinal plants (Table 1.5) and herbal formulations (Table 1.6) in cervical cancer. In most of the studies, the anti-cancer action of plant extracts has been shown in human cervical cancer cell line, HeLa. The other cervical cancer cell lines such as SiHa, C33A and CaSki as well as murine cervical carcinoma cell line U14 have also been used. Table 1.5 contains detailed information of plants and their parts used in various types of cervical cancer cell lines along with their mode of action. Very few herbal formulations have been studied in cervical cancer, of which some have been tested at clinical level as well (Table 1.6).

Table 1.5: Anti-cancer activity of the extracts from different plants in cervical cancer

Botanical name	Plant part	Extract	Cell line	Mechanism of action	References
Rubus occidentalis	Fruit	Ethanol	HeLa, SiHa, C33A	Antiproliferative activity	Zhang et al., 2011
Croton lechleri	Leaf	Methanol	HeLa	Cytotoxic effect	Alonso-Castro et al., 2012
Cremanthodium humile	Flower	Ether	HeLa	Release of cytochrome c, activation of caspase-3, 7 and 9, generation of ROS and induction of apoptosis	Li et al., 2007
Zingiber officinale	Rhizome	Aqueous	HeLa	Inhibition of microtubule structure and functions, increase in sub-G_0/G_1phase and apoptotic cell population	Choudhury et al., 2010
Cordyceps pruinosa	Fruiting body	Butanol	HeLa	Decrease of Bcl-2 protein, increase of Bax protein, release of cytochrome-c and AIF, activation of caspases-3 and -9 resulting in apoptosis	Kim et al., 2010
Ficus hirta	Root	Aqueous, Ethylacetate, butyl alcohol	HeLa	Inhibition of cell viability, induction of morphology changes and increase of sub-G_1phase	Zeng et al., 2012
Scutellaria lindbergii	Root	Methanol	HeLa	Antiproliferation activity through induction of sub-G_1 phase arrest	Tayarani-Najaran et al., 2010

Table 1.5: Continued…

Botanical name	Plant part	Extract	Cell line	Mechanism of action	References
Pinellia pedatisecta	Rhizome	Lipid soluble extract	CaSki, HeLa	Decreased HPV E6 mRNA and protein expression, increased caspase-8, 3, Bax, p53 and p21; with decrease of Bcl-2 mRNA and protein resulting in apoptosis	Li et al., 2007
Mangifera indica	Peel	Ethanol	HeLa	Increase in sub-G_1 phase and apoptosis cell population, downregulation of Bcl-2 expression, activation of caspase	Kim et al., 2012
Nigella sativa	Seed	Methanol, n-hexane chloroform	HeLa	Regulation of the expression of pro- and anti-apoptotic genes and immune-modulatory activity	Shafi et al., 2009
Corallina pilulifera	Seaweed	Ethanol	HeLa	Induction of apoptosis through the mitochondria-dependent pathway and downregulation of DNA topoisomerase IIa gene expression	Kwon et al., 2007
Citrus grandis	Leaf	Chloroform	HeLa	Downregulation of Bcl-2 expression, activation of caspases and degradation of PARP protein leading to apoptosis	Kim et al., 2010
Cassia tora	Leaf	Methanol	HeLa	Reduction of DNA content and caspase-3 activity	Rejiya et al., 2009

Table 1.5: Continued…

Botanical name	Plant part	Extract	Cell line	Mechanism of action	References
Pterocarpus santalinus	Stem	Methanol	HeLa	Release of cytochrome c, activation of caspases-9 and 3	Kwon et al., 2006
Crocus sativus	Stigma	Ethanol	HeLa	Induction of a sub-G_1 phase arrest and ROS production leading to apoptosis	Tavakkol-Afshari et al., 2008
Pinus massoniana	Bark	Aqueous	HeLa	Decrease in the migration rate, induction of cell cycle arrest and apoptosis, increase of Bax, downregulation of Bcl-2 expression and activation of caspase-9 and -3	Wu et al., 2011; Ma et al., 2008
Phaseolus vulgaris	Seed	Methanol	HeLa	Increase of Bax and caspase-3 expression	Aparicio-Fernández et al., 2008
Duchesnea indica	Whole plant	Phenolic	C-33A, HeLa, U14	Upregulation of Bax, downregulation of Bcl-2 expression, release of cytochrome c and induction of apoptosis. S-phase arrest, decrease of PCNA and ki-67 expression. Reduced tumor weight.	Peng et al., 2009
Solanum nigrum	Whole plant	Aqueous	U 14	Modulated immune response of tumor-bearing mice, caused G_0/G_1 phase arrest and Induction of apoptosis	Li et al., 2008

Table 1.5: Continued…

Botanical name	Plant part	Extract	Cell line	Mechanism of action	References
Triticum aestivum	Wheat sprout	Hydro alcoholic	HeLa	Induction of all proteasome activities gradual inhibition	Bonfili et al., 2009
Livistona chinensis	Seed	Ethanol	HeLa	Induction of apoptosis	Huang et al., 2007
Abrus precatorius	Seed	Abrus agglutinin peptide fraction	HeLa	Induced ROS generation, decreased Bcl-2/Bax ratio, mitochondrial permeability transition, activate caspase-3, DNA fragmentation and cell apoptosis	Bhutia et al., 2008
Phryma leptostachya	Whole plant	Ethanol	HeLa	Downregulation of Bcl-2 and activation of caspase-3	Yu et al., 2012
Artemisia afra	Leaf	Ethanol	HeLa	Caspase activation and G_2/M phase arrest	Spies et al., 2013
Agrimonia eupatoria	Leaf, stem flower	Aqueous and methanol	HeLa	Antiproliferative activity	Ad'hiah et al., 2013

Table 1.6: Anti-cancer activity of different herbal formulations in cervical cancer

Herbal formulation	Composition	Cell type	Mechanism of action	References
Guizhi-Fuling decoction	*Cinnamomum cassia Blume, Paeonia lactiflora Pall, Paeonia suffruticosa Andrews, Prunus persica Batsch, Poria cocos Wolf.*	HeLa	Enhancement of TIMPs expression and activation, downregulation of MMPs expression and activation	Yao et al., 2008
Ge-Jee-Bok-Ryung-Hwan	*Cinnamomi Ramulus, Pachyma hoelen Rumphius, Moutan Cortex Radicis*	HeLa	Induction of apoptosis in HeLa cells via ER stress-pathway associated mitochondria-dependent pathway	Chae et al., 2004
Tien-Hsien Liquid	*Cordyceps sinensis, Oldenlandia diffusa, Indigo pulverata levis, Polyporus umbellatus, Panax ginseng, Solanum nigrum, Pogostemon cablin, Atractylodis macrocephalae*	C33A	Cytotoxicity	Sun et al., 2005
Compound matrine capsule	*Sophora flavescens Ait., Rhodiola rosea, Acanthopanax senticosus*	HeLa	Induction of sub-G_1 peak and S phase arrest	He et al., 2012
Erhuangsan	*Coptis chinensis*	HeLa	Downregulation of Bcl-2 protein	Chen et al., 2010

Table 1.6: Continued…

Herbal formulation	Composition	Cell type	Mechanism of action	References
Kung Ching Tang	*Achyranthes bidentata, Angelica sinensis, Coix lacryma-jobi, Curcuma zedoaria, Cyperus rotundus, Dipsacus asper, Laminaria japonica, Prunela vulgaris, Prunus persica, Sparganium stoloniferum, Vaccaria segetalis.*	Uterine cervical neoplasia (Clinical study)	Disappearance of symptoms and reduction in tumor size	Wu et al., 1981
Praneem polyherbal formulation	Purified extracts of *Azadirachta indica*	LSIL (Clinical study)	Clearance of viral DNA from pre-cancer lesions	Shukla et al., 2009
Basant	Diferuloylmethane (curcumin), purified extracts of *Emblica officinalis* (Amla), purified saponins from *Sapindus mukorossi*, Aloe vera and rose water	HeLa cells, LSIL (Clinical study)	Inhibition of oncogenic and viral transcription through AP-1 and NF-λB pathway, Block viral entry in cervical cells	Talwar et al., 2008; Basu et al., 2013
Rasagenthi Mezhugu (RGM)	Preparation contain 38 different botanicals and 8 inorganic compounds such as mercury, sulphur, calomel (mercury chloride), yellow orpiment (arsenic sulphide),lodestone (iron), blue vitriol (copper sulfate), calamine (zincoxide) and litharge (lead oxide), all prepared into a paste inpalm sugar and hen's egg base	ME-180 and SiHa	Induced DNA damage and Mitochondria-mediated apoptosis	Riyasdeen et al., 2012

SECTION 3

Objectives and Organization of the Thesis

1.3.1. Rationale of the study

Recent developments in the use of herbal medicines for the prevention and/or treatment of cancer has aroused a lot of interest in this area. There are a number of herbs and plants which are traditionally used in managing various gynaecological disorders, however, their efficacy and mechanistic actions in cervical cancer have not been tested specifically. This formed the basis of the present study.

The medicinal plants used in our study include Cinnamon **(*Cinnamomum zeylanicum* and *Cinnamomum cassia)* and *Ficus religiosa.*** Both of these plants have been used traditionally for various disorders. We undertook a multifaceted approach to study the anti-cancer potential of Cinnamon and Ficus in cervical neoplasia. **We hypothesized that the plants would target important signaling pathways in cancer as well as biological markers whose deregulation leads to cervical cancer.** This study would help in evaluation of medicinal plants for prevention, cure and better management of cervical cancer.

1.3.2. Aims and Objectives

1. To analyze the antineoplastic potential of *C. zyelanicum* and *C. cassia* in vitro in cervical cancer cell lines.
2. To analysis the antineoplastic potential of *Ficus religiosa* in vitro in cervical cancer cell lines.
3. To develop a novel herbal formulation having antineoplastic potential in vitro in cervical cancer cell lines and *in vivo* in mouse melanoma model.

1.3.3. Organization of the thesis

Chapter 1: Introduction and review of literature

Chapter 1 gives information regarding cervical cancer and herbal medicines for the treatment of cervical cancer by providing facts and figures of cervical cancer, its etiology, process of development and current therapies for treatment. This is followed by importance of Complementary and Alternative Medicine (CAM) with a special emphasis on herbal medicine in the treatment of cervical cancer. The objectives and organization of the thesis are given at the end.

Chapter 2: Elucidating the anti-cancer potential of Cinnamon in SiHa cells

Chapter 2 analyzes the anti-cervical cancer potential of the bark of *Cinnamomum cassia*. The studies include different molecular and cellular level experiments in human cervical cancer cell line SiHa to detail out anti-cancer mechanism of aqueous cinnamon extract (ACE-*c*). The chapter discusses the important findings wherein, cinnamon was found to induce apoptosis in cervical cancer cells.

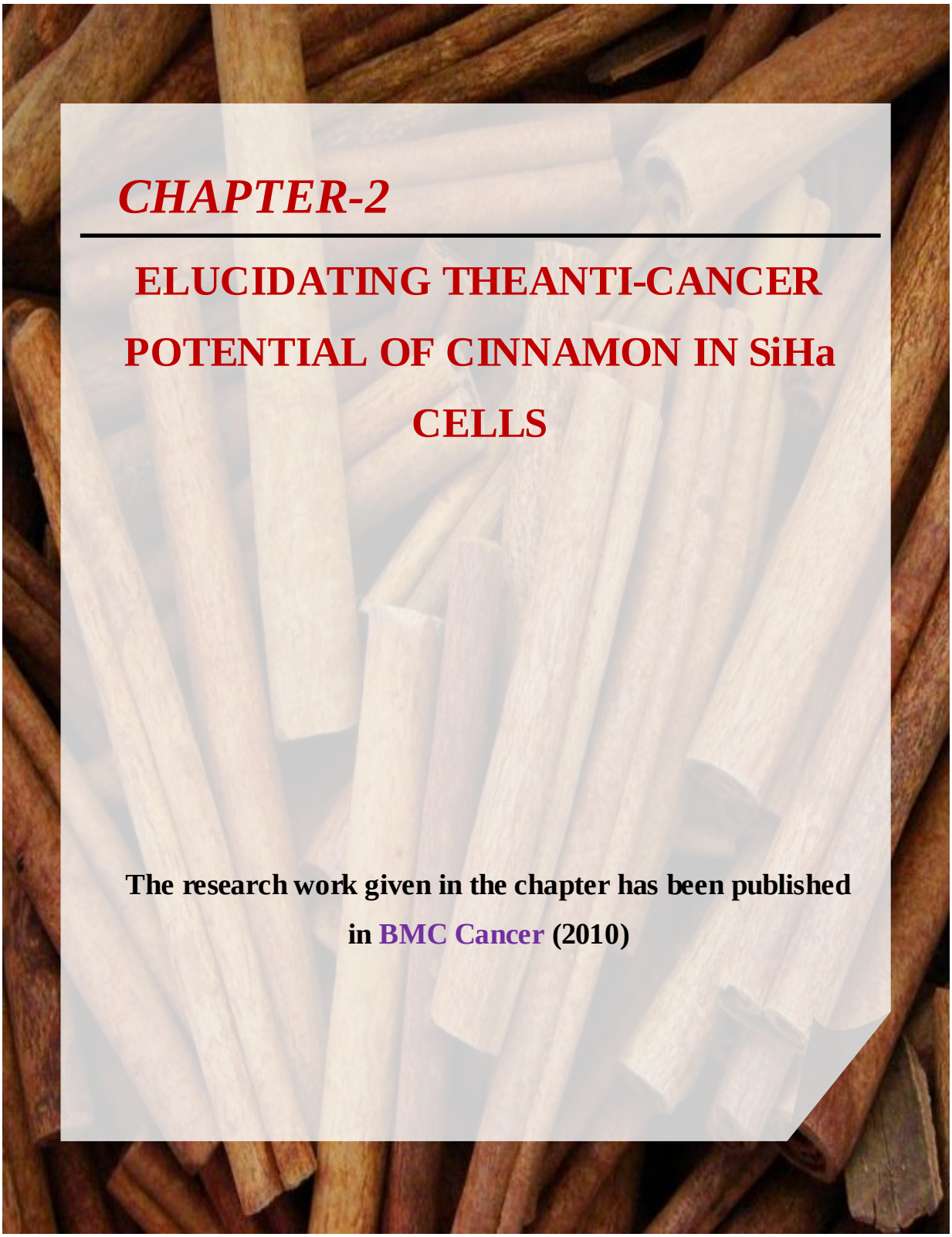
Chapter 3: Elucidating the anti-cancer potential of *Ficus religiosa* in SiHa and HeLa cells

Chapter 3 describes the anti-cervical cancer potential of the bark of *Ficus religiosa*. Different molecular and cellular level studies were carried out in human cervical cancer cell lines SiHa and HeLa to assess the anti-cancer mechanism of the aqueous extract of *Ficus religiosa* (FR_{aq}). Important observations about how Ficus induce G_1 phase arrest in SiHa and apoptosis in HeLa cells have been included in the studies.

Chapter 4: Analyzing the anti-neoplastic potential of a novel herbal formulation (FC): In vitro and *in vivo* study

Chapter 4, comprises of development of novel herbal formulation (FC) and analyzing its anti-cancer potential in cervical cancer cells (in-vitro) and $B_{16}F_{10}$ induced melanoma tumor in C57BL/6 mice. The data on induction of apoptosis in both SiHa and HeLa cells, and inhibition of tumor growth *in vivo* is also included.

The overall conclusion and future perspectives have been given at the end.



CHAPTER-2

ELUCIDATING THEANTI-CANCER POTENTIAL OF CINNAMON IN SiHa CELLS

The research work given in the chapter has been published in BMC Cancer (2010)

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-cubebene, α-terpineol, terpinolene and α-thujene, has been reported (Tung et al., 2008).

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

(Ravindran et al., 2003). 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 ($\Delta\psi_m$) 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-3conjugate (Millipore, MA) and Annexin V-FITC apoptosis kit #3 from Invitrogen (CA, USA). All other common reagents were procured from Qualigens fine chemical (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% CO_2 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 10^5/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% CO_2 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 ($OD_{570\text{-}630nm}$). 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 x 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 x 10^3cells/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 x 10^3cells/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% CO_2 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% CO_2 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, Banglore, 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'-taccactggcatcgtgatggact-3'; β-actin-R: 5'-tttctgcatcctgtcggaaat-3'; MMP-2-F: 5'-ggctggtcagtggcttggggta-3'; MMP-2-R: 5'-agatcttcttcttcaaggaccggtt-3'. PCRs were performed in 25 µl volume in which 1X PCR buffer, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, 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 $CaCl_2$, 50 mM Tris-HCl pH 7.5, 1% Triton X- 100, 0.02%NaN_3) 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 5×10^5 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 ($\Delta\psi m$) 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 (1×10^5) 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 (5×10^5) 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 x 10^5cells/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 α 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).

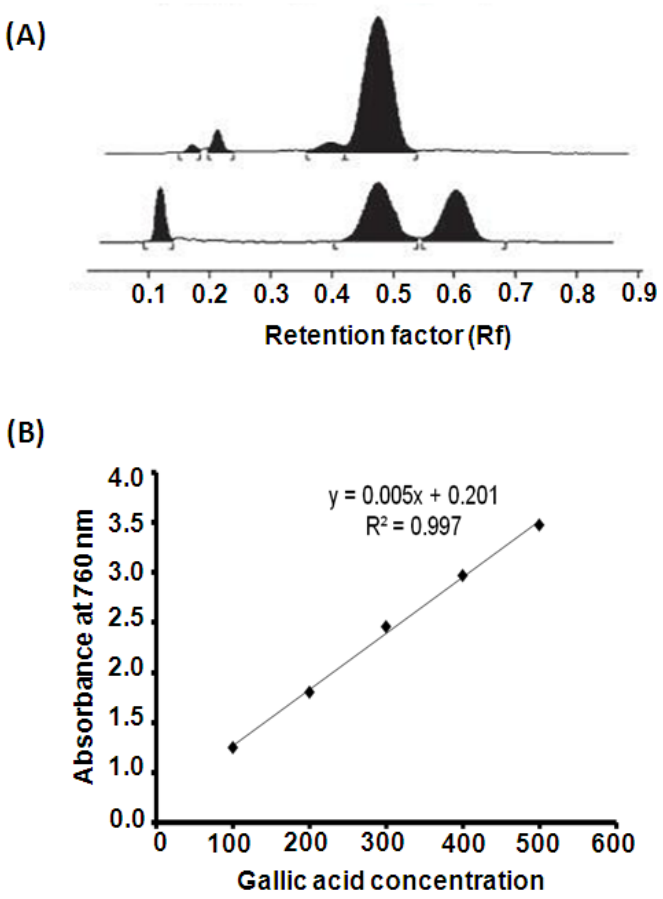


Figure 2.2: Detection and quantification of cinnamaldehyde in aqueous cinnamon extract from C.cassia (ACE-c). *(A) The figure shows HPTLC chromatogram of standard mixture of piperine, cinnamaldehdye and eugenol (I) as well as ACE-c (II). (B) Calibration curve for quantification of total polyphenolic 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.

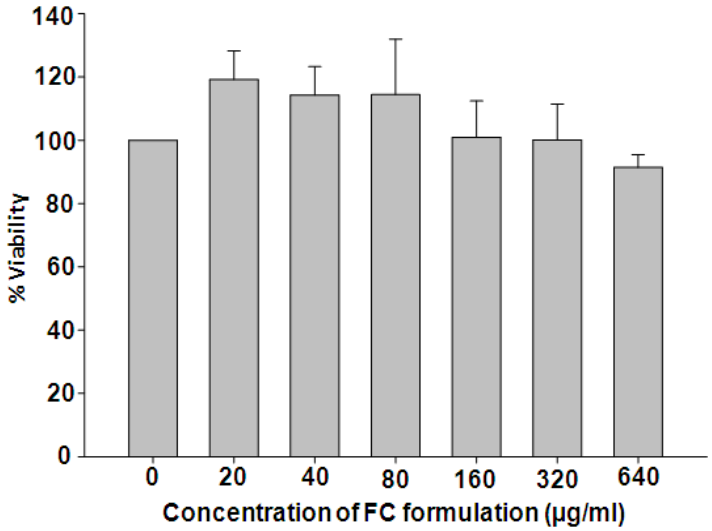


Figure 2.3: Cytotoxic effect of ACE-c on human cervical cancer cells. *SiHa cells were treated with different concentrations (0-320 µg/ml) of ACE-c for 24 h. The cell viability was measured by MTT assay.*

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 ($p \leq 0.05$ for 24 h; $p \leq 0.001$ for 48 h and 72 h).

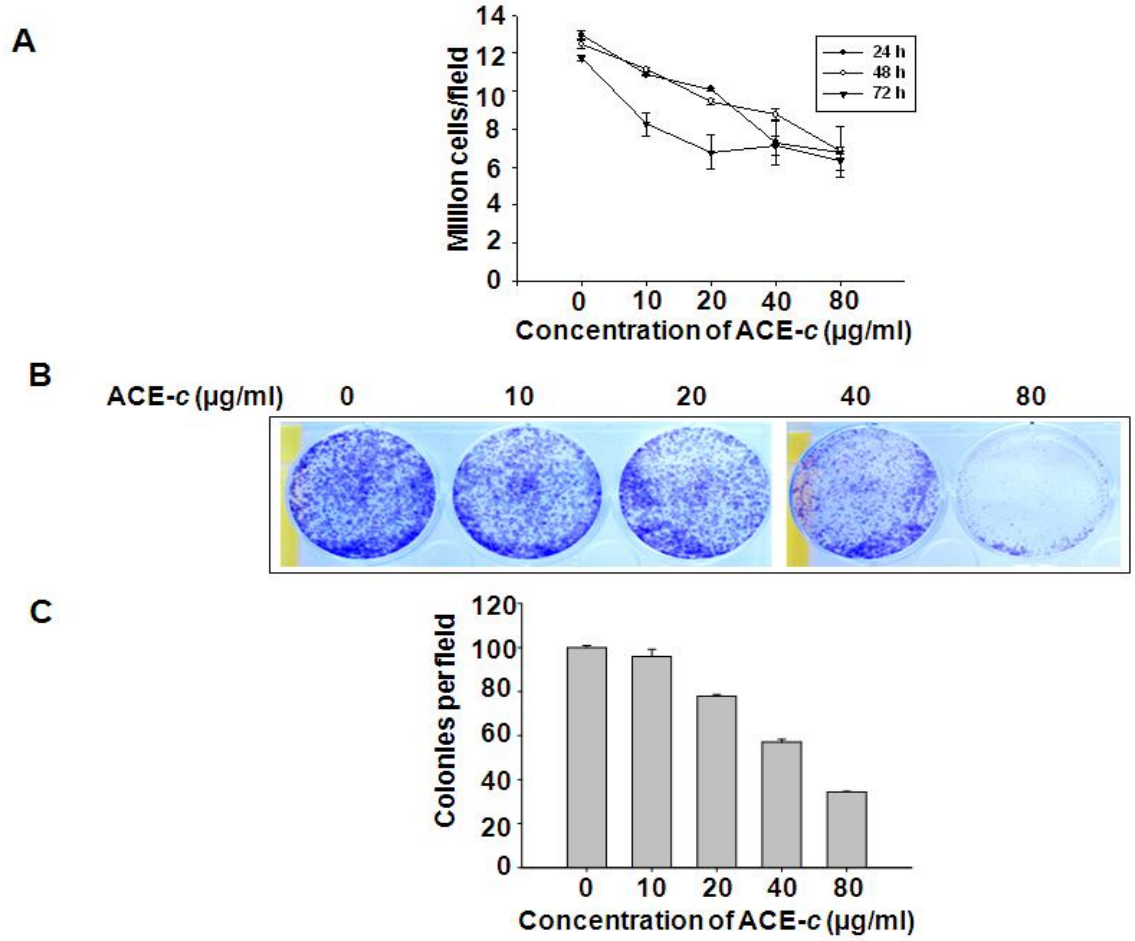


Figure 2.4: Cinnamon altered growth kinetics of cervical cancer cells. *(A) The growth kinetics has been presented in the figure taken at different time points. Data represent means ±SD of five different experiments. (B) The cells (1 x 10^3/ml) were grown in 6-well plates and treated with various concentrations (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 x10^3) 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 ($p \leq 0.001$) (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 ($p \leq 0.001$) 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.

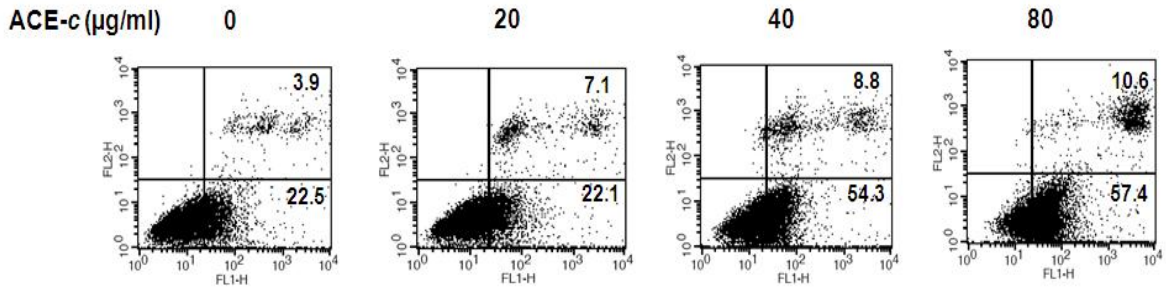


Figure 2.5: Cinnamon induced apoptosis. *SiHa cells were treated with different concentrations of ACE-c (0-80 µg/ml) followed by Annexin V-FITC and PI staining to analyse the effect of cinnamon in apoptosis. This was determined by FACS analysis showing the percentage of early (lower right quadrant) and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

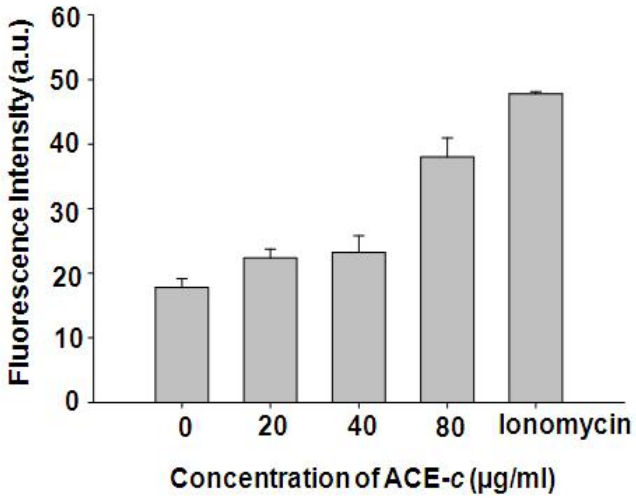


Figure 2.6: Cinnamon increased intracellular calcium. *Flow cytometric analysis of the rapid calcium release in SiHa cells after treatment with cinnamon. Cells (5 x 10^3 cells) were treated with different doses (0-80 µg/ml) of ACE-c for 24 h. This was followed by loading the cells with Fluo-3/AM for 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.*

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 µM) (Figure 2.7 B).

As clearly observed from the figure, cinnamon induces significant depolarization at 80 µg/ml ACE-*c* concentration wherein there was ~5-fold reduction in the ratio of red-green fluorescence intensity ($p \leq 0.001$). 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.

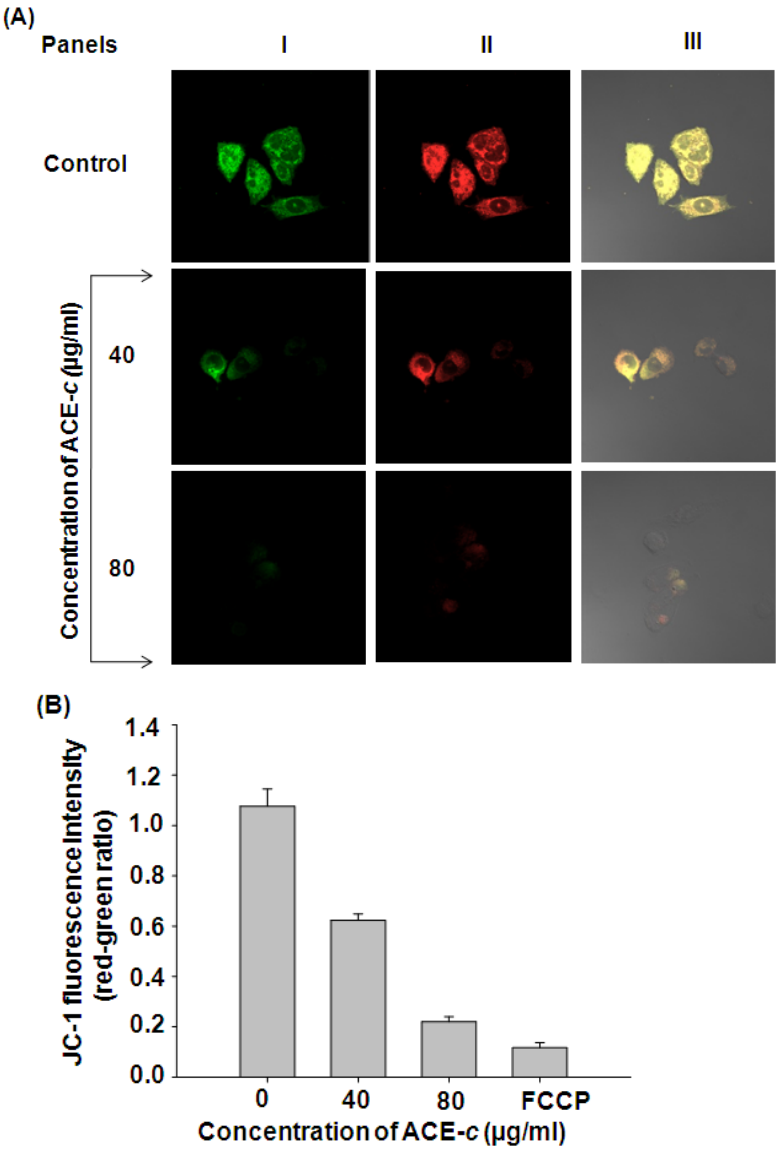


Figure 2.7: Cinnamon dysregulated mitochondrial membrane potential. *(A) Confocal images showing mitochondrial membrane depolarization induced by cinnamon. Control and cinnamon-treated SiHa cells were stained with JC-1 and the staining pattern was monitored by confocal laser scanning microscopy. For 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 flowcytometer. 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; $p \leq 0.001$) 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 ($p \leq 0.001$) 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.

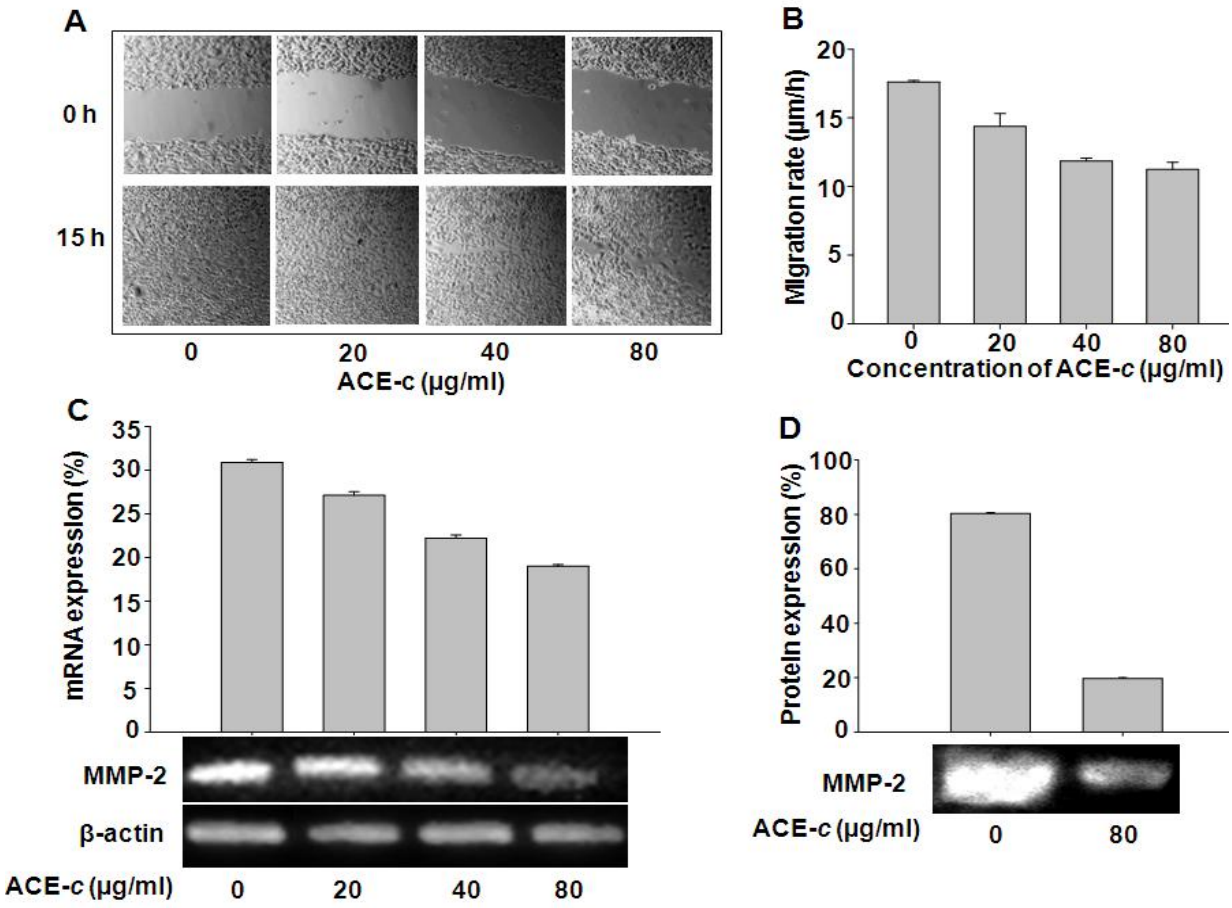


Figure 2.8: Cinnamon inhibited migration of SiHa cells. *(A) Time-Lapse image at the end of 15 h in a wound-healing assay in cells treated with different concentrations (0-80 µg/ml) of ACE-c treatment. The upper panel of the image shows the wound made at 0 h. The lower panel shows cell movement corresponding 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; $p \leq 0.001$).

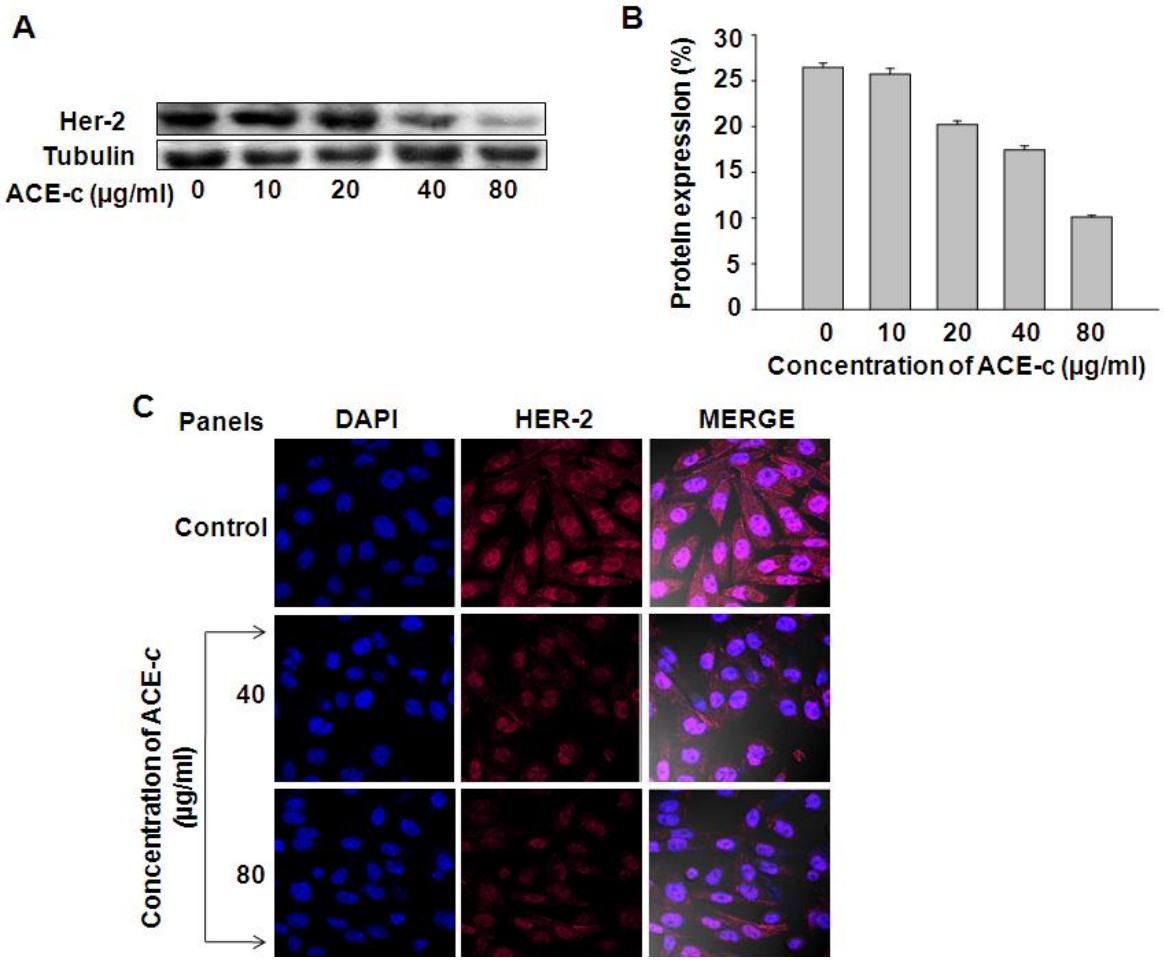


Figure 2.9: Cinnamon decreased the expression of HER-2 oncoprotein. *(A) Western blot analysis shows the expression levels of HER-2. Tubulin was used as a loading control. (B) Densitometric analysis of the western blot showing fold change in HER-2 protein levels upon ACE-c treatment. The data represents 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.

CHAPTER-3

ELUCIDATING THE ANTI-CANCER POTENTIAL OF *FICUS RELIGIOSA* IN SiHa AND HeLa CELLS

The research work given in the chapter has been published in Biotechnol. Bioinf. Bioeng (2011) and Plos One (2013)

Abstract:

In the present study, we have explored the anti-oxidant and anti-neoplastic potential of *F. religiosa* bark. The aqueous (FR_{aq}) and ethanolic (FR_{et}) extracts of the bark exhibited significant 'total antioxidant capacity' as determined by Oxygen Radical Absorbance Capacity (ORAC) method, however FR_{aq} showed higher ORAC than FR_{et}. Both FR_{aq} and FR_{et} exhibited significant increase in anti-lipid peroxidative (ALP) activity with IC_{50} values of 29.06 and 34.39µg/ml, respectively. The total phenol content present in one milligram of FR_{aq} and FR_{et} was found to be around 497.77 and 375.23 µg, respectively, equivalent to gallic acid control. Interestingly, both FR_{aq} and FR_{et} showed significant cytotoxicity in cervical cancer cell lines SiHa (HPV 16+) and HeLa (HPV18+).

Based on the antioxidant potential, polyphenol content and ALP activity, FR_{aq} was selected for further experiments. Its anti-cancer activity was further delineated with underlying molecular mechanisms in human cervical cancer cell lines, SiHa and HeLa. FR_{aq} altered the growth kinetics of SiHa and HeLa cells in a dose-dependent manner. It blocked the cell cycle progression at G_1/S phase in SiHa that was characterized by an increase in the expression of p53, p21 and pRb proteins with a simultaneous decrease in the expression of phospho Rb (ppRb) protein. On the other hand, in HeLa, FR_{aq} induced apoptosis through an increase in intracellular Ca^{2+} leading to loss of mitochondrial membrane potential, release of cytochrome-c and increase in the expression of caspase-3. Moreover, FR_{aq} reduced the migration as well as invasion capability of both the cervical cancer cell lines accompanied with downregulation of MMP-2 and HER-2 expression. Interestingly, FR_{aq} reduced the expression of viral oncoproteins E6 and E7 in both the cervical cancer cell lines. All these data suggest that *F. religiosa* could be explored for its chemopreventive potential in cervical cancer.

3.1 Introduction

F. religiosa L. family Lauraceae, the most popular member of the genus Ficus, is native of the sub-Himalayan tract, Bengal and central India. It has been extensively distributed worldwide through cultivation (McFarland et al., 1944; Galil et al., 1984). It is found in the areas up to1500 m elevation having annual rainfall varying from 50 to 500 cm during the monsoon season and tolerates a wide variation in temperature (below 0°C and above 40°C) (Pullaiah et al., 2006) Chemically *F. religiosa* had been found to contain phytosterols, amino acids, furanocoumarins, phenolics components, hydrocarbons, aliphatic alcohols, volatile components and a few other classes of secondary metabolites (Singh et al., 2011). The bark of *F. religiosa* shows the presence of bergapten, bergaptol, lanosterol, β-sitosterol, stigmasterol, lupen-3-one, β-sitosterol-d-glucoside (phytosterolin) and vitamin K1 (Singh et al., 2011). The bark also contains tannin, wax, saponin, β-sitosterol, lupeol, ceryl behenate, lupeol acetate, α-amyrin acetate, leucoanthocyanidin, leucoanthocyanin, leucocyanidin-3-0-β-D-glucopyrancoside, leucopelargonidin-3-0-β-D-glucopyranoside and leucopelargonidin-3-0-α-L-rhamnopyranoside, (Husain et al., 1992).

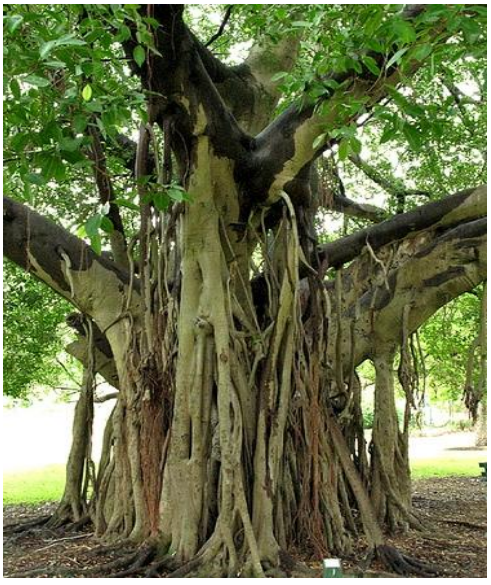


Figure 3.1: A typical Ficus religiosa tree in its natural habitat

Ficus religiosa L. (Figure 3.1), has been extensively used in traditional medicine to treat various disorders (Panda et al., 2005; Kirtikar et al., 1993). The bark forms the part of many Ayurvedic formulations such as "Pancha Valkala Kashaya" (decoction containing *F. religiosa, F. benghalensis, F. glomerata., F. infectoria and A*zadirachta. *indica*) and "Pancha Valkaladi Tailum" (oil containing *F. religiosa*, *Ficus benghalensis* L., *Ficus glomerata* Roxb., *Ficus infectoria* Willd., *A indica*, *Curcuma longa* L. and *Hemidesmus indicus* R. Br.) (Panda et al., 2005; Singh et al., 2005). It has been shown to exhibit diverse pharmacological activities (Haneef et al., 2012) including wound healing (Choudhary et al., 2006), anti-bacterial (Nair et al., 2007), anti-convulsant (Singh et al., 2009), anti-diabetic (Deshmukh et al., 2007; Kirana et al., 2009), anti-inflammatory (Sreelekshmi et al., 2007), acetyl cholinesterase inhibitory activity (Vinutha et al., 2007), and anti-anxiety activity (Ratnasooriya et al., 1998). The acetone extract of *F. religiosa* leaves has been shown to induce apoptosis in breast cancer cell lines (Haneef et al., 2012).

In the present study, we have investigated the antioxidant potential of aqueous (FR_{aq}) and ethanolic (FR_{et}) extracts of *F. religiosa* bark. Based on the antioxidant potential, polyphenol content and Anti Lipid Peroxidation (ALP) activity, FR_{aq} was selected for further experiments. The putative molecular mechanism underlying the antineoplastic potential of (FR_{aq}) was elucidated in cervical cancer cell lines, SiHa and HeLa. Our data suggests that Ficus inhibits the growth of SiHa and HeLa by inducing cell cycle arrest and apoptosis, respectively. Interestingly, FR_{aq} significantly reduces the expression of viral oncoproteins E6 and E7, thereby suggesting the therapeutic potential of *F. religiosa* in cervical cancer.

3.2. Materials and Methods

3.2.1. Chemicals and Reagents

Tissue culture plasticware was purchased from BD Biosciences (CA, USA) and Axygen Scientific Inc (CA, USA). Dulbecco's Modified Eagles Medium (DMEM) powder, penicillin and streptomycin were obtained from Invitrogen/Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), FCCP, Ionomycin and JC-1 were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibody against p53 (DO-1), p21 (187), caspase-3 (H-277), cyto-c (7H8), HER-2 (F-11), pRb (C-15), ppRb (SER 807/811), HPV16 E6/18 E6 (C1P5), HPV16 E7 (ED17), HPV18 E7 (N-19) or tubulin (B-7) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Annexin V-FITC apoptosis kit #3 was purchased from Invitrogen (CA, USA). All other common reagents were procured from Qualigens Fine Chemicals (Mumbai, India).

3.2.2. Preparation of aqueous and ethanolic extract of *Ficus religiosa*

Bark of *Ficus religiosa* L. was collected from Pune District, Maharashtra, India. Botanical identification of plant material was carried out with the help of standard flora (18) and a voucher specimen (MPCC 2417) of authentic plant species have been deposited at the herbarium of Medicinal plants Conservation Center (MPCC), Pune, Maharashtra, India. Bark was chopped into small pieces, shade dried at ambient temperature and ground into coarse powder in a grinder. Aqueous and ethanolic extracts were prepared as per standard Indian Pharmacopoeia and soxhlet method, respectively. The extract obtained, was centrifuged at 13000 rpm for 15min and the supernatant was filtered through Swiney filter (pore size, 0.45 µm) and the extract was stored at -80ºC until further use.

3.2.3. Estimation of total phenolic content by Folin-Ciocalteu method

The total phenolic content of FR_{aq} and FR_{et} extracts was determined spectrophotometrically by Folin-Ciocalteu method (Pourmorad et al., 2006). The extract (100 µg mL^{-1}) was mixed with 5 ml of Folin-Ciocalteu reagent, previously diluted in distilled water (1:10), and 4 mL of sodium carbonate (1M in H_2O). The mixture was incubated at 37ºC for 15 min for the color development. The absorbance was measured at 765 nm using a Perkin Elmer spectrophotometer (lambda EZ201). Samples of the extracts were evaluated at a final concentration of 1 mg mL^{-1}. Total phenolic content was expressed as mg g^{-1} gallic acid equivalent using the equation obtained from the standard calibration curve: $y = 0.004x+0.0201$, $R^2 = 0.9953$.

3.2.4. Oxygen radical absorbance capacity (ORAC) assay

The total antioxidant capacity of the extract was determined using ORAC method (Huang et al., 2002). A freshly prepared fluorescein (150 µl of a 5 nM solution) was mixed with 25µl of various concentrations of the extract (0-200 µg/ml) in the flat-bottom black 96-well plate and incubated for 30 min at 37ºC. After incubation, fluorescence measurements (excitation, 485 nm; emission, 520 nm) were taken every 100 seconds to determine the background signal. After 3 cycles, 25 µl (250 mM) of AAPH (2,2'-azobis-2-methyl-propanimidamide) was added manually with a multi-channel-pipette. The test was resumed and fluorescent measurements were taken upto 150 min using FLUOstar omega multiplate reader, BMG labtech (Offenburg, Germany). The net area under the curve (AUC) of the standards and samples was calculated. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the three measurements for each concentration. The AUC was calculated as AUC = 1 + $f1/f0$ + ... $fi/f0$ + ... + $f149/f0$ + ($f150/f0$), where, $f0$ = initial fluorescence reading at 0 min and fi =

fluorescence reading at time *i*. The data were analyzed in Microsoft Excel to calculate the AUC. The net AUC was obtained by subtracting the AUC of the blank from that of the sample.

3.2.5. Anti-lipid peroxidation activity by TBARS method

Inhibition of lipid peroxidation activity by both aqueous and ethanolic extracts was determined using goat liver homogenate and thiobarbituric acid-malondialdehyde (TBA-MDA) (Wade et al., 1985). Animal tissue (goat liver) was perfused with KCl (0.15M in H_2O) and the perfused tissue (10%w/v) was homogenized in ice cold KCl (0.15M) using mortar and pestle. The reaction mixture was set up using 0.5 ml of homogenate, 0.5 ml of different concentrations (0-200µg/ml) of FR_{aq} and FR_{et} extracts and 1 ml KCl (0.15 M). Lipid peroxidation was induced by adding 100µl $FeCl_3$ (1mM in H_2O) and the reaction mixtures were incubated at 37ºC for 30 min. Trichloroacetic acid (15% in 0.25N HCl), thiobarbituric acid (0.38%) and 200µl butylated hydroxyl toluene (0.05%) were added to stop the reaction. The reaction mixtures were heated at 80ºC for 60 min, cooled at room temperature and centrifuged at 6000 rpm for 15 min. Supernatant was collected and measured at 532 nm. The percent inhibition of lipid peroxidation was calculated by using the formula:

$$\%\ \text{inhibition of lipid peroxidation} = \left[\frac{\text{OD of induced sample} - \text{OD of test sample}}{\text{OD of induced sample}}\right] * 100$$

3.2.6. Cell Culture

The human cervical carcinoma cell lines, SiHa (HPV-16), HeLa (HPV-18) and C33A (HPV-negative) were obtained from National Center for Cell Science (NCCS), Pune, Maharashtra, India. The cells were grown in DMEM supplemented with 10% FBS, 2mM

L-glutamine, and antibiotics (100 units/ml penicillin and streptomycin). The cells were incubated in a humidified 5% CO_2 incubator at 37ºC.

3.2.7. Cell viability

The anti proliferative activity of FR_{aq} and FR_{et} was determined by MTT dye uptake method on SiHa, HeLa as describe in chapter-2 section 2.2.4 (Koppikar et al., 2010). The anti proliferative activity of FR_{aq} was further evaluated on C33A (HPV negative cell line)

3.2.8. Preliminary phytochemical investigation of aqueous extract of Ficus religiosa (FR_{aq})

The freshly prepared FR_{aq} extract was qualitatively tested for the presence of flavonoids, phenols, saponins, tannins and carbohydrates using standard procedures of analysis (Khandelwal et al., 2005).

3.2.9. Cell growth analysis

The assay was performed on FR_{aq} (0-80 µg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.5 (Koppikar et al., 2010; Kaul et al., 2003).

3.2.10. Colony formation assay

The assay was performed on FR_{aq} (0-80 µg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.6 (Koppikar et al., 2010).

3.2.11. Soft agar assay

The assay was performed on FR_{aq} (0-80 µg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.7 (Koppikar et al., 2010; Kaul et al., 2003).

3.2.12. Wound healing assay

The assay was performed on FR_{aq} (0-80 µg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.8

3.2.13. Matrigel transmembrane invasion assay

For invasion studies, 24-well BioCoat Matrigel Invasion Chambers (BD Bioscience, Bedford, MA) were used (Kim et al., 2012). SiHa and HeLa cells (5×10^4) with or without FR_{aq} treatment (0-80 µg/ml) were seeded in serum-free medium into the upper invasion chambers and allowed to invade across the Matrigel-coated membrane for 24 h. The medium containing 10% FBS was added to the lower chamber which served as a chemo attractant. After 24 h of incubation, non-invading cells were removed from the top of each membrane with wet cotton swabs; invading cells attached to the bottom of the membrane were fixed with 4% formalin and stained using 0.5% crystal violet. The cell numbers were counted in ten random high-power (20X) fields using Axiovert 200M microscope (Carl Zeiss, Germany) equipped with a Sony Cyber-shot 3.3 mega pixels camera.

3.2.14. Gelatin zymography

SiHa and HeLa cells were seeded at a density of 4×10^5 cells/ml in 6-well plates and allowed to adhere overnight at 37°C in 5% CO_2 incubator. Next day, the cells were treated with various concentrations of FR_{aq} (0-80 µg/ml) prepared in serum-free medium and incubated for 24 h. The following day, the culture medium was collected and centrifuged at 14,000 rpm for 20 min at 4°C to remove the cellular debris. The activity of MMP-2 in the conditioned medium was determined by gelatin zymography describe in chapter-2 section 2.2.7 (Koppikar et al., 2010).

3.2.15. Immunoblotting

SiHa and HeLa cells were plated at a seeding density of 4 x 10^5cells/ml in 6-well plates and allowed to adhere overnight at 37°C in CO_2 incubator. Next day, the cells were exposed to various concentrations of FR_{aq} (0-80 µg/ml) and incubated for 24 h. Following incubation, the cells were harvested by trypsinization, washed with 1X PBS and protein was extracted as described in chapter-2 section 2.2.11 (Koppikar et al., 2010). For cytochrome-c release, cytosolic and mitochondrial fractions were prepared as described previously (Sánchez-Alcázar et al., 2010). The protein was estimated using Bradford reagent (Biorad Laboratories Inc, CA, USA). Equal amount of protein was loaded on to either 10% or 12% (for E6 protein) 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 4ºC overnight with primary antibody against p53, p21, caspase-3, cyto-c, HER-2, pRb, ppRb, HPV16 E6/18 E6, HPV16 E7, HPV18 E7 or tubulin (Santacruz, CA, USA) at a 1:500 dilution. The membrane was washed in TST and incubated with secondary IgG HRP conjugate at 1:5000 dilution. Proteins were visualized with a chemiluminescence kit (Amersham ECL Advance western blotting detection kit, GE Healthcare, UK) and densitometry analysis was performed on scanned immunoblot images using the Image J gel analysis tool.

3.2.16. Assessment of cell cycle arrest

For cell cycle analysis, HeLa, SiHa and C33A cell lines were plated at a seeding density of 5× 10^5 cells/well in 6-well plates and allowed to adhere for 24 h at 37°C in CO_2 incubator. Next day, the cells were treated with FR_{aq} (0-80 µg/ml) for 24 h. The cells were harvested by trypsinization and fixed in ice-cold 70% ethanol at -20°C for 30 min.

Following washing with 1X PBS, the cells were treated with RNAse A (100 mg/ml) at room temperature for 30 min and stained with Propidium Iodide (20 µg/ml). Stained cells were analyzed for DNA-PI fluorescence using a flowcytometer (FACS Calibur, BD). A minimum of 10,000 events were counted per sample; data were analyzed using FACS Calibur-cell quest software (Becton Dickinson) for the proportions of cells in G_0/G_1, S phase and G_2/M phases of the cell cycle.

3.2.17. Assessment of apoptosis

The assay was performed on FR_{aq} (0-80 µg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.12.

3.2.18. Detection of Intracellular calcium using Fluo-3/AM

The assay was performed on FR_{aq} (0-80 µg/ml) treated and untreated HeLa cells as describe in chapter-2 section 2.2.13 (Koppikar et al., 2010).

3.2.19. Analysis of mitochondrial membrane potential ($\Delta\psi m$)

The assay was performed on FR_{aq} (0-80 µg/ml) treated and untreated HeLa cells as describe in chapter-2 section 2.2.14

3.2.20. Statistical analysis

All the experiments were performed in triplicates and repeated at least three times and the data has been presented as mean±SD. Statistical analysis was conducted with the SigmaStat 3.5 program (Systat Software, Inc.) using one-way ANOVA with $\alpha = 0.05$.

3.3. Results

3.3.1 Comparison of total antioxidant capacity of FR_{aq} and FR_{et} by ORAC method

The antioxidant activity of *Ficus religiosa* has been reported by various groups (Panchawat et al., 2010; Rathee et al., 2010; Kumar et al., 2011). However, we have for the first time compared the 'total antioxidant capacity' of aqueous (FR_{aq}) and ethanolic (FR_{et}) extracts of bark of *F. religiosa* by ORAC method. It was found that both the extracts possessed significant free radical scavenging capabilities. However, FR_{aq} inhibited fluorescein decay in a dose-dependent manner closer to that of the positive control, Trolox (Figure 3.2).

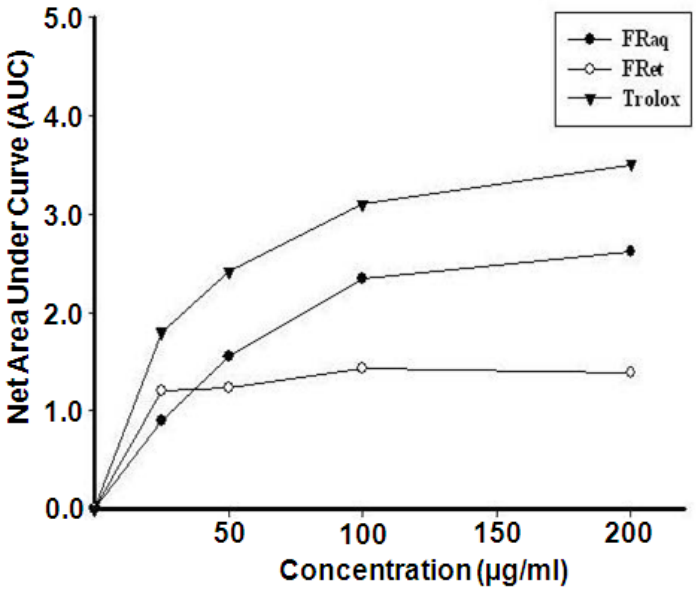


Figure 3.2: Oxygen Radical Absorbance Capacity (ORAC) assay. *The graph represents the ORAC of the FR_{aq} and FR_{et} extracts which is expressed as Net Area Under Curve and compared with Trolox. (n=3 experiments)*

3.3.2 Total phenolic content in FR_{aq} and FR_{et}

The antioxidant activity exhibited by both aqueous and ethanolic *F religiosa* bark extract may be due to phenolic compounds present in them. Folin-Ciocalteu method was

used to determine the total phenol content present in both the extracts and was compared with standard gallic acid (Figure 3.3). The results were expressed in terms of mg/g of extract. The total phenolic content present in FR_{aq} and FR_{et} was 497.77 and 375.23 mg/g equivalent of gallic acid, respectively.

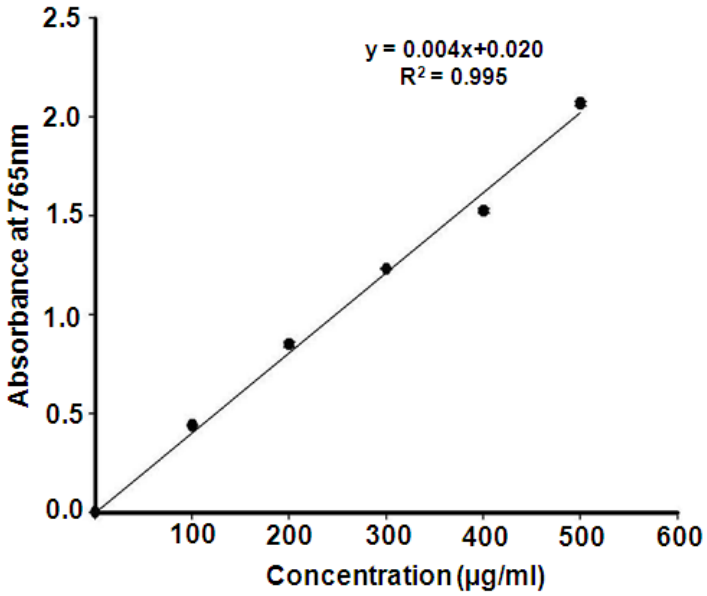


Figure 3.3: Standard Gallic Acid Curve. *The graph represents the absorbance of gallic acid at known increasing concentrations for Total phenol determination of FR_{aq} and FR_{et} extract. (n=3 experiments)*

3.3.3 FR_{aq} and FR_{et} inhibited lipid peroxidation

We used modified TBARS method to analyze the anti-lipid peroxidation capacity of both FR_{aq} and FR_{et} extracts. Animal tissue (liver) homogenate was mixed with different concentrations of the extracts and lipid peroxidation was induced with $FeCl_3$. It was observed that the aqueous extract showed greater anti-lipid peroxidation (ALP) activity compared to the ethanolic extract (Figure 3.4), with IC_{50} values of 29.06 and 34.39 µg/ml, respectively, for FR_{aq} and FR_{et}.

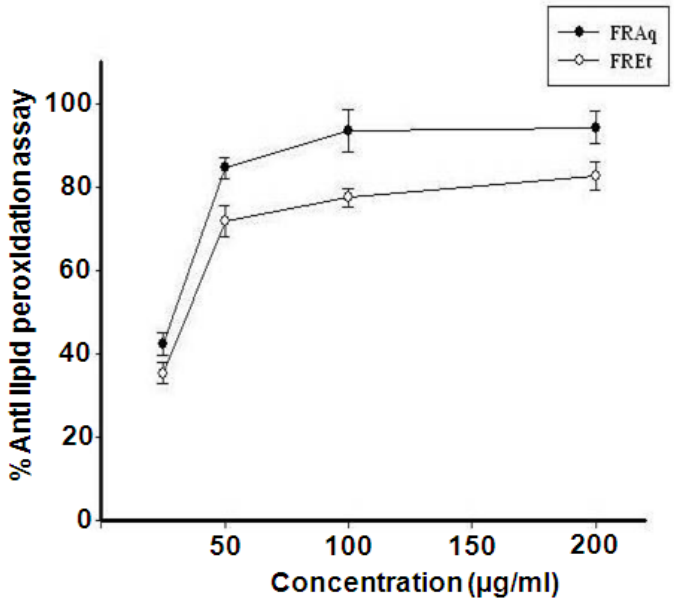


Figure 3.4: Anti-lipid peroxidation by TBARS Method. *The graph represents the inhibition of lipid peroxidation activity by the FR_{aq} and FR_{et} extracts expressed in percentage. (n=3 experiments)*

3.3.4 FR_{aq} and FR_{et} exhibited significant cytotoxic activity in cervical cancer cell lines

We evaluated the cytotoxic potential of both FR_{aq} and FR_{et} extracts in cervical cancer cell lines, SiHa and HeLa. It was observed that FR_{aq} exhibited 100% viability in both SiHa and HeLa upto 160 µg/ml (Figure 3.5 A), beyond which it was cytotoxic to both the types of cell lines. However, FR_{et} showed 100% survival till 160 and 80 µg/ml in SiHa and HeLa cell lines, respectively, beyond which it was cytotoxic to both the cell types (Figure 3.5 B). Thus, our results showed that besides antioxidant potential, *F. religiosa* aqueous and ethanolic bark extracts exhibited significant cytotoxic activity in both the cervical cancer cell lines.

Based on the antioxidant potential, polyphenol content and ALP activity, FR_{aq} was selected for further experiments. Before evaluating its anticancer mechanism, we evaluated the cytotoxic potential of FR_{aq} on C33A (HPV negative) cell line. Ficus did not induce any cytotoxicity up to 160 µg/ml concentration in C33A cells (Figure 3.5 C),

which was similar to that observed in SiHa and HeLa. However, at higher concentrations, FR_{aq} induced cytotoxicity in all the three cell lines.

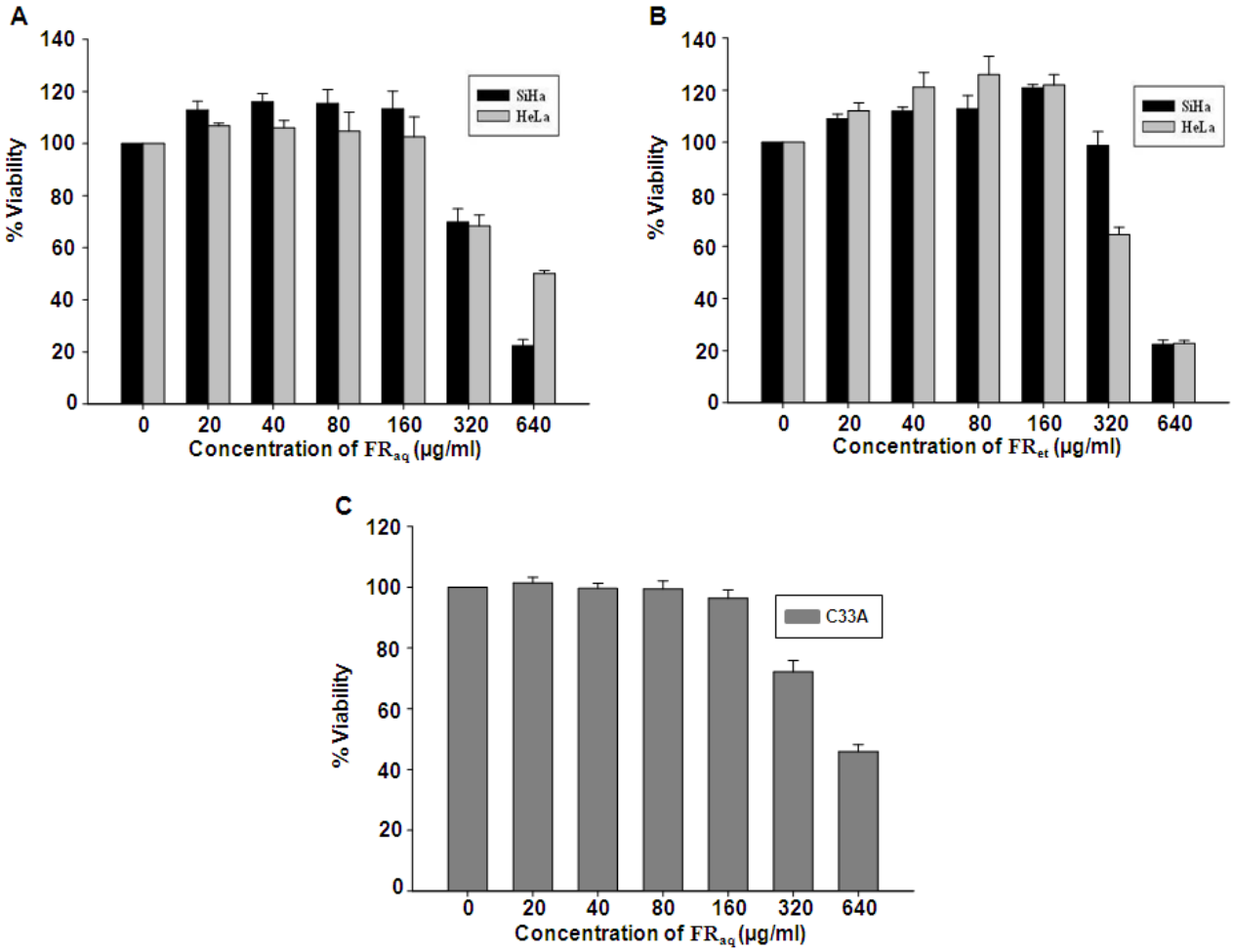


Figure 3.5: Cytotoxic effect of (A)FR_{aq} and (B)FR_{et} extract in cervical cancer cell lines. *SiHa and HeLa were treated with different concentrations (0-620 µg/ml) of extract for 24 h. The viability was measured by MTT assay.* ***(C)*** *Cytotoxic effect of FR_{aq} on C33A (HPV negative) cell line after 24 h of treatment.*

3.3.5 Phytochemical analysis of *F. religiosa*

The freshly prepared FR_{aq} extract was qualitatively tested for the presence phytochemical and showed the presences of flavonoids, phenols, saponins, tannins and carbohydrates (Table 3.1).

Table 3.1: Preliminary phytochemicals analysis of FR_{aq} extract.

Sr. No	Phytochemical	Occurrence
1.	Flavonoids	+
2.	Phenols	+
3.	Saponins	+
4.	Tannins	+
5.	Carbohydrate	+

'-' =Absent '+'= Present

The freshly prepared FR_{aq} extract was qualitatively tested for the presence of flavonoids, phenols, saponins, tannins and carbohydrates using standard procedures of analysis (Khandelwal et al., 2005).

3.3.6 Ficus modulated the growth kinetics of cervical cancer cells

To test the effect of Ficus on the growth kinetics, SiHa and HeLa cells were treated with different concentrations of FR_{aq} (0, 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 FR_{aq}-treated cells compared to the untreated control cells. It was observed that FR_{aq} decreased the growth of the cells in a dose- and time-dependent manner.

In SiHa, FR_{aq} decreased the cell growth at 80 µg/ml concentration by ~4.78- ($p=0.008$), ~4.72- ($p=0.001$) and ~3.42-fold ($p=0.053$) at 24, 48 and 72 h, respectively, compared to the untreated control cells (Figure 3.6 A). Similarly, at 80 µg/ml concentration of FR_{aq}, HeLa cells exhibited ~5.53- ($p\leq0.001$), ~5.94- ($p=0.010$) and ~6.37-fold ($p=0.001$) decrease in the cell growth at 24, 48 and 72 h, respectively, compared to the untreated control cells (Figure 3.6 B). This was further supported by colony formation and soft agar assays wherein a dose-dependent decrease in the number

of colonies was observed in both the cervical cancer cell lines (Figure 3.6 C and D, respectively). Interestingly, at 80 µg/ml concentration, FR_{aq} significantly reduced the number of colonies in HeLa (~4.97 fold; $p \leq 0.001$) and SiHa (~2.95 fold; $p \leq 0.001$) compared to their respective untreated control cells (Figure 3.6 D). Thus, Ficus regulated the growth kinetics of cervical cancer cells in a significant manner.

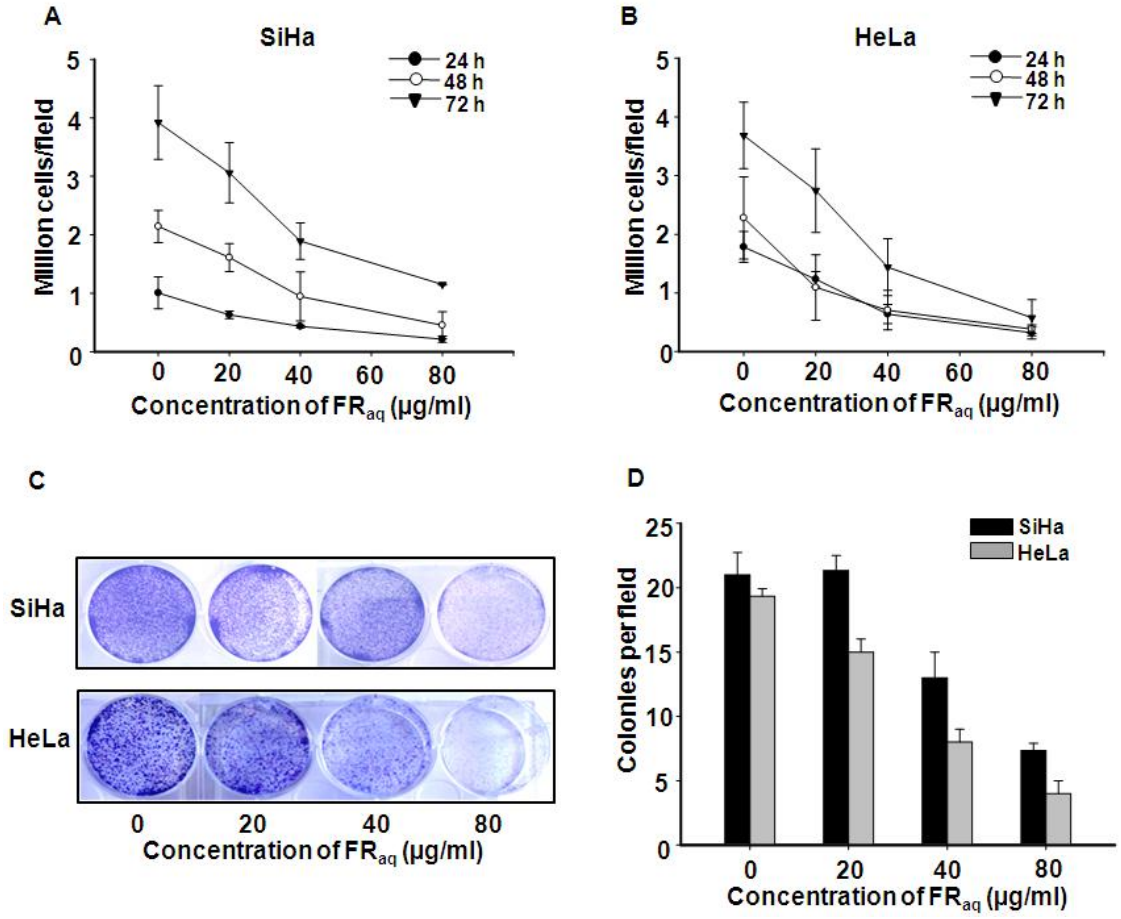


Figure 3.6: Ficus regulated the growth of cervical cancer cells. *SiHa* ***(A)*** *and HeLa* ***(B)*** *were treated with* FR_{aq} *(0-80 µg/ml) for 24-72 h and the number of viable cells were counted using the trypan blue dye exclusion method. Data represent mean ± SD of three independent experiments.* ***(C)*** *The cervical cancer cell lines (SiHa and HeLa) were treated with* FR_{aq} *(0-80 µg/ml) for one week. The colonies were stained with crystal violet and photographed. The experiments were repeated three times. (**D**) Both SiHa and HeLa* ($5x10^3$) *along with* FR_{aq} *(0-80 µg/ml) were grown in soft agar for two weeks. Colonies were counted from at least 10 different areas and the average of each has been plotted. The data represents mean ± SD of 5 independent experiments.*

3.3.7 Ficus induced cell cycle arrest in SiHa

To analyze the mechanism behind the Ficus mediated regulation of growth kinetics in cervical cancer cells, we investigated the cell cycle distribution in SiHa, HeLa and C33A. Flow cytometry analysis showed that in presence of FR_{aq}, SiHa exhibited an increase in G_1 population with a simultaneous decrease in S phase in a dose-dependent manner (Figure 3.7 A).

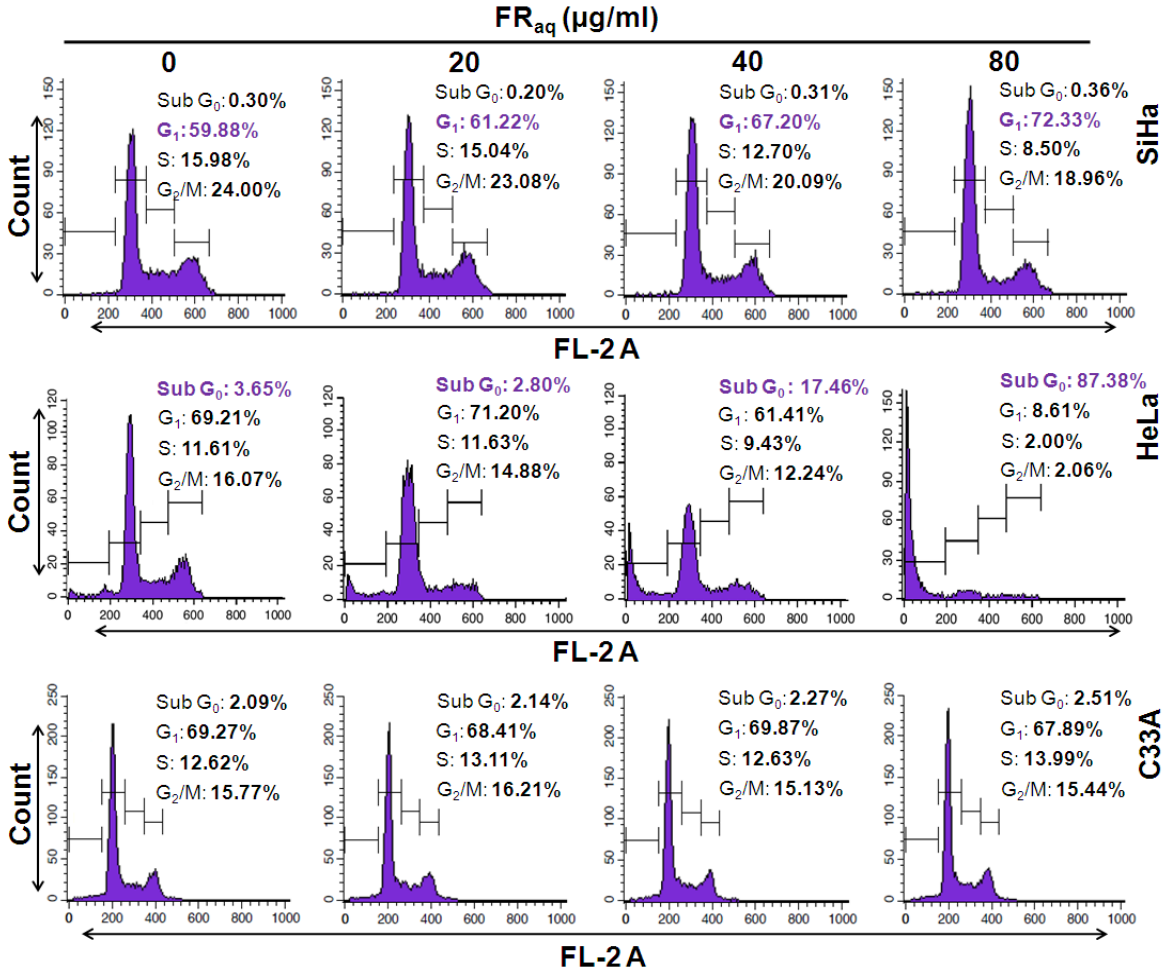


Figure 3.7: Ficus arrested the SiHa cell at G_1/S phase. *Representative FACS pictograms of (**A**) SiHa, (**B**) HeLa and (**C**) C33A cells treated with FR_{aq} (0-80 µg/ml) are shown. Cells were treated with different concentrations of FR_{aq} (0-80 µg/ml) for 24 h. Enhanced accumulation of the cells in G_1 phase with a concomitant decrease in S-phase population was observed after treatment with Ficus in SiHa (as indicated by histograms).The data represents mean ± SD of three independent experiments.*

Interestingly, at 80 µg/ml concentration, there was an increase in the percentage of cells in G_1 phase (from 59.88 to 72.33%) with a simultaneous decrease in the S phase population (from 15.98 to 8.50%; $p<0.050$). On the other hand, in HeLa (Figure 3.7 B), there was a significant increase in sub-G_0 population (from 3.65 to 87.38%; $p<0.001$) indicating apoptotic population. Interestingly, at non-toxic doses, FR_{aq} did not affect the growth of HPV negative C33A (Figure 3.7 C) cells.

3.3.8 Ficus altered the expression of cell cycle regulating protein in SiHa

We investigated the mechanism of G_1/S phase arrest in SiHa by evaluating the expression of G_1 checkpoint proteins such as p53, pRb, phospho Rb (ppRb) and p21. There was a significant increase in the expression of p53 (Figure 3.8 A and C) as well as its downstream effector, p21 (Figure 3.8 B and D) after treatment of the cells with FR_{aq}. The expression of pRb was analyzed since dephosphorylated pRb is known to form complexes with E2F to repress the transcription of cell proliferative genes (Giacinti et al., 2006). FR_{aq}, significantly increased the expression of pRb (Figure 3.8 A and C) with a simultaneous decrease in the levels of ppRb (Figure 3.8 B and D) in a dose-dependent manner. These results suggest that Ficus induced G_1/S arrest in SiHa by modulating the expression of the cell cycle regulatory proteins.

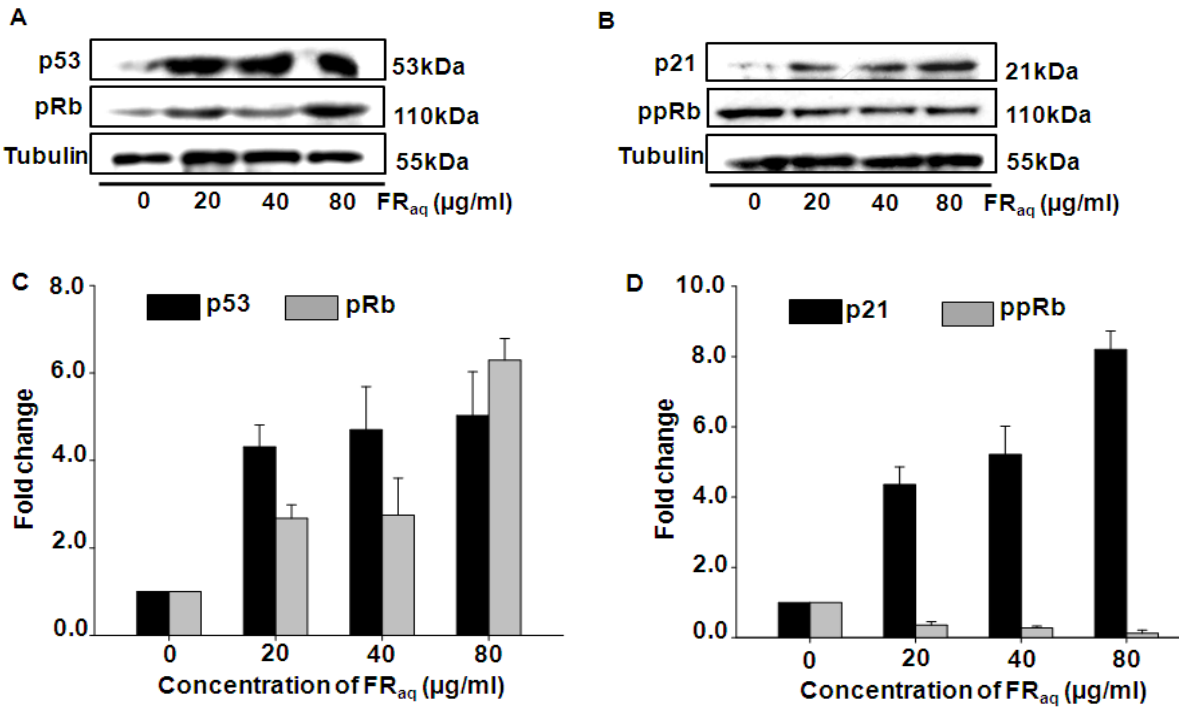


Figure 3.8: Ficus modulated the expression of cell cycle regulatory proteins. *Western blot shows the expression levels of p53 and pRb (**A**) as well as p21 and ppRb (**B**). Tubulin was used as a loading control. (**C, D**) Densitometric analysis of the western blot showing fold change in protein levels upon FR_{aq} treatment. The bands were quantified by densitometry scanning using Image J 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij). The data represents mean ± SD of three independent experiments.*

3.3.9 Ficus induced apoptosis in HeLa

We found that in HeLa, Ficus treatment resulted into increase in the number of cells in sub-G_0 phase, indicative of apoptotic population (Figure 3.7 B). On staining with Annexin V-FITC, the cells showed a dose-dependent increase in both early as well as late apoptotic cell population (Figure 3.9 A). Interestingly, at 80 µg/ml FR_{aq} concentration, there was ~4.4-fold ($p \leq 0.050$) and ~5.5-fold ($p \leq 0.050$) increase in both early as well as late apoptotic cell population, respectively, compared to the untreated

control cells. On the other hand, no apoptosis was observed in FR_{aq} treated SiHa (Figure 3.9 B) or C33A (Figure 3.9 C) cells.

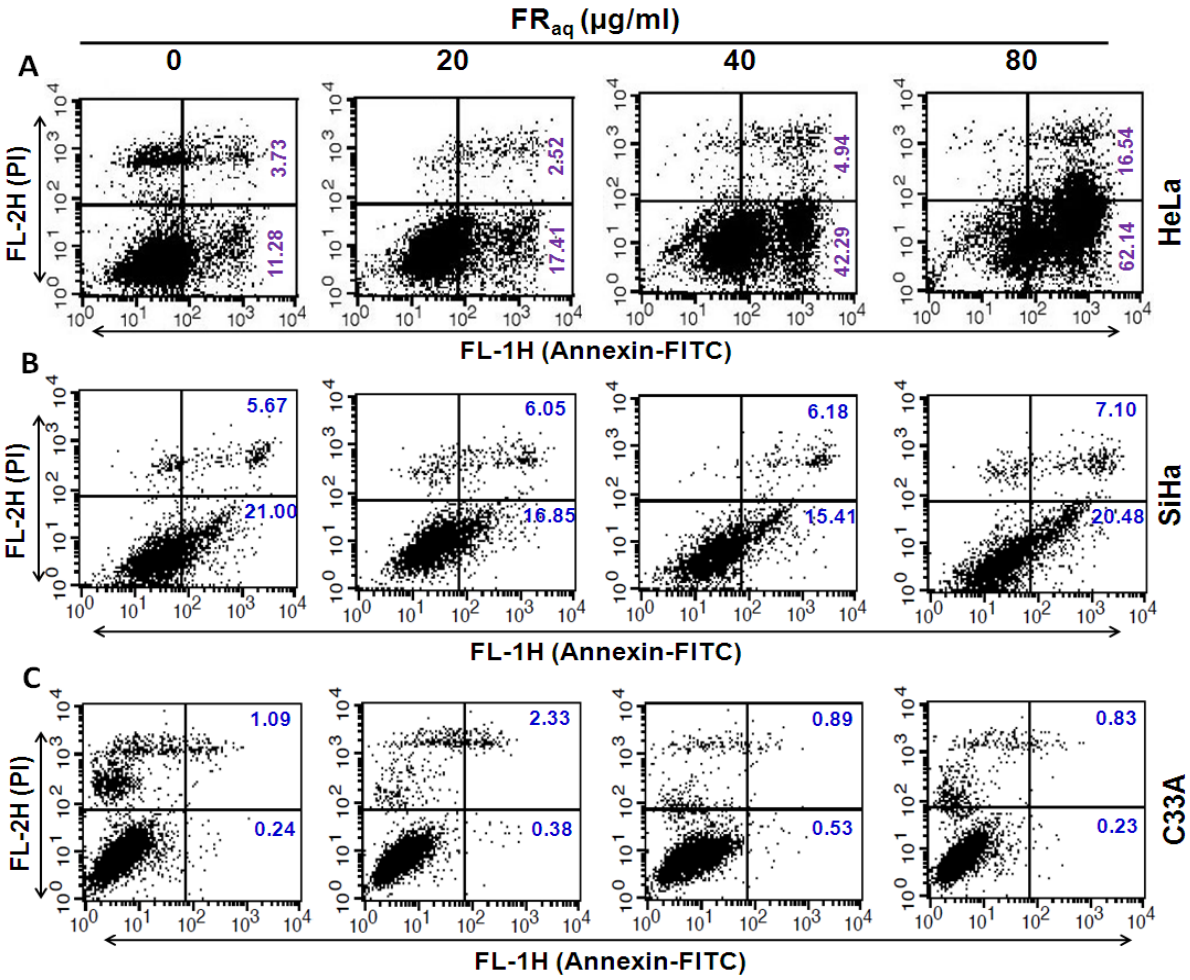


Figure 3.9: Ficus induced apoptosis in HeLa. *Representative FACS pictograms of (**A**) HeLa, (**B**) SiHa and (**C**) C33A cells treated with FR_{aq} (0-80 µg/ml) are shown. Percent of annexin V-positive (early-apoptotic cells, lower right quadrant) and Annexin V/PI-double-positive cells (late-apoptotic cells, upper right quadrant) are indicated. The data represents mean ± SD of three independent experiments.*

3.3.10 Ficus increased intracellular calcium and decreased mitochondrial membrane potential in HeLa

We studied Ca^{2+} signaling mechanism in cells treated with FR_{aq} and observed that it induced a dose-dependent increase in the intracellular calcium levels (Figure 3.10 A). Ionomycin was used as a positive control. Interestingly, the increase in intracellular

calcium resulted into disruption of the mitochondrial membrane potential ($\Delta\psi m$) that was observed by decrease in red fluorescence intensity, after staining the cells with JC-1 dye (Figure 3.10 B). There was ~3-fold reduction in the red fluorescence intensity ($p \leq 0.001$) at 80 µg/ml concentration of $FR_{aq.}$ FCCP was used as a positive control in the study.

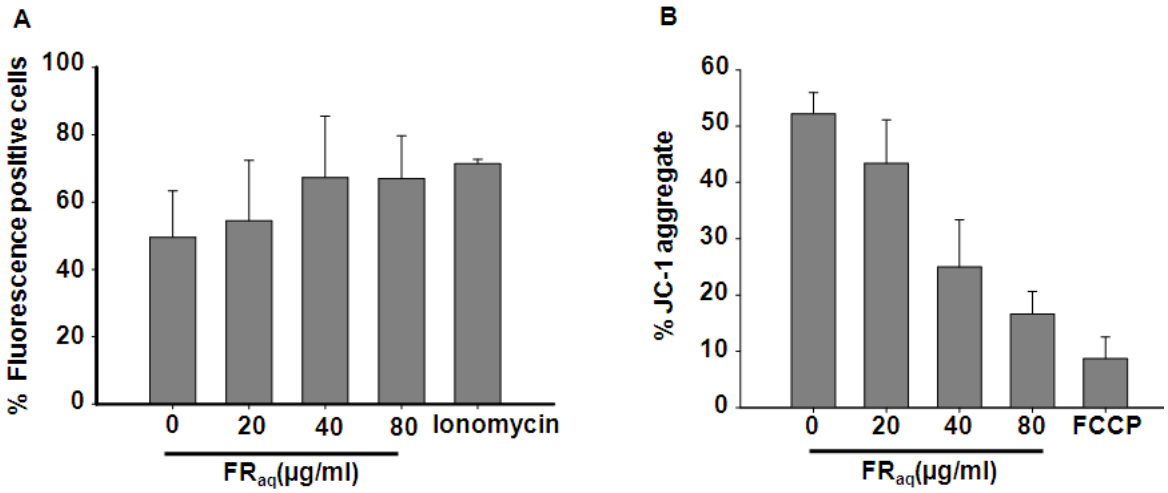


Figure 3.10: Ficus increase intracellular calcium and decreased mitochondrial membrane potential. *(**A**) Flow cytometric analysis of the rapid calcium release in HeLa cells after treatment with FR_{aq}(0-80 µg/ml) has been shown. Ionomycin was used as a positive control. The data represents mean ± SD of three independent experiments. (**B**) FACS analysis following JC-1 staining of HeLa showed alteration of the mitochondrial membrane potential after FR_{aq} (0-80 µg/ml) treatment compared to untreated control cells. The data represents mean ± SD of three independent experiments.*

3.3.11 Ficus increased p53, caspase 3 and cytosolic cytochrom-c expression in HeLa

The mitochondrial membrane depolarization was associated with a dose-dependent increase in the cytosolic cytochrome c (Figure 3.11 A and C) that was accompanied by an increase in the expression of caspase 3 and p53 (Figure 3.11 B and D). These results indicate that Ficus induced apoptosis in HeLa through mitochondrial dependent pathway.

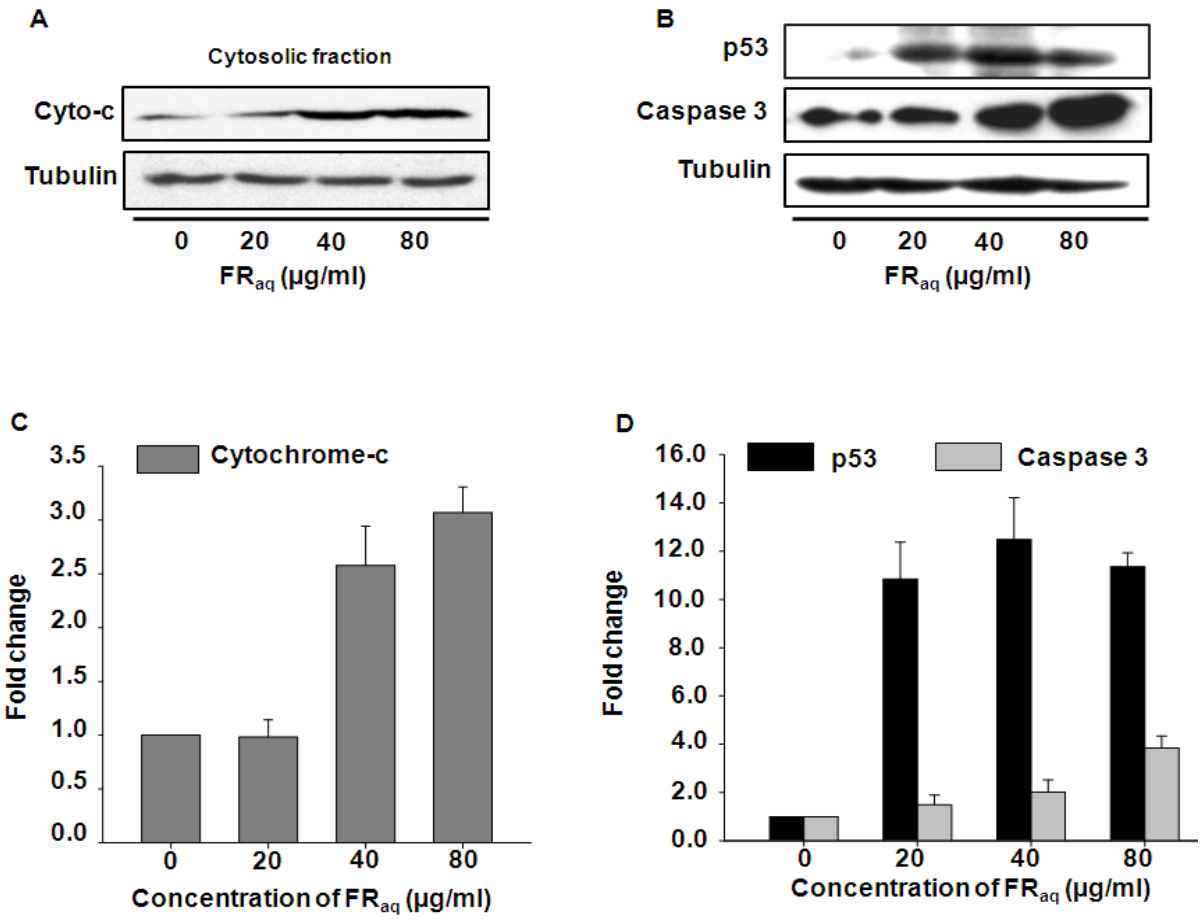


Figure 3.11: Ficus altered p53, caspase 3 and cytosolic cytochrome-c expression in HeLa *(**A**) Western blot shows the expression of cytochrome c from cytosolic fraction. Tubulin was used as a loading control. (**B**) Total protein was isolated and analysed for expression of p53 and caspase 3 by immunoblotting. Tubulin was used as a loading control. (**C and D**)Densitometric analysis of the western blot showing fold change in protein levels. The bands were quantified by using Image J 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).*

3.3.12 Ficus decreased invasion and migration of SiHa and HeLa

Wound healing assay was performed in both the cell lines and it was observed that Ficus effectively inhibited the migration of both SiHa (Figure 3.12 A) and HeLa (Figure 3.12 B) in a dose- and time-dependent manner compared to the untreated control cells. After 16 h, the untreated SiHa and HeLa cells were able to cover up ~82% of the wound, whereas at 80 µg/ml of FR_{aq} treatment, the cells covered up the wound by ~33%

(p<0.001) and 22% (p<0.001), respectively (Figure 3.12 C). At this particular dose, Ficus reduced the invasive capability of both SiHa and HeLa by ~2.45- (p≤0.001) and ~3.8-folds (p≤0.001), respectively, compared to the untreated control cells (Figure 3.12 D).

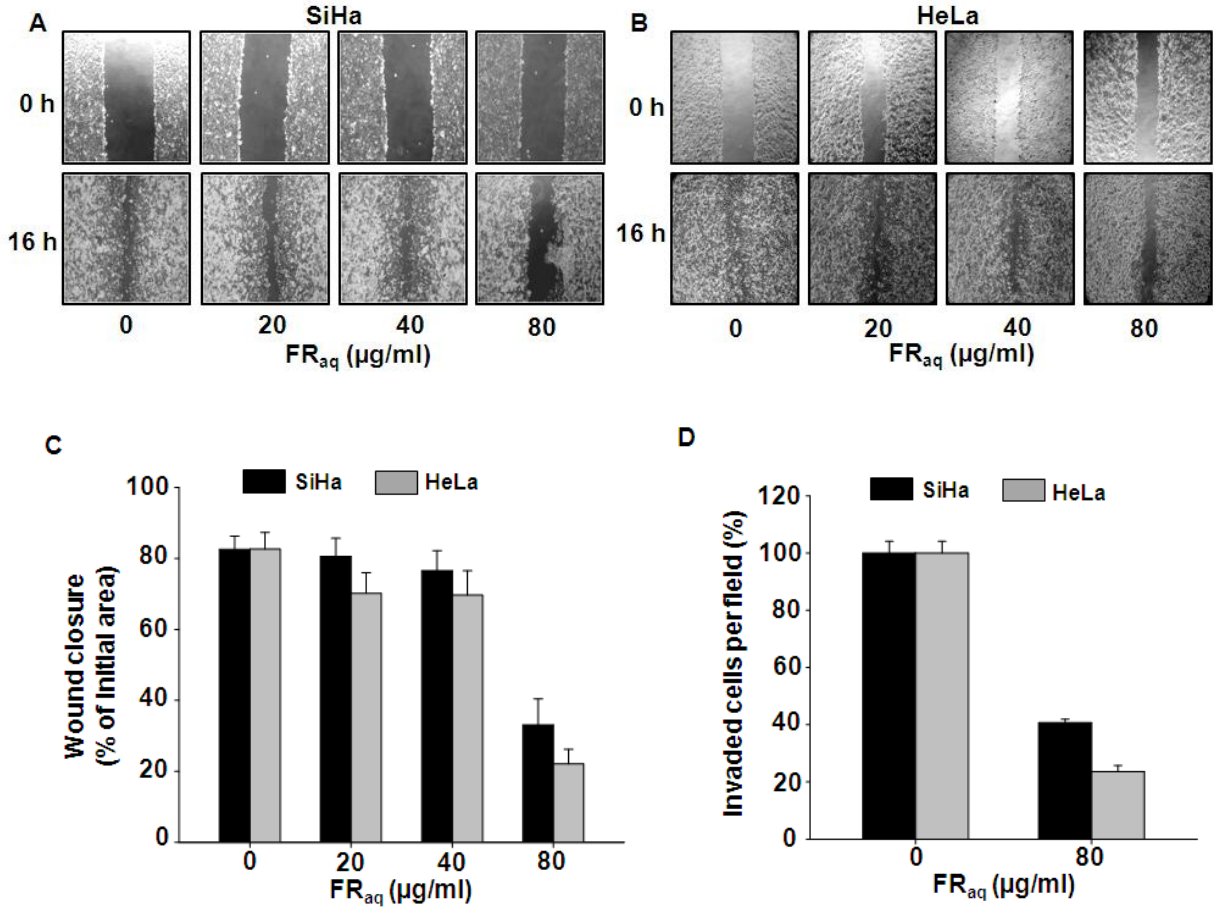


Figure 3.12: Ficus regulated invasion and migration of cervical cancer cells. *Analysis of cell migration in SiHa (**A**) and HeLa (**B**) treated with FR_{aq} (0-80 µg/ml) was measured by wound-healing assay. The upper panel of the image shows the wound made at 0 h. The lower panel shows the migration of cells corresponding to the distance travelled at 16 h. (**C**) Graphical representation of wound closure in SiHa and HeLa cells at 16 h after FR_{aq} treatment has been shown. Values were represented as the percent wound closure and expressed as mean ± SD for three independent experiments. (**D**) Cell invasion assay showing the percentage of cells invaded per field in the presence or absence of FR_{aq}. The invaded cells were counted in ten random fields and the values have been expressed as mean ± SD for three independent experiments.*

3.3.13 Ficus reduced the expression ofMMP-2 and HER-2 expression

It is well known that increased expression of MMPs in tumor tissues is associated with cancer cell matrix degradation, invasion as well as metastasis (Deryugina et al., 2006). We observed that FR_{aq} significantly down-regulated the expression of MMP-2 in both SiHa and HeLa cells (Figure 3.13 A) compared to the untreated control cells.

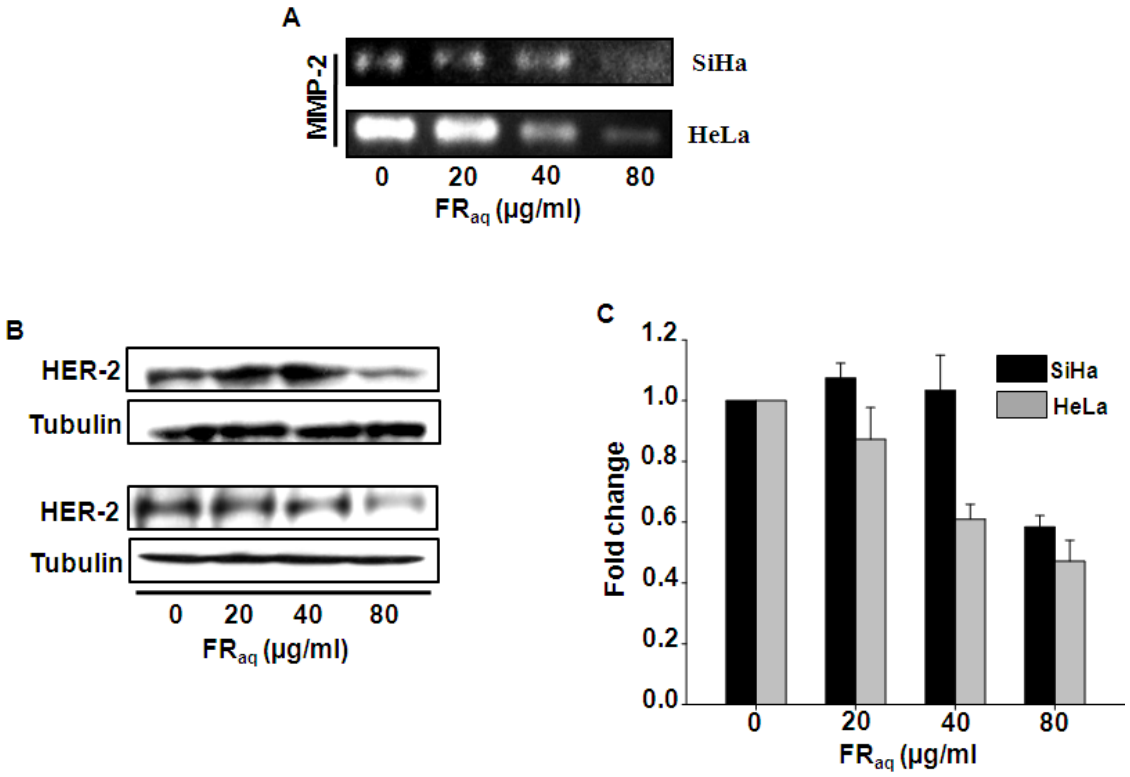


Figure 3.13: Ficus reduced the expression of MMP-2 and HER-2 expression. *(**A**) Gelatin zymography showing downregulation of MMP-2 expression in FR_{aq} (0-80 µg/ml) treated SiHa and HeLa. (**B**) Western blot analysis showing decrease in HER-2 expression in SiHa and HeLa treated with FR_{aq} (0-80 µg/ml). Tubulin was used as a loading control. (**C**) Densitometric analysis of the western blot showing fold change in HER-2 protein levels in SiHa and HeLa. The bands were quantified by densitometry using Image J 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).*

HER2/*neu* has been reported to enhance the metastatic potential of cancers cells (Yu et al., 1994) and is positively correlated with MMP-2 expression (Pellikainen et al., 1994). We found that FR_{aq} decreased the expression of HER-2 in a dose-dependent

manner in both SiHa and HeLa (Figure 3.13 B and C). The data suggest that Ficus reduced the migration as well as invasion of cervical cancer cells by modulating the expression of HER-2 and MMP-2 proteins.

3.3.14 Ficus reduced the expression of viral oncoproteins E6 and E7

Since, Ficus exhibited significant antineoplastic potential in both HPV16 (SiHa) and HPV18 (HeLa) positive cell lines, we investigated the expression of the viral proteins E6 and E7 in the treated and untreated cells. It was observed that, FR_{aq} significantly reduced the expression of E6 and E7 oncoproteins in both SiHa and HeLa (Figure 3.14 A and B, respectively).

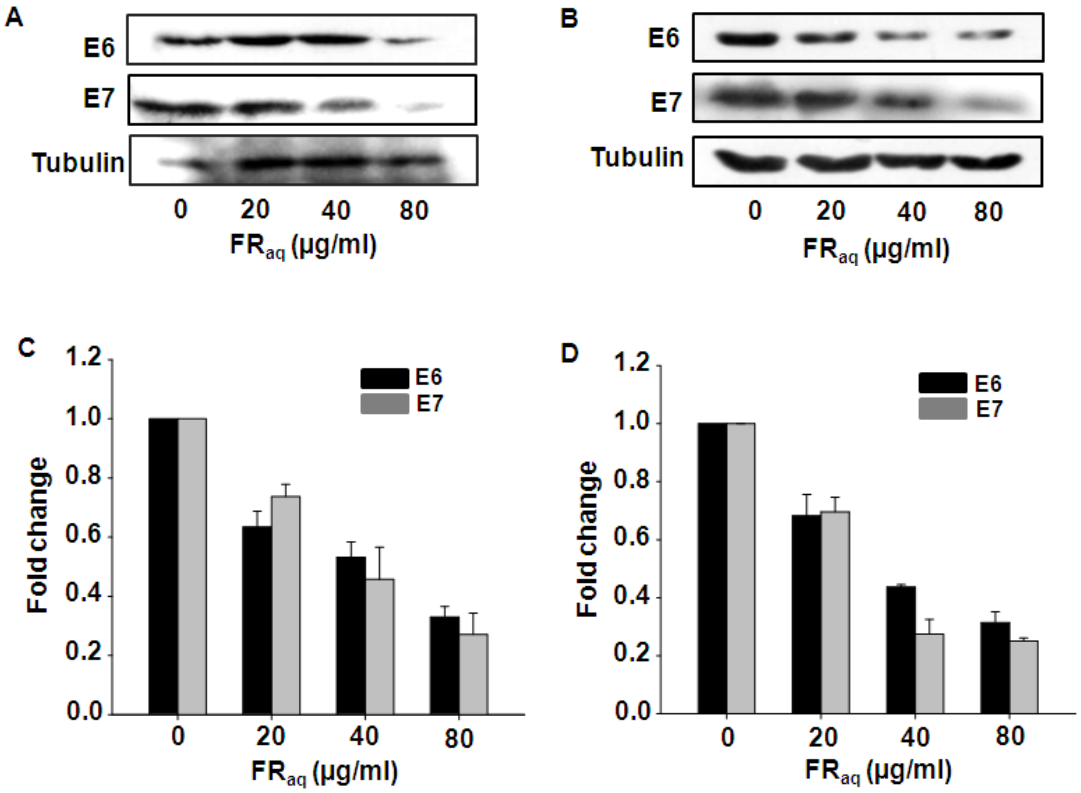


Figure 3.14: Ficus decreased the expression of E6 and E7 proteins. *The expression of E6 and E7 oncoproteins was determined by immunoblotting with E6 and E7 antibodies in SiHa (**A**) and HeLa (**B**) treated with FR_{aq} (0-80 µg/ml). Tubulin was used as a loading control. Densitometric analysis of the western blot showing fold change in E6 and E7 protein levels upon FR_{aq} treatment*

*in SiHa (**C**) and HeLa (**D**). The bands were quantified by densitometry using ImageJ 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).*

At 80 µg/ml FR_{aq} concentration, the expression of E6 and E7 proteins were decreased by ~3.0- ($p \leq 0.001$) and 3.7-folds ($p \leq 0.001$), respectively, in SiHa (Figure 3.14 A and C) and by ~3.2- ($p \leq 0.001$) and 4.0-folds ($p \leq 0.001$), respectively, in HeLa compared to the untreated control cells (Figure 3.14 B and D). Thus, Ficus decreased the expression of the viral oncoproteins E6 and E7, which potentiates its therapeutic significance in cancer regulation.

3.4 Discussion

In the present study, we have investigated the antioxidant potential of aqueous (FR_{aq}) and ethanolic (FR_{et}) extracts of *F. religiosa* bark. We have further elucidated the anti-neoplastic activity of the aqueous extract of *F. religiosa* bark (FR_{aq}), based upon its higher antioxidant potential, polyphenol content and ALP activity compared to FR_{et} extract. FR_{aq} regulated the growth kinetics of the cervical cancer cells lines in a statistically significant manner and thus, Ficus exhibited a promising anticancer potential.

p53, a master tumor suppressor, is the most frequently mutated gene in almost all kinds of human cancers (Rivlin et al., 2011). Moreover, loss of p53 function is responsible for the progression to more aggressive cancer phenotype (Muller et al., 2011). In cervical cancer, E6 from high-risk HPV types (16 and 18) initiates degradation of p53 and thus, restoration of its function could be an effective therapeutic approach (Scheffner et al., 1991). Reactivation of p53 in cervical cancer cells can lead to inhibition of cell proliferation as well as induction of apoptosis (Kochetkov et al., 2006). Most of

the chemopreventive drugs regulate the growth of cancer cells either by arresting them at G_1/S or G_2/M phase or by induction of apoptosis by p53-dependent or independent mechanisms (Sa et al., 2008). In our studies, we found that FR_{aq} exerted its anti-proliferative activity in each of the cervical cancer cell line by different mechanisms. In HPV-16 positive SiHa cells, FR_{aq} induced G_1/S phase arrest through increase in the expression of p53 and p21 with a simultaneous decrease in the phosphorylation of pRb tumor suppressor protein. $p21^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor, is a p53-inducible protein that blocks the cell cycle progression in the G_1/S phase (Niculescu et al., 1998). Thus, up-regulation of p21 by FR_{aq} might have resulted into activation of downstream effectors of p53-dependent G_1/S arrest. The hypophosphorylated form of retinoblastoma protein (pRb), a tumor suppressor, forms a complex with E2F transcription factor resulting into repression of cell proliferative genes (Henley et al., 2012). The viral E7 oncoprotein is known to inactivate the complex formation between pRb and E2F, thereby resulting into destabilization of pRB that eventually leads to deregulation of the cell cycle (Jones et al., 1997). In our studies, we observed that Ficus reduced the levels of ppRb that might have resulted into increased expression of pRb as well as eventual arrest of cells in G_1/S phase.

Apoptosis is an important mechanism to kill the tumor cells and (Korbakis et al., 2012) can be induced by increase in the mitochondrial calcium that results into loss of membrane potential ($\Delta\psi m$), expansion of the matrix and rupture of the outer mitochondrial membrane (Fulda et al., 2010). This results into release of cyt c into the cytosol, either by inhibition of anti-apoptotic factors or activation of pro-apoptotic proteins leading to the activation of caspase 3/9 (Elmore et al., 2007). During cell death, mitochondria are known to accumulate Ca^{2+}, resulting into activation of the permeability transition pore (PTP) that leads to transient mitochondrial depolarization (Duchen et al.,

2000). This leads to release of cyt c along with a large number of other factors from the inter-membrane space (Giorgi et al., 2012). In HPV-18 positive HeLa cells, FR_{aq} induced p53-dependent apoptosis through increase in intracellular calcium and depolarization of mitochondrial membrane potential that lead to release of cytosolic cyt c and increase in caspase 3 expression. Interestingly, at non-cytotoxic dose, Ficus did not induce either arrest or apoptosis in C33A (HPV-negative, p53 mutated), thereby suggesting alternate mechanisms of cell death.

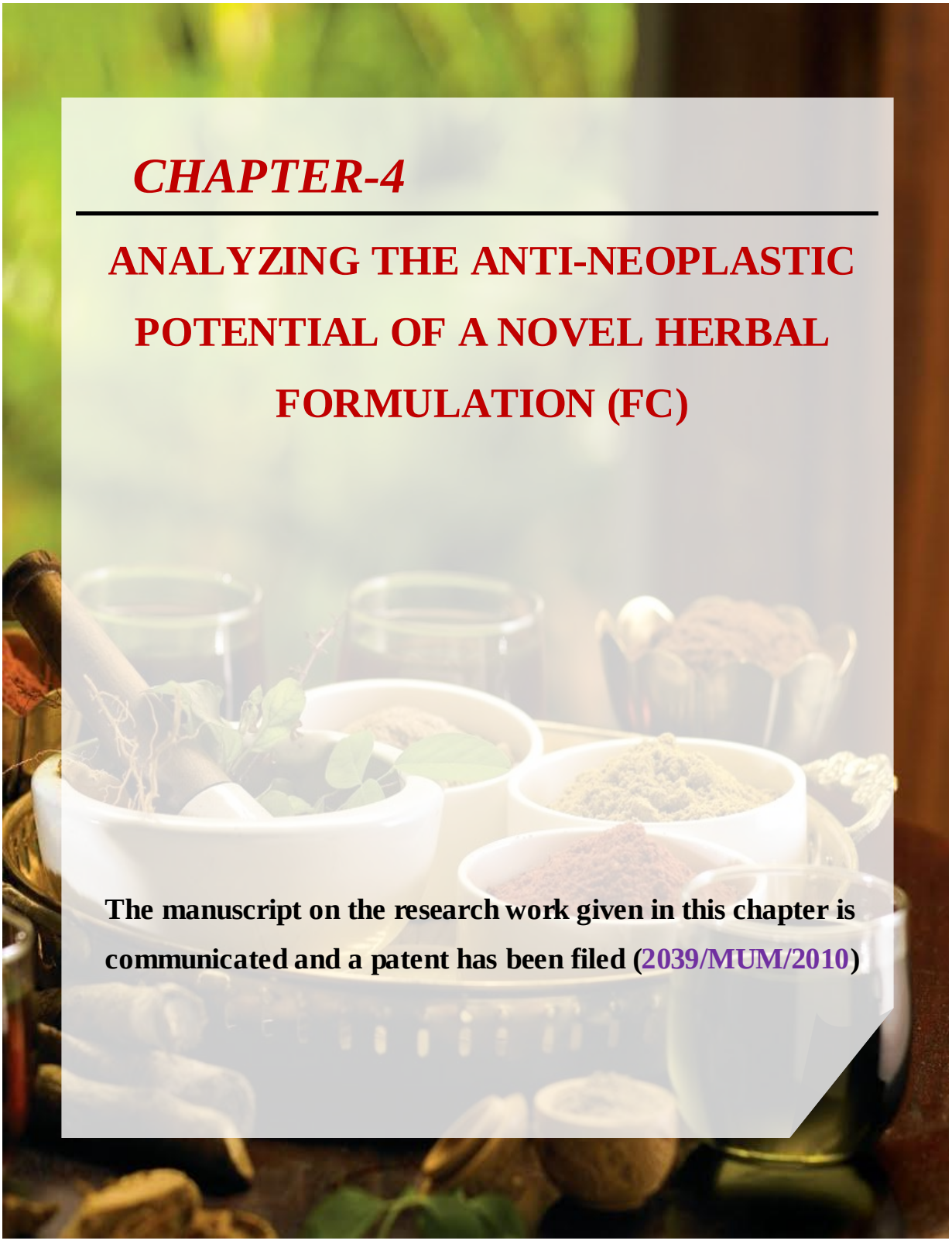
The observed dichotomy in the regulation of growth in SiHa and HeLa could be due to variation in p53 activation that may decide the fate of a cell to either initiate apoptosis or undergo cell cycle arrest. It has already been reported that low levels of p53 induce cell cycle arrest whereas high levels of p53 induce apoptosis (Vousden et al., 2012; Zhang et al., 2000). Our results show that p53 was activated more in HeLa compared to SiHa in response to FR_{aq} treatment, thereby, resulting into apoptosis in the former and cell cycle arrest in the latter. The other reason for the altered response of SiHa and HeLa towards Ficus treatment could be the difference in their genetic make-up that includes their HPV status as well as the viral copy number (Meissner et al., 1999). For example, SiHa (squamous cell carcinoma) contains around 1-2 integrated copies of HPV 16 genome whereas HeLa (adenocarcinoma) has around 10-50 integrated copies of HPV 18 (Meissner et al., 1999; Bravo-Cuellar et al., 2010). Moreover, the rate of replication is also different in both the cell types.

HER2/neu oncogene is frequently amplified in cervical cancer and can be considered as a potent therapeutic target (Mitra et al., 1994; Chavez-Blanco et al., 2004). Its overexpression has been found to be associated with up-regulation of MMP-2 and MMP-9 that play an important role in cancer cell invasion and metastasis (Pellikainen et al., 2004). Interestingly, Ficus significantly reduced the expression of both HER-2 and

MMP-2 that might have resulted in the observed decrease in the migration as well as invasion of cervical cancer cells.

E6 and E7 are the two viral oncoproteins known to induce cervical cancer by inactivating the tumor suppressor proteins, p53 and pRb, respectively (Scheffner et al., 1991). p53 gene is mutated irreversibly in most of the cancers; however, cervical carcinomas and cell lines have been reported to retain wild-type p53 and pRb genes whose function gets masked by the viral E6 and E7 proteins (Abdulkarim et al., 2002). We observed that Ficus decreased the expression of E6 and E7 in both the cervical cancer cell lines. The down regulation of E6 and E7 oncoproteins might have led to the restoration of tumor suppressor functions of p53 and pRb proteins, respectively. This might have led to the activation of downstream signaling molecules resulting into either cell cycle arrest or apoptosis. Even though a direct effect of E6/E7 on HER-2 has not been reported, however, their coexpression has been demonstrated to be critical for induction of head and neck squamous cell carcinomas (HPV positive) (Al Moustafa et al., 2004) as well as breast cancer (Woods et al., 2005). These data suggest that *F. religiosa* has the potential to target HPV E6 and E7 proteins that could have a significant therapeutic potential in cervical cancer.

Thus, our findings provide a strong basis for further exploration of *F. religiosa* as a therapeutic drug against cervical cancer, either alone or as an adjuvant to standard chemotherapeutic agents.



CHAPTER-4

ANALYZING THE ANTI-NEOPLASTIC POTENTIAL OF A NOVEL HERBAL FORMULATION (FC)

The manuscript on the research work given in this chapter is communicated and a patent has been filed (2039/MUM/2010)

Abstract:

A novel herbal formulation (FC) was developed from the bark materials of *Ficus religiosa* and *Cinnamomum zeylanicum*. FC was standardized biochemically and its anticancer properties were delineated through in vitro and *in vivo* approaches. FC induced apoptosis in SiHa and HeLa cells through early generation of reactive nitrogen and oxygen species (RNOS) and loss of mitochondrial membrane potential (MMP). In C57BL/6 mouse melanoma model, the oral administration of FC inhibited tumor growth significantly. Interestingly, FC treatment led to an appreciable increase in the serum levels of Th1 cytokines (IFN-γ and IL-2) and subsequent decrease in the levels of Th2 cytokine (IL-4). These data suggest that FC formulation could be explored further for its chemopreventive potential in cervical cancer.

4.1. Introduction

Cancer is the leading cause of death worldwide, accounting for about 8.2 million deaths in 2012 (Globocan., 2012). Survival and progression of cancer cell strongly depends upon its ability to overcome apoptosis as well as host immune response (Brandacher et al., 2006).

In the recent years, there is a renewed interest in the use of herbal medicines in the management of wide range of human diseases, including cancer. The herbal formulations from Ayurveda, traditional Chinese medicines, Japanese (Kampo) and others are composed of mixture of different herbs, which may work synergistically to produce maximum therapeutic efficacy with minimum or no side effects (Ahmed et al., 2013). Moreover, extensive research has revealed that these formulations can induce apoptosis in cancer cells and may also stimulate the immune system (Liu et al., 2012a). For example, many polyherbal formulations such as PHY906 (Liu et al., 2012b), PC-SPES (Wang et al., 2013) and YWKLF (Li et al., 2008) have revealed their efficacy against cancer and some of them are even in phase II clinical trials. Likewise, herbal formulation of *Withania somnifera* (WSF) has been reported to have both anticancer and immune modulatory activities (Malik et al., 2008).

In previous chapter, we have elucidated the anticancer mechanism of aqueous extract of *Cinnamomum cassia* (Chapter-2) and *Ficus religiosa* (Chapter-3) in cervical cancer cells. Moreover, earlier work from our group had shown that *Cinnamomum zeylanicum* exhibited cytotoxic activity in a panel of cancerous cell lines (Singh et al., 2009), including cervical cancer cell line. We wanted to analyze whether combination of Ficus and Cinnamon could reduce the drug dose through the synergistic activity of both the plant materials. Thus, we prepared a herbal formulation (FC) of *C. zeylanicum* with

F. religiosa. We did not take *C.cassia* since it is known to contain high amounts of coumarins that have been reported to exhibit toxicity in humans (Wang et al., 2013).

Our data suggests that FC inhibited the growth of SiHa and HeLa by inducing apoptosis. Interestingly, FC retarded tumor growth significantly and modulated the immune system in C57BL/6 melanoma tumor model through regulation of Th1 and Th2 cytokines.

4.2. Materials and Methods

4.2.1. Plant material and extract preparation

The bark of *Cinnamomum zyelanicum (CZ)* was purchased from Green Pharmacy Pune Maharashtra, India. The bark of *Ficus religiosa (FR)* was collected from Pune District, Maharashtra, India. Barks of both *CZ* and *FR* were chopped into small pieces, shade dried at ambient temperature and ground into coarse powder in a grinder. The aqueous extract was prepared as per the standard Indian Pharmacopoeia by combining the two bark materials (*FR: CZ*) in different ratios of 1:1,1:2 and 2:1. The extract obtained was centrifuged at 13000 rpm for 15 min and the supernatant was filtered through swiney filter (pore size, 0.45 µm). The extract was stored at -80ºC until use. *(Note: FR:CZ composition at ratio of 2:1, is termed as FC formulation)*

4.2.2. HPLC analysis

The HPLC fingerprint of FC formulation was determined by Shimadzu High Performance Liquid Chromatographic System LC 2010 CHT with UV detector in combination with Class LC solution software. Chromatographic separation was performed with a Kromasil C18 reversed-phase column (4.6 mm id x 250 mm) with a 5 µm particle size (Sigma-Aldrich, USA). Column temperature was set at 40ºC. Gradient

flows for the two solvent systems (solvent A, 0.14% anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in water; solvent B, acetonitrile) were as follows: 0 min, 5% B; 12 min, 15% B; 35 min, 70% B; 40 min, 5% B; and hold at 5% B for 5 min. The flow rate of the mobile phase was maintained at 1.5 ml/min. The injection volume was 20 ul and the chromatogram was monitored at wavelengths of 254 nm throughout the experiment. The reference standards protocatechuic acid, catechin, cinnamic acid, cinnamyl alcohol and cinnamaldehyde, were used to confirm their presence in FC formulation.

4.2.3. Cell culture

Human cervical carcinoma cell lines SiHa (HPV-16) and HeLa (HPV-18) were obtained from National Center for Cell Science (NCCS), Pune, Maharashtra, India. PBMCs were isolated from a healthy donor and used as primary cells. The cells were grown in DMEM with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 units/ml penicillin and streptomycin). Cells were maintained at 37ºC in 5% CO_2/air atmosphere.

4.2.4. Cytotoxic activity

Cytotoxic potential of each component (*FR* and *CZ*) and three various combinations (1:1, 1:2, 2:1) of *FR:CZ* were determine in SiHa and HeLa, by MTT dye uptake (Choudhari et al., 2011). Briefly, SiHa, and HeLa, were seeded at 1 x 10^5/ml density in a 96-well plate. Next day, the cells were incubated with various concentrations (0-640 µg/ml) of *FR* and *CZ* (used alone or in combination) in triplicates for 24 h in 5% CO_2 incubator at 37°C. An untreated group was kept as a control in all the cell lines used. 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% CO_2 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 (O.D) with the ELISA microplate reader (Biorad, Hercules, CA) at OD 570-630 nm. The percentage viability was calculated as:

$$\% \text{ Viability } = \left[\frac{\text{OD of treated cells}}{\text{OD of control cells}}\right] * 100$$

4.2.5. Isolation of peripheral blood mononuclear cells from human blood and cytotoxicity assay

Peripheral blood mononuclear cells (PBMCs) were separated from whole heparinized blood by Ficoll-Hypaque density gradient centrifugation method (Fuss et al., 2009). Briefly, 6 ml of Ficoll–Hypaque (Sigma, St. Louis, MO) was stratified under 10 ml of peripheral blood and centrifugation was performed at 400 × *g* for 30 min at room temperature. Recovered PBMCs were washed three times with 1X PBS and the number of living cells was counted by trypan blue (Sigma, St. Louis, MO) staining. PBMCs were seeded at 1 x 10^5 cells/ml density in a 96-well plate. Next day, the cells were incubated with various concentrations (0-640 µg/ml) of FC. The experiment was repeated thrice and for each experiment freshly isolated PBMCs were used.

4.2.6. Assessment of apoptosis by Annexin FITC/PI staining

Apoptosis was dertermined by Annexin FITC/PI staining (Malo et al., 2010). Briefly, HeLa and SiHa cells were plated at a seeding density of 1 x 10^5 cells/ml in a 96 well black culture plate and allowed to adhere overnight at 37°C in a CO_2 incubator. Next day, the cells were treated with FC (0-40 µg/ml) for 24 h at 37°C in 5% CO_2. Following incubation, cells were stained with Annexin V-FITC according to the

manufacturer's instructions (Annexin V-FITC apoptosis kit #3, Invitrogen, Grand Island, NY). The plate was centrifuged at 400 g for 5 min at 37°C and the supernatant was aspirated and discarded. Hundred microliters of assay binding buffer was added to each well and the fluorescence intensity was measured using the Fluostar Omega microplate reader (BMG Labtech). Dead cells were stained by propidium iodide which displays strong fluorescence intensity with excitation and emission at 560 nm and 595 nm, respectively. Early stage apoptotic cells, stained by Annexin V FITC, were detected at the excitation and emission wavelength of 485 nm and 535 nm, respectively.

4.2.7. Estimation of cellular Reactive Oxygen Species (ROS)

Cellular ROS production was determined by dihydrodichlorofluorescein diacetate probe (DCF-DA) (Zhou et al., 2010). Briefly, SiHa and HeLa cells were seeded at a density of 1 x 10^5 cells/ml in a 96 well black culture plate and allowed to adhere overnight at 37°C in a CO_2 incubator. Next day, the medium was removed and the cells were incubated with fresh culture media containing DCF-DA (10 µM) dye (Molecular Probes) for 30 min in the dark followed by washing twice with 1X PBS. The cells were then treated with various concentrations of FC (10-40 µg/ml). The fluorescence intensity generated from intracellular ROS was detected using a Fluostar Omega microplate reader (BMG Labtech) with excitation and emission wavelengths of 480 nm and 520 nm, respectively. ROS was determined by comparing the changes in fluorescence intensity at a give point (F) with that of the baseline (control) fluorescence intensity (F_0).

4.2.8. Analysis of intracellular Nitric Oxide (NO)

Nitric oxide production in cervical cancer cells was measured using 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) (Vardi et al., 2006). Briefly, SiHa and

HeLa cells were seeded at a density of 1 x 10^5 cells/ml in a 96 well black culture plate and allowed to adhere overnight at 37°C in a CO_2 incubator. Next day, the medium was removed and the cells were incubated with fresh culture media containing DAF-FM (2.5 μM) dye (Molecular Probes) for 30 min in the dark, followed by washing twice with 1X PBS. The cells were then treated with various concentrations of FC (10-40 μg/ml). The fluorescence intensity generated from NO was detected by using a Fluostar Omega microplate reader (BMG Labtech) with excitation and emission wavelengths of 480 nm and 520 nm, respectively. NO was determined by comparing the changes in fluorescence intensity at a give point (F) with that of the baseline (control) fluorescence intensity (F0).

4.2.9. Analysis of Mitochondrial Membrane Potential (Δψm)

Cervical cancer cell lines were seeded at a density of 1 x 10^5 cells/ml in a 96 well black culture plate and allowed to adhere overnight at 37°C in a CO_2 incubator. Next day, the cells were treated with different concentrations of FC (10-40 μg/ml). After 24 h of incubation, the medium was removed and the cells were washed twice with 1X PBS followed by incubation with fresh culture media containing JC-1 (2.5 μg/ml) dye (Sigma-Aldrich, St. Louis, MO) for 30 min in the dark. Fluorescence intensity was measured using the Fluostar Omega microplate reader (BMG Labtech) at 520 nm for JC-1 monomers and at 590 nm for JC-1 aggregates.

4.2.10. Tumor retardation study

The *in vivo* anti-cancer study was performed at Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Navi-Mumbai. Briefly, $B_{16}F_{10}$ melanoma (1 x 10^6 cells/mouse) cells were inoculated subcutaneously (s.c) into the right flanks of six weeks old C57BL/6 mice. Eight days after the injection, animals were

randomized into five groups, each group having six mice. Group I was kept as a negative control and received normal saline; Group II received Doxorubicin (4 mg/kg body weight (bw)) intravenously on 1st, 5th and 9th day; Group III and IV were given oral gavage of FC at concentrations of 100 and 200 mg/kg bw, respectively, daily for 15 days. During the treatment period, the tumor size was measured with vernier calipers after every 2 days, and the average tumor volume was calculated using the formula: $1/2(\text{length} \times \text{width}^2)$.

The percent tumor growth inhibition in treated groups was calculated as follows:

$$\%\ \text{Tumor inhibition} = \left[\frac{\text{Av. tumor volume of control group} - \text{Av. tumor volume of test group}}{\text{Av. tumor volume of control group}}\right] * 100$$

After 15 days of treatment, animals were scarified and blood samples were collected for cytokine analysis.

4.2.11. Th1/Th2-like cytokine determination in tumor bearing mice sera

Blood samples were collected from FC treated and un-treated tumor-bearing mice on day 15 of FC treatment. The concentrations of serum cytokines (IFN- γ, IL-2 and IL-4) were measured by using mouse Th1/Th2 enzyme-linked immunosorbent assay (ELISA) Ready SET Go Kit (BD Bioscience, San Diego, CA, USA), The detection procedures were performed according to the manufacturer's instructions. The sensitivity of assay for each cytokine was as follows: 4 ρg/ml for IL-2 and IFN-γ; and 2 ρg/ml for IL-4.

4.3 Results:

4.3.1 Effect of individual extracts and its combination on cell viability

The cervical cancer cell lines, SiHa and HeLa were treated with *Ficus religiosa* (*FR*), *Cinnamon zyelanicum* (*CZ*) and three different combinations (1:1, 1:2, 2:1) of *Ficus religiosa*:*Cinnamomum zyelanicum* (*FR:CZ*) at various concentrations (0-640 µg/ml) for 24 h. The cytotoxicity of *FR*, *CZ* and *FR:CZ* (1:1, 1:2, 2:1) in SiHa and HeLa has been shown in Table 4.1and 4.2, respectively.

In SiHa, *FR* exhibited significant cytotoxicity at 320 µg/ml concentration whereas *CZ* showed cytotoxicity at 640 µg/ml concentration (Table 4.1). However, when the cells were treated with different ratios (1:1, 2:1 and 1:2) of *FR:CZ*, all the different compositions exhibited significant cytotoxicity at relatively lower doses compared to either *FR* or *CZ*.

Tabel 4.1: Cytotoxicity of *FR*, *CZ* and *FR:CZ* (1:1, 1:2, 2:1) in SiHa cells

Concentration (µg/ml)	*C. zyelanicum* (CZ) % viability	*F. religiosa* (FR) % viability	FR:CZ (1:1) composition % viability	FR:CZ (1:2) composition % viability	FR:CZ (2:1) composition % viability
0	100.00± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
20	106.20± 2.43	112.86± 3.4	113.85 ± 5.13	112.26± 4.23	103.94 ± 5.28
40	106.76± 1.03	116.02± 3.14	120.80± 8.21	122.22± 7.45	100.01± 1.77
80	107.14± 4.6	115.37 ± 5.31	122.97± 8.52	141.43 ± 3.43	86.86 ± 5.02*
160	108.60± 6.22	113.35± 6.79	87.25± 3.64*	85.47± 3.43*	57.75± 2.17*
320	101.27± 3.64	69.88± 5.10*	78.62± 0.96*	66.36± 7.06*	43.25± 2.14*
640	83.66± 1.16*	22.32± 2.43*	29.78± 1.66*	32.84± 4.70*	32.31± 1.08*

Similarly, in HeLa (Table 4.2), *FR* induced significant cytotoxicity at 320 µg/ml, whereas, *CZ* showed cytotoxicity at 640 µg/ml concentration. However, when the cells

were treated with different ratios (1:1, 2:1 and 1:2) of *FR:CZ*, the different compositions exhibited significant cytotoxicity at relatively lower doses compared to either *FR* or *CZ*.

Table 4.2: Cytotoxicity of *FR*, *CZ* and *FR:CZ* (1:1, 1:2, 2:1) in HeLa cells

Concentration (µg/ml)	*C. zyelanicum* (CZ) % viability	*F. religiosa* (FR) % viability	FR:CZ (1:1) composition % viability	FR:CZ (1:2) composition % viability	FR:CZ (2:1) composition % viability
0	100.0± 0.00	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00
20	110.65±1.44	104.43±1.47	102.77±10.78	112.39±2.44	97.06±3.91
40	111.79±2.09	100.93±2.43	103.15±11.15	108.35±1.65	91.37.±2.05
80	109.82±2.03	102.49±0.78	104.91±6.91	111.93±3.12	70.46±3.21*
160	104.09±2.84	91.09±2.79	112.49±9.9	71.94±5.52*	38.75±3.69*
320	99.26±3.03	76.49±1.71*	44.46±11.57*	44.76±11.21*	35.58±4.54*
640	84.11±2.10*	57.97±1.38*	11.56±2.21*	9.80±1.41*	25.92±9.47*

Interestingly, at 2:1 ratio of *FR:CZ* significant cytotoxicity was observed, in both SiHa and HeLa at relatively lower concentration compared to other ratios. Thus, further studies were carried out with 2:1 ratio of FR and *CZ*. (*Note: FR:CZ composition at ratio of 2:1, has been termed as FC formulation in further work*)

4.3.2 FC selectively inhibited growth of cervical cancer cells

Since FC showed toxicity at relatively lesser concentration compared to the individual plant materials and other combinations, its cytotoxicity was investigated in normal human peripheral blood mononuclear cells (PBMCs). The cells were treated with various concentrations (0-640 µg/ml) of FC for 24 h and cytotoxicity was measured by MTT assay. The cytotoxicity of FC in SiHa, HeLa and PBMCs has been shown in Figure 4.1. Compared to IC_{50} value of 203.16 and 119.70 µg/ml in SiHa and HeLa, respectively,

the IC_{50} value of FC in PBMCs was > 640 µg/ml concentration. This suggests that FC was safe to the normal cells.

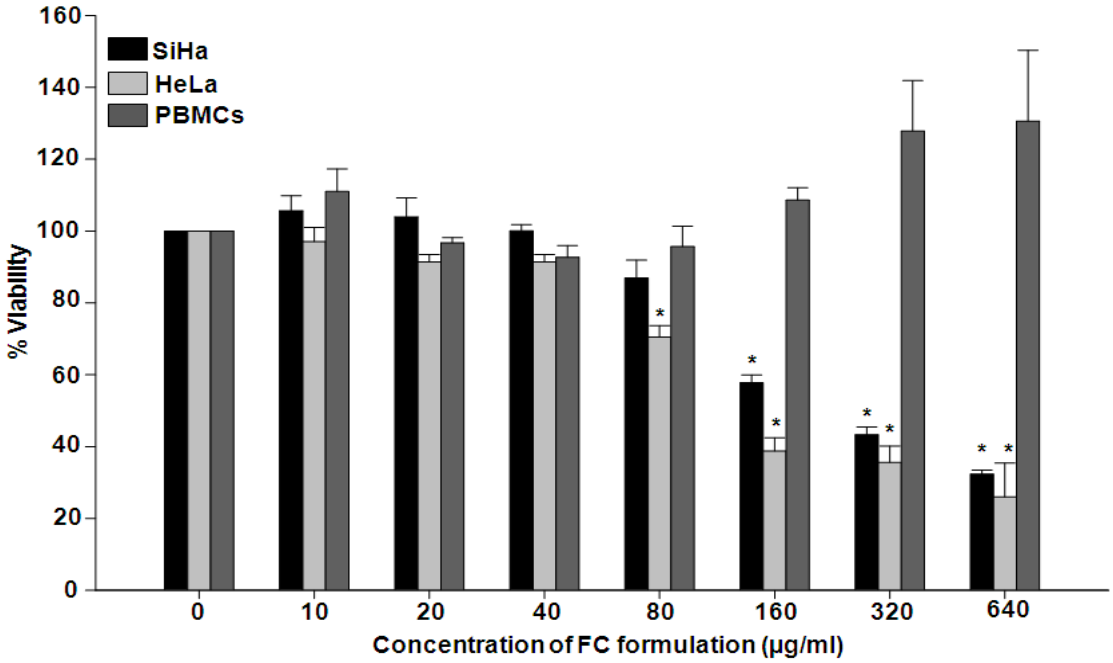


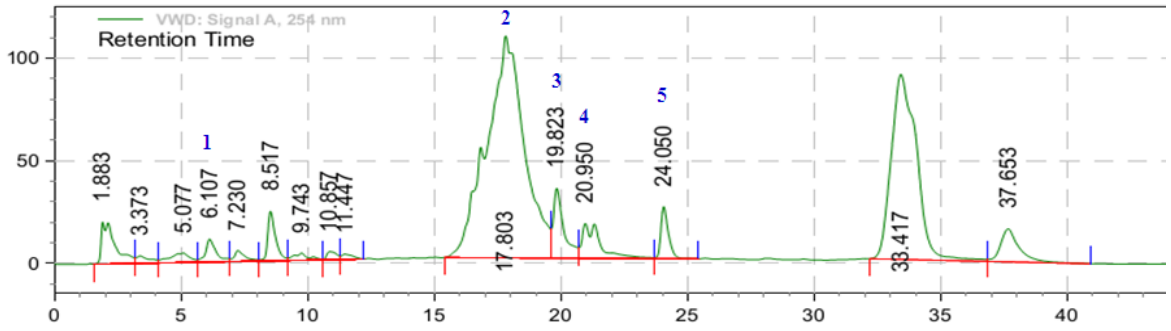
Figure 4.1: Cytotoxic effect of FC in cervical cancer lines and normal cells. *SiHa, HeLa and PBMCs were treated with different concentrations (0-640 µg/ml) of FC for 24 h. The viability was measured by MTT assay.*

4.3.3 Standardization of FC formulation

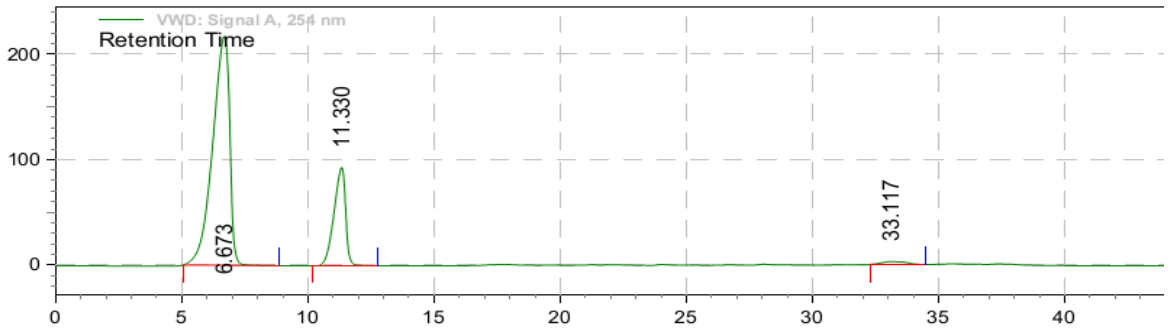
Before evaluating the therapeutic efficacy of FC, it was important to standardize it with respect to its marker compounds. Thus, chemo-profiling of FC was performed based upon five standard marker compounds (protocatechuic acid, cataechin, cinnamic acid, cinnamyl alcohol and cinnamaldehyde) present in it employing HPLC. Comparisons of the retention times of sample peaks (Figure 4.2 A) were done with respect to the standard peaks (Figure 4.2 B-F). The retention times of protocatechuic acid, catechin, cinnamic acid, cinnamyl alcohol and cinnamaldehyde were 6.97, 17.15, 20.50, 21.26, 23.93 minutes, respectively (Figure 4.2 B-F). Catechin was the most abundant marker component (Average Content (AC) was around 140.67 mg/g), followed

by cinnamaldehyde (AC, 1.20 mg/g), protocatechuic acid (AC, 0.89 mg/g), cinnamic acid (AC, 0.80 mg/g) and cinnamyl alcohol (AC, 0.30 mg/g).

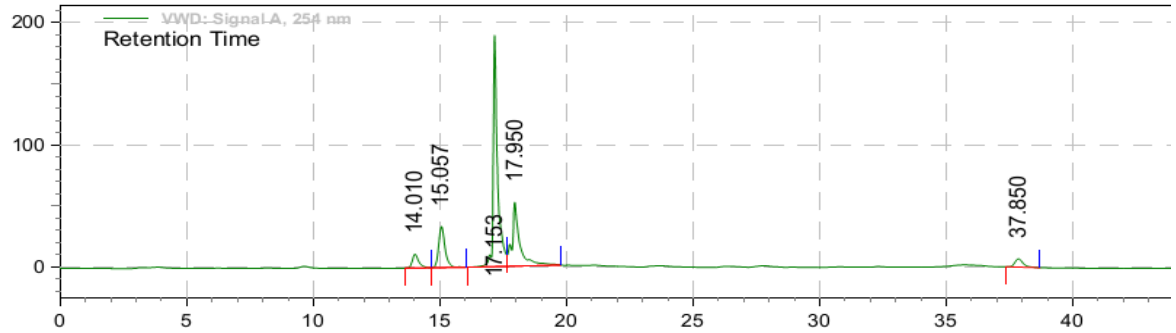
A) FC formulation



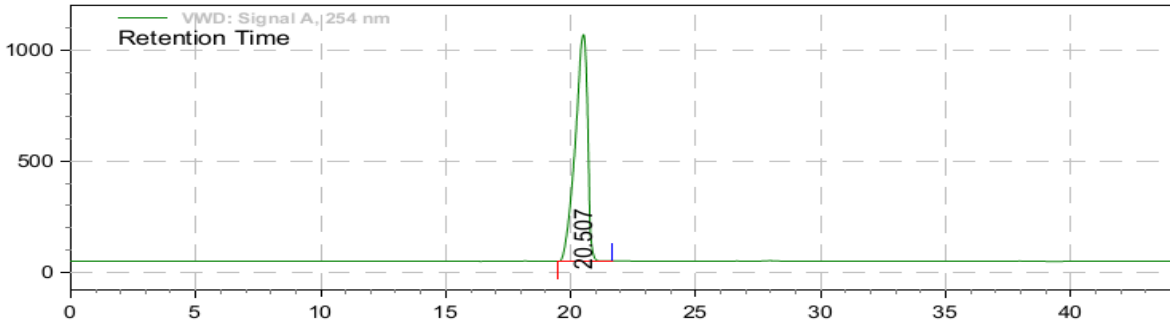
B) Protocatechuic acid



C) Catechin



D) Cinnamic acid



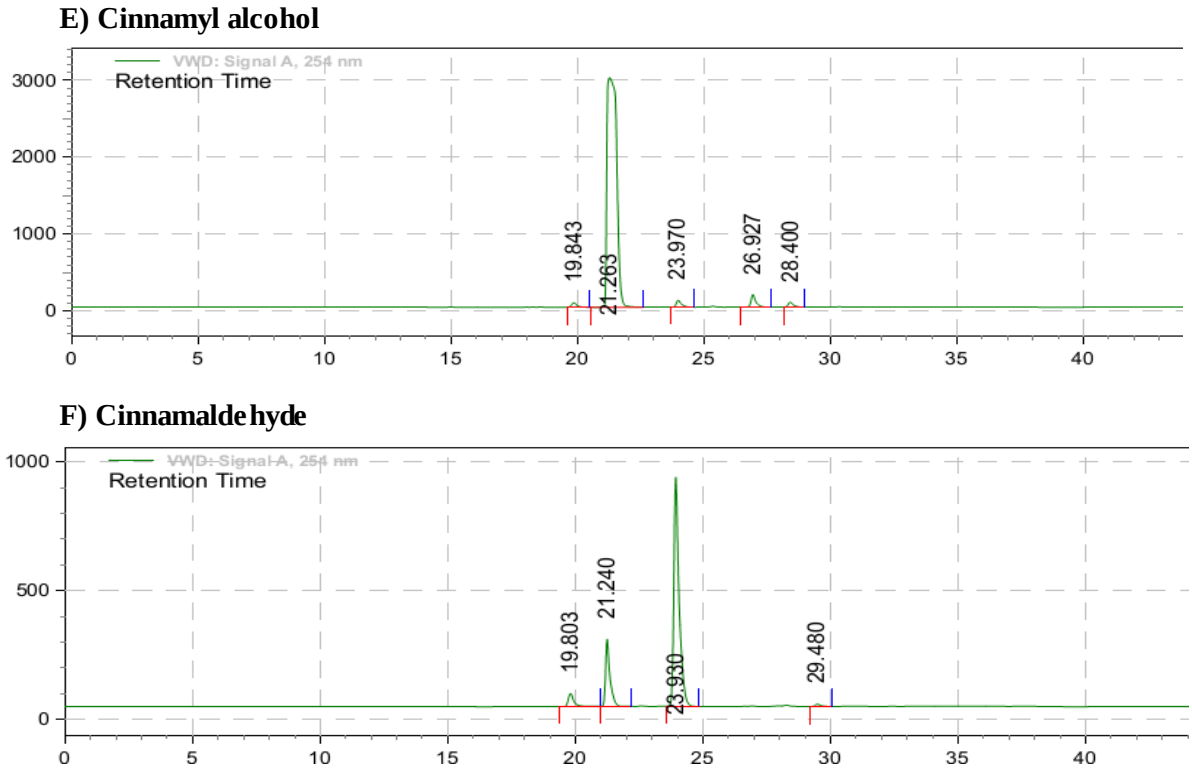


Figure 4.2: Representative chromatogram of (A) FC formulation and standard (B) protocatechuic acid (C) catechin (D) cinnamic acid, (E) cinnamyl alcohol, (F) cinnamaldehyde. *HPLC was performed using potassium dihydrogen orthophosphate (KH_2PO_4): acetonitrile gradient. Gradient flows for the two solvent systems (solvent A, 0.14% anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in water; solvent B, acetonitrile) were as follows: 0 min, 5% B; 12 min, 15% B; 35 min, 70% B; 40 min, 5% B; and hold at 5% B for 5 min, flow rate 1.0 ml/min, detection wavelength UV 254 nm, injection volume 20 µl.*

4.3.4 FC induced apoptosis in cervical cancer cells

To analyze the mechanism behind the FC mediated cell death in cervical cancer cells, we investigated apoptosis in SiHa and HeLa after treating the cells with different concentrations (0-40 µg/ml). On staining with Annexin V-FITC, both SiHa and HeLa showed a dose-dependent increase in both early as well as late apoptotic cell population (Figure 4.3). Interestingly, in SiHa, at 40 µg/ml FC concentration, there was ~1.69-fold ($p=0.039$) and ~2.31-fold ($p\leq0.001$) increase in both early as well as late apoptotic cell

population, respectively, compared to the untreated control cells (Figure 4.3A). On the other hand, at 40 µg/ml concentration of FC, HeLa cells exhibited ~5.1-fold (p=0.47) and ~1.47-fold (p≤0.001) increase in early and late apoptotic cell population, respectively, compared to the untreated control cells (Figure 4.3B).

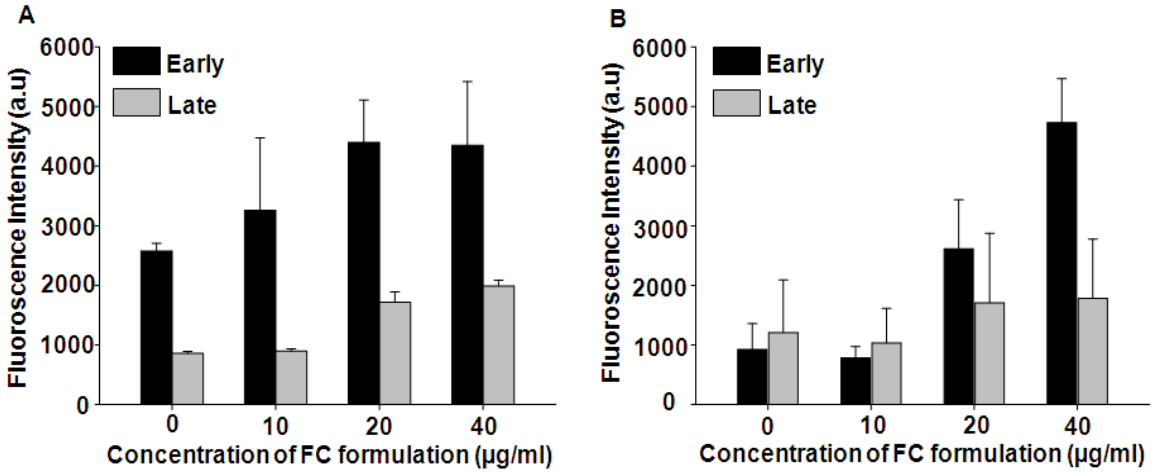


Figure 4.3: FC induced apoptosis. *(**A**) SiHa and (**B**) HeLa cells treated with different concentrations (0-40 µg/ml) of FC for 24 h. Apoptosis was measured by Annexin V-FITC/ Propidum iodide staining. Dead cells were stained by propidium iodide which displays strong fluorescence intensity with excitation and emission at 560 nm and 595 nm, respectively. Early stage apoptotic cells stained by Annexin V FITC were detected at the excitation and emission wavelength of 485 nm and 535 nm, respectively.*

4.3.5 FC generated intracellular Reactive Oxygen Species (ROS) in cervical cancer cells

Elevated amounts of intracellular ROS are sufficient to trigger cell death, and it has been suggested that ROS are biochemical mediators of apoptosis (Brodská et al., 2011). Therefore, to determine whether ROS is involved in the regulation of apoptosis induced by FC, we performed the ROS kinetic assay using DCF-DA, a selective probe for ROS measurement. Cells were loaded with DCF-DA, followed by treatment with FC (0-40 µg/ml). ROS was measured every 10 min after FC treatment for 6 h and then at 24 h. Compared with the untreated control group, a dose dependent increase in the ROS

content was observed in FC-treated SiHa and HeLa cells. FC significantly increased endogenous ROS within 6 h of the treatment with a maximal at 40 µg/ml concentration in SiHa (~11.63±2.00 fold; p≤0.001) and HeLa (~13.39±1.41 fold; p≤0.001) (Figure 4.4A and B, respectively). However, the fold increases in ROS when measured after 24 h treatment with FC, at 40 µg/ml concentration, was ~7.06±1.46 (p≤0.05) and ~17.58±1.27 (p≤0.05), for SiHa and HeLa, respectively, compared to the untreated cells (Figure 4.4C and D, respectively).

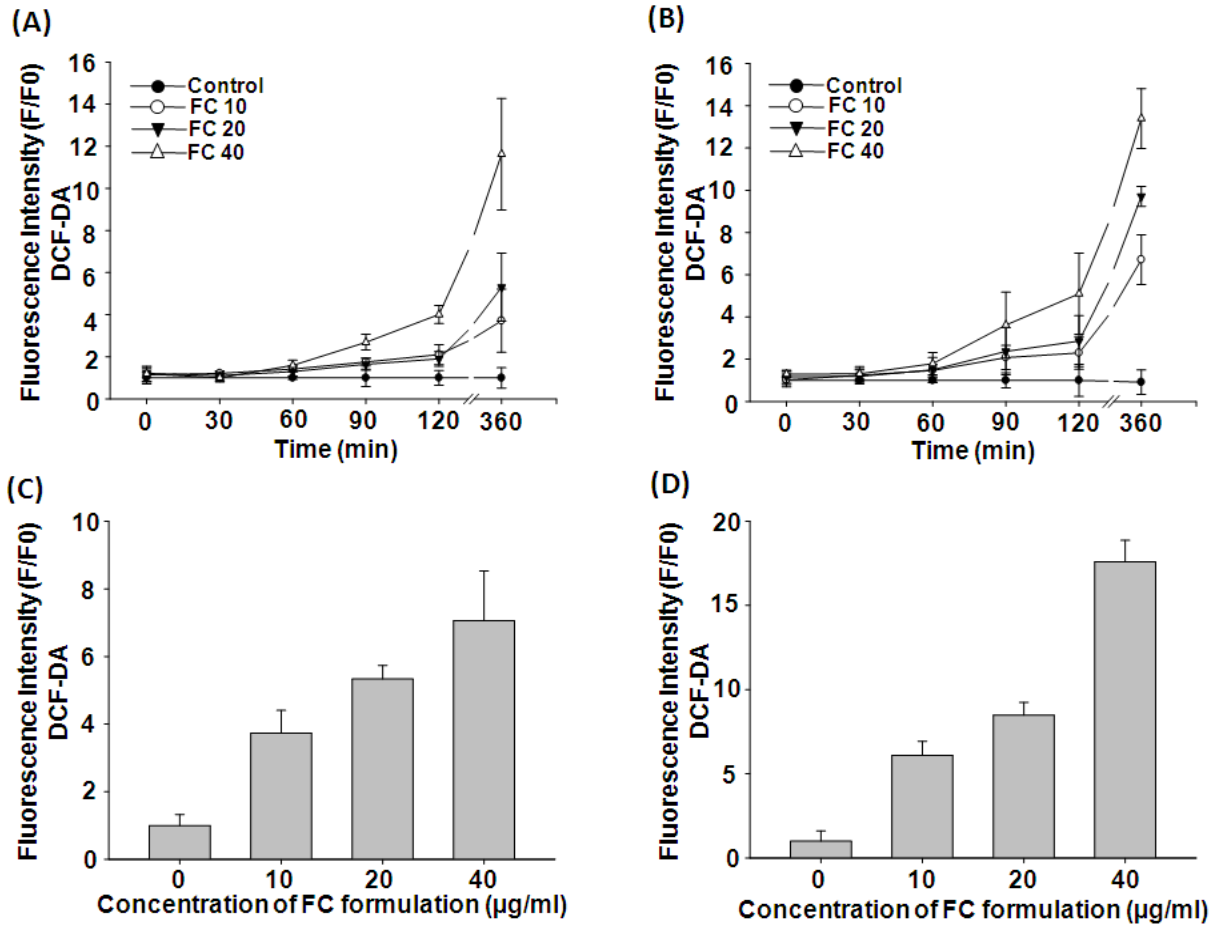


Figure 4.4: FC treatment increased intracellular Reactive Oxygen Species. *(**A**) SiHa and (**B**) HeLa cells were loaded with DCF-DA (2.5 µM) and exposed to FC, during which measurements (495/515 nm) were recorded every 1 min for 6 h. Representative graphs of intracellular ROS in (**C**) SiHa and (**D**) HeLa after 24 hours of FC treatment; (n = 4) are shown for three independent experiments.*

4.3.6 FC causes increase in nitric oxide (NO)

The extent of NO generation in cells was analyzed by performing its kinetics using DAF-FM, a selective probe for NO measurement. Cells were loaded with DAF-FM, followed by treatment with FC (0-40 µg/ml) and measurement of NO was done after every 10 min for 6 h and then at 24 h. NO levels increased significantly in cells treated with FC compared to the untreated control cells (Figure 4.5).

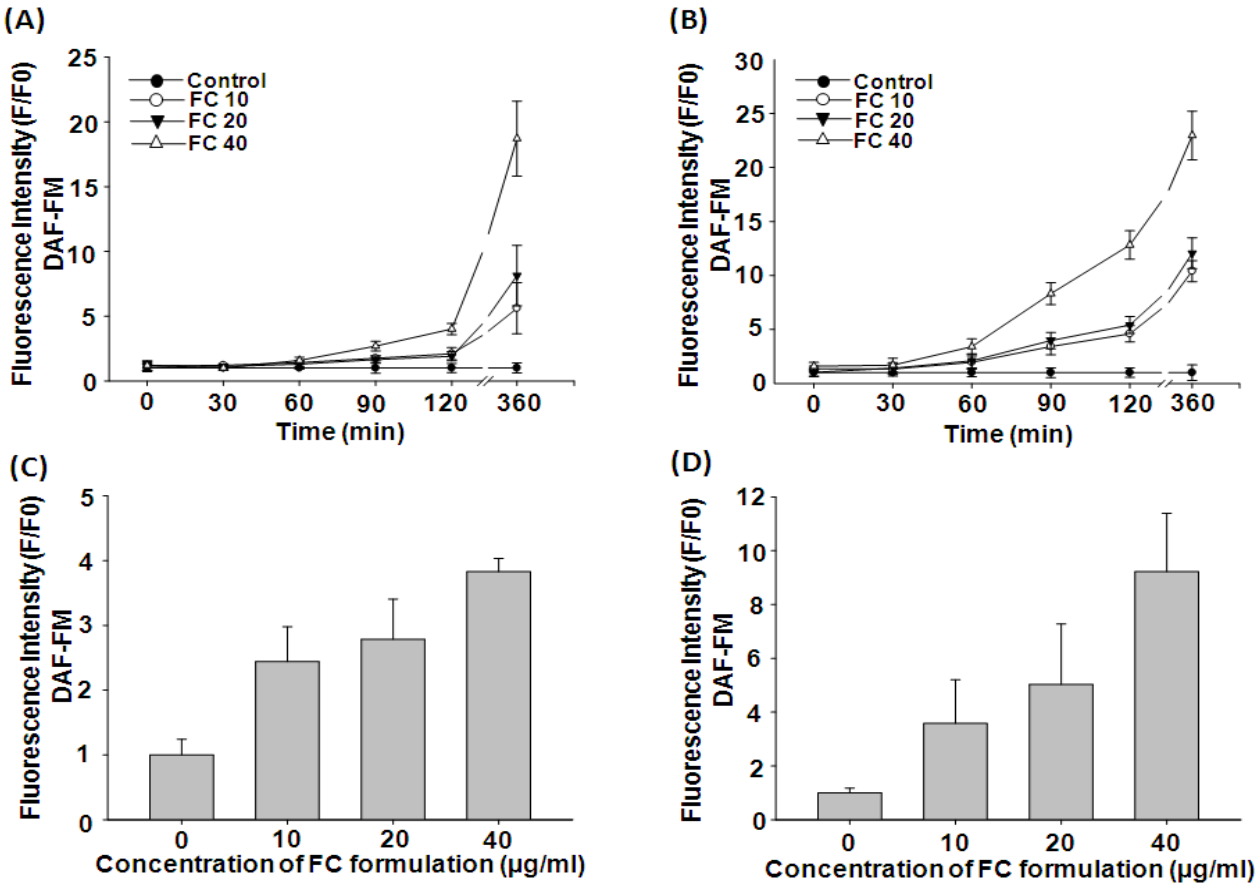


Figure 4.5: FC treatment increased intracellular Nitric oxide. *(**A**) SiHa and (**B**) HeLa cells were loaded with DAF-FM diacetate (2.5 µM) and exposed to FC, during which measurements (495/515 nm) were recorded every 1 min for 6 h Representative graphs of intracellular NO in (**C**) SiHa and (**D**) HeLa after 24 hours of FC treatment; (n = 4) are shown for three independent experiments.*

FC significantly increased intracellular NO within 6 h of the treatment with a maximal at the concentration of 40 µg/ml in both SiHa (~19.54±1.54 fold; $p \leq 0.001$) and HeLa

(~22.96±2.2 fold; p≤0.05) (Figure 4.5A and B, respectively). However, at 40 µg/ml of FC, the fold increase in NO at 24 h was ~3.28±0.20 (p≤0.05) and ~9.22±2.16 (p≤0.05), for SiHa (Figure 4.5C) and HeLa (Figure 4.5D), respectively, compared to the untreated control cells.

4.3.7 FC decreased mitochondrial membrane potential

It is well known that loss of mitochondrial membrane potential (ΔΨm) is an important step in initiation and activation of apoptotic process in cells (Ahn et al., 2014). Since generation of reactive nitrogen and oxygen species (RNOS) is related with mitochondrial dysfunction (Ahn et al., 2014), the effect of FC on ΔΨm was examined in SiHa and HeLa. The cells were treated with various concentrations of FC (0-40 µg/ml) for 24 h and ΔΨm was measured by using JC-1 dye. FC caused concentration dependent decrease in ΔΨm within 24 h of the treatment with maximum decrease at 40 µg/ml concentration in both SiHa (~1.25-fold; p=0.010) and HeLa (~1.37-fold; p=0.008) (Figure 4.6).

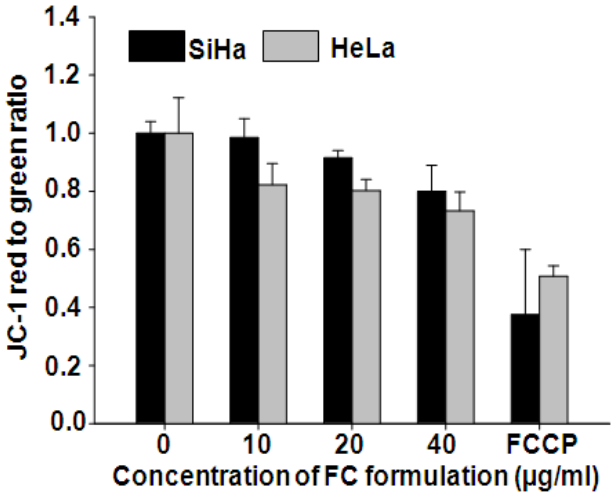


Figure 4.6: FC decreased mitochondrial membrane potential. *SiHa and HeLa cells treated with different concentrations (0-40 µg/ml) of FC for 24 h. The mitochondrial membrane potential was measured by JC-1 (2.5 µM). The data represents mean ± SD of three independent experiments.*

4.3.8 FC inhibited tumor growth in mice melanoma model

For analyzing the anti-tumorigenic potential of FC, B16F10 mouse melanoma model was used. Subcutaneous tumors of B16F10 melanoma were induced in mice by the injection of 1×10^6 viable tumor cells. One week after development of subcutaneous tumors, mice were given oral gavage of FC at 100 and 200 mg/kg bw for 15 consecutive days. It was observed that the untreated control mice showed tumor volume of 4.74±0.58 cm^3 and the positive control Adriamycin (ADR) (2 mg/kg i.v) showed a tumor volume of 0.89 ±0.28cm^3 ($p<0.001$). On the contrary, mice treated with 100 and 200 mg/kg of FC showed a tumor volume of 2.97±0.30cm^3 ($p=0.026$) and 1.58±0.25 cm^3 ($p<0.001$), respectively (Figure 4.7A). The percent tumor growth inhibition in 100 and 200 mg/kg of FC treated animals was found to be 37% and 66%, respectively. ADR treatment (2 mg/kg i.v) resulted into 81% tumor growth inhibition compared to the untreated tumor control. Treatment as well as control groups showed 100% survival by day 15, except for one mouse in FC (200 mg/kg) that died quite early during the study (day 5), due to unknown reasons (Figure 4.7 B). There was no significant difference between the body weight of the FC treated group and that of the untreated control group (Figure 4.7 C).

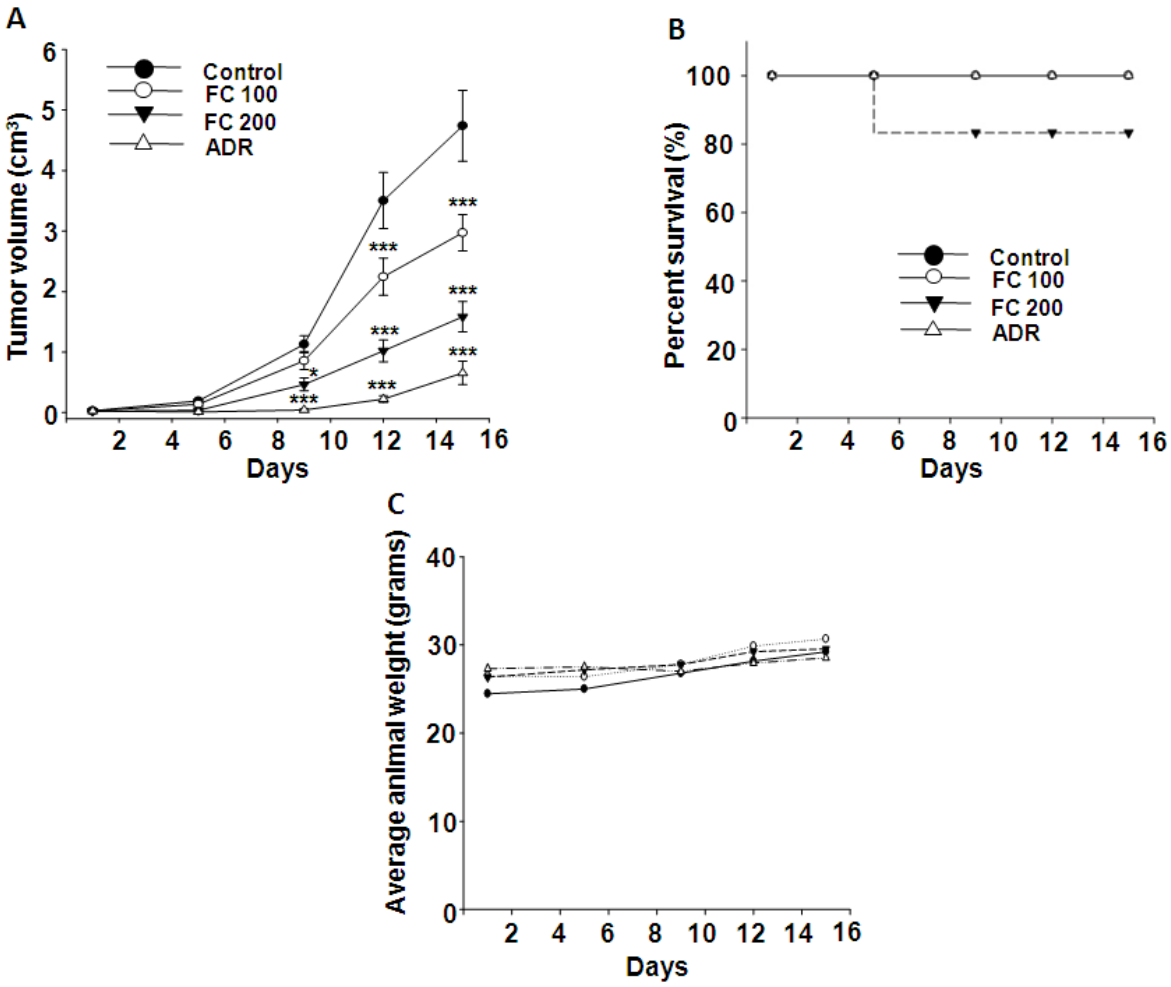


Figure 4.7: FC exhibited anti-tumor activity in the B16F10 mouse melanoma model. (A) Effect of FC on tumor growth. *$B_{16}F_{10}$ melanoma (1×10^6 cells /mouse) was inoculated subcutaneously (s.c) into the flanks of C57BL/6 mice (6 weeks old male). Eight days after the injection, mice were divided into five groups and each group consisted of six mice. The mice were treated with FC (100 and 200mg/kg) for 15 consecutive days. Adriamycin (2mg/kg) was used as positive control which was administrated i.v at 1, 4 and 9th day. The tumor volume was measured every 3 days; ***p<0.001, *p<0.05 between FC and tumor control at same day (**B**) **Animal survival.** Kaplan-Meier survival analysis was compared using the log-rank test (**C**) **Body weight.** The mean ± SD body weight of mice in each group was calculated.*

4.3.9 FC modulated Th1 and Th2 cytokine response in mice

To investigate the effect of FC on Th1 and Th2 cytokines, we measured the levels of IFN-γ, IL-2 and IL-4 cytokines in the serum samples of FC treated and untreated mice (Figure 4.8). With 200 mg/kg FC treatment, B16F10 tumor bearing mice showed a

significant increase in IL-2 (~5.60-fold; p<0.001) and IFN-γ (~5.66-fold; p<0.001) levels (Figure 4.8 A and B) with a significant decrease in IL-4 (~3.77-fold; p=0.006) level compared to the tumor control group (Figure 4.8 C). Interestingly, ADR did not alter the levels of Th1 cytokine but decreased the Th2 cytokine levels. These observations showed that FC not only retarded the tumor growth but also helped in the activation of Th1 cytokine response in mice.

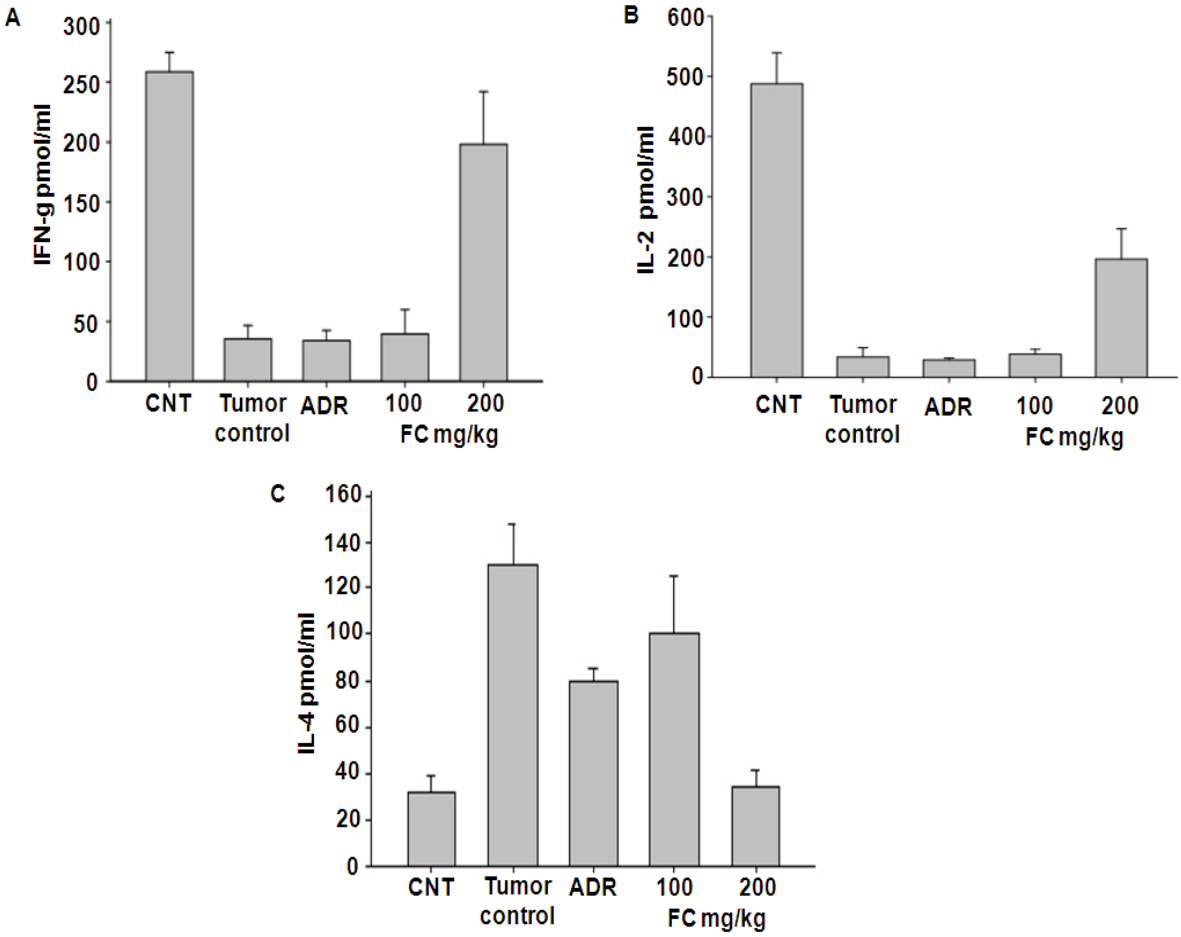


Figure 4.8: FC exhibited immunomodulatory activity. *Levels of* ***(A)*** *IFN-γ,* ***(B)*** *IL-2 and* ***(C)*** *IL-4, in the sera of mice were determined by ELISA on day 15 of the treatment. The data represents mean ±SD of mice in each group*

4.4. Discussion:

A novel formulation, FC, was developed from the bark materials of *Ficus religiosa* and *Cinnamon zeylanicum* based on our previous experimental work related to their anti-cancer potential in cervical cancer. The standardized FC formulation strongly inhibited the cell viability of cervical cancer cells at relatively lower doses compared to either *Ficus religiosa* or *Cinnamon zeylanicum*. Interestingly, FC did not induce any cytotoxicity in normal cells which promoted us to further elucidate the molecular mechanism underlying the anti-cancer potential of FC in SiHa and HeLa cells.

Apoptosis or programmed cell death is an important mechanism to kill the tumor cells and is recognized as a strategy for identifying anti-cancer drugs (Ricci et al., 2006). It can be induced by generation of reactive nitrogen and oxygen species (RNOS) that result into loss of membrane potential ($\Delta\psi m$), expansion of the matrix and rupture of the outer mitochondrial membrane (Ahn et al., 2014). Depolarization of mitochondrial membrane potential ($\Delta\psi m$) is an important phenomenon responsible for the release of cytochrome c from mitochondria into the cytosol and subsequent activation of caspase (Ly et al., 2003). In cervical cancer cells, at an effective concentration of FC (40 µg/ml), we observed a significant proportion of cells undergoing apoptosis compared to the untreated control cells. Interestingly, at similar concentration, we observed increased intracellular RNOS and significant decrease in mitochondrial membrane potential ($\Delta\psi m$).

Since FC induced apoptosis in cervical cancer cells, we examined its anti-cancer activity in C57BL/6 melanoma tumor model. This is a widely used model in cancer research to investigate the antitumor as well as immune response of anticancer drugs and their role in disease progression, as well as to test new therapies (Hannani et al., 2013). Daily oral administration of 100 and 200 mg/kg of FC significantly inhibited the growth

of B16F10 cells in mice. Adriamycin, was used as a positive control in the study. Interestingly, FC did not induce any adverse effect on the body weight of the treated mice. The treatment and the control groups did not show any major toxic effect till the end of the experiment, except for one mouse in FC (200 mg/kg) that died quite early during the study (day 5), due to unknown reasons.

Tumour reactive immune system is another checkpoint in cancer cell proliferation (Dobbelstein et al., 2014). The tumor inhibitory immune response is generally mediated by T-helper 1 (Th1) cells which express cytokines responsible for the destruction of tumor cells, whereas the expression of T-helper 2 (Th2) cytokines create a tolerogenic environment in which the tumor cells could grow (Smyth et al., 2001). In cancer patients or tumour-bearing animals, cell mediated immunity is weak with gradual shift from Th1 to Th2 cell phenotype leading to immune suppressive environment and tumor reactive immune dysfunction (Lauerova et al., 2001; Moretti et al., 2001). Plant extracts and herbal formulations have been shown to reduce cancer cell proliferation by modulating the immune system (Malik et al., 2009; Kamiyama et al., 2005). Consistent with this notion, our results showed significant up-regulation of Th1 (IFN-γ and IL-2) and down regulation of Th2 (IL-4) cytokine in FC treated groups. IFN-γ and IL-2 are important lymphokines that can activate Natural Killer (NK) cells, cytotoxic T lymphocytes, and tumoricidal macrophages, thereby, increasing the ability to kill tumor cells (Weigent et al., 1983). On other hand, IL-4, acts as the natural antagonist, and suppresses the function and production of IFN-γ (O'Garra et al., 2008). Thus, FC not only reduced the tumor size but also helped in the activation of Th1 immune system, whose down-regulation otherwise worsens the severity of the disease.

In conclusion, this study has demonstrated that standardized FC formulation caused NO and ROS production that could be responsible for the induction of apoptosis

through mitochondrial dependent pathway. Interestingly, FC retarded tumor growth in mice and simultaneously activated the immune system, favoring Th1 response. The immunomodulatory activity is a valuable addition to the therapeutic potential of FC. Our results clearly indicate that FC is a potent immuno-chemotherapeutic agent which may find usefulness in the management of cervical cancer either alone or as an adjunct to conventional radio or chemotherapy.

CONCLUSION

Summary

The present study entitled **"Analyzing the Chemopreventive Potential of Medicinal Plants in Cervical Cancer and to Study Underlying Molecular Mechanisms Governing the Anticancer Activity"** includes in vitro experiments to elucidate the anti-cancer potential of aqueous extract of the bark of *Cinnamon cassia* and *Ficus religiosa* in cervical cancer cell lines. In addition, this study also includes in vitro and *in vivo* experiments with novel herbal formulation (FC) to elucidate its anti-cancer potential. We hypothesize that *Cinnamomum (common and cassia)* and *Ficus religiosa* alone as well as in combination possess anti-cancer activity and may target important signaling pathways and biological markers whose deregulation leads to cervical cancer.

Cervical cancer is a major woman health problem, with invasive cervical cancer being a leading cause of cancer death in women worldwide (Ferlay et al., 2008). It is the most common cancer among women in developing countries where 86% of the global cervical cancer burden of approximately 529,409 cases and 274,883 deaths are found annually (Bruni et al., 2014a). The effective cervical cancer screening programs, which can detect and treat the precancerous lesions at an early stage, have dramatically reduced the disease incidence as well as death (Dickinson et al., 2012). However, prognosis for the advanced cancer remains poor despite several efforts to improve treatment outcomes. For locally advanced disease, radiation is combined with low-dose chemotherapy; however, this modality often leads to severe toxicity (Kirwan et al., 2003). Considering this scenario of current treatment modalities, newer approaches of cancer management deserve immediate attention.

Traditional medicines have been used from time immemorial to treat various chronic ailments including cancer. Recently, complementary and alternative medicine (CAM), particularly herbal medicine, is becoming popular as an adjunct to chemotherapy

(Helyer et al., 2006). Plants, vegetables, herbs and spices used in folk and traditional medicine have been accepted as one of the main sources of chemopreventive drugs (Cragg et al., 2005; Pandey et al., 2009). A wide variety of naturally occurring substances from plants have been shown to offer protection from carcinogenic exposure (Aziz et al., 2008). Various scientific studies support herbal medicines, an important part of traditional medicines, as potent anti-cancer drug candidates (Pandey et al., 2009).

In this contribution, we proposed to evaluate anti-cancer activity of the bark of Cinnamon **(*Cinnamomum zeylanicum* and *Cinnamomum cassia)*** and ***Ficus religiosa*** alone or in combination in cervical cancer.

To test our hypothesis, we aimed at following objectives

1. To analyze the antineoplastic potential of Cinnamon in vitro in cervical cancer cell line
2. To analyze the antineoplastic potential of Ficus in vitro in cervical cancer cell lines
3. To develop a novel herbal formulation (FC) and test its antineoplastic potential in vitro in cervical cancer cells and *in vivo* in mouse melanoma model

NOVEL FINDINGS OF THE STUDY

- **The thesis for the first time demonstrates the following interesting findings with *Cinnamomum cassia* in SiHa**
  - ✓ Cinnamon treatment alters growth kinetics the cells
  - ✓ Cinnamon extract induces apoptosis in the cervical cancer cells through increase in intracellular calcium signaling as well as loss of mitochondrial membrane potential
  - ✓ Cinnamon decreases cell migration through reduction in MMP-2 expression

- ✓ Cinnamon significantly reduces the expression of Her-2 oncoprotein

- **The thesis for the first time demonstrates the following interesting finding with *Ficus religiosa* in SiHa and HeLa cells**
  - ✓ Ficus modulates the growth kinetics of cervical cancer cells
  - ✓ Ficus induces cell cycle arrest and altered the expression of cell cycle regulating protein in SiHa
  - ✓ Ficus induces apoptosis through increase in intracellular calcium and decrease in mitochondrial membrane potential in HeLa
  - ✓ Ficus increases p53, caspase-3 and cytosolic cytochrom-c expression in HeLa
  - ✓ Ficus decreases invasion and migration, and alters the expression of MMP-2 and HER-2 oncoproteins of SiHa and HeLa cells
  - ✓ Ficus reduces the expression of viral oncoproteins E6 and E7

- **The thesis also for the first time developed a novel herbal formulation (FC) and demonstrated the following interesting in vitro and *in vivo* finding**
  - ✓ FC induces apoptosis in cervical cancer cells
  - ✓ FC induces cell death involved early generation of Reactive Oxygen Species (ROS)
  - ✓ FC causes robust increase in nitric oxide (NO) in parallel to ROS
  - ✓ FC decreases mitochondrial membrane potential
  - ✓ Oral administration of FC extract inhibits melanoma growth *in vivo*.
  - ✓ FC stimulates Th1 cytokine expression in tumor-bearing mice

Implications

This study attempts to elucidate the anti-cancer potential of two plant bark materials namely *Cinnamomum cassia* and *Ficus religiosa* in cervical cancer cells by examining their effects at cellular and molecular level. Further, the study with *Cinnamomum cassia* throws light on mechanism by which ACE-c induced apoptosis and inhibited migration of the SiHa cells. The study with *Ficus religiosa* shows differential mechanism involved in regulation of anti-cancer activity by FR_{aq} in SiHa and HeLa. The study also provides in vitro anti-cancer mechanism of novel herbal formulation (FC) in SiHa and HeLa cells. It has also established *in vivo* anti-cancer activity along with initial data on immunomodulatory activity in C57BL/6 mouse model. The novel information gathered from the current study clearly indicates the potential of *Cinnamon cassia* and *Ficus religiosa* and herbal formulation FC as phytomedicines for the treatment of cervical cancer.

Societal Relevance

Cervical cancer is the second most common malignancy worldwide and the leading cause of cancer related deaths among women in India. The failure of conventional chemotherapy to reduce mortality invites attention towards new alternative approaches that would reduce morbidity as well as side effects conferred by conventional chemotherapy. This study may provide a clue which would enable development of effective herbal interventions in the management and prevention of cervical cancer, and would also reduce the side effects of conventional therapy.

Future Perspectives

The herbal formulation developed from the above study could be tested in clinical trials in cervical cancer patients. This would further validate our experimental studies and would translate our findings from bench to bedside.

References

Abdulkarim B, Sabri S, Deutsch E, Chagraoui H, Maggiorella L, Thierry J, Eschwege F, Vainchenker W and Bourhis J (2002). **Antiviral agent Cidofovir restores p53 function and enhances the radiosensitivity in HPV-associated cancers.** *Oncogene*, *21*(15), 2334-2346.

Abdullah ASM, Lau Y and Chow LWC (2003). **Pattern of alternative medicine usage among the Chinese breast cancer patients: Implication for service integration.** *American Journal of Chinese Medicine, 31,* 649–658.

Ad'hiah AH, Al-Bederi ON and Al-Sammarrae KW (2013). **Cytotoxic effects of *Agrimonia eupatoria* L. against cancer cell lines *in vitro*.** *Journal of the Association of Arab Universities for Basic and Applied Sciences*, *14*(1), 87-92.

Ahmed D, Sharma M, Mukerjee A, Ramteke PW and Kumar V (2013). **Improved glycemic control, pancreas protective and hepatoprotective effect by traditional poly-herbal formulation "Qurs Tabasheer" in streptozotocin induced diabetic rats.** *BMC complementary and alternative medicine*, *13*(1), 10.

Ahn HJ, Kim KI, Hoan NN, Kim CH, Moon E, Choi KS,Yang SS and Lee JS (2014). **Targeting Cancer Cells with Reactive Oxygen and Nitrogen Species Generated by Atmospheric-Pressure Air Plasma.** *PloS one*, *9*(1), e86173.

Al Moustafa AE, Foulkes WD, Benlimame N, Wong A, Yen L, Bergeron J, Batist G, Alpert L and Alaoui-Jamali MA (2004). **E6/E7 proteins of HPV type 16 and ErbB-2 cooperate to induce neoplastic transformation of primary normal oral epithelial cells.** *Oncogene*, *23*(2), 350-358.

Alonso-Castro AJ, Ortiz-Sánchez E, Domínguez F, López-Toledo G, Chávez M, Ortiz-Tello ADJ, and García-Carrancá A (2012). **Antitumor effect of *Croton lechleri* Mull. Arg. (Euphorbiaceae).** *Journal of ethnopharmacology*, *140*(2), 438-442.

Alqasoumi S (2012). **Anti-secretagogue and antiulcer effects of 'Cinnamon' *Cinnamomum zeylanicum* in rats.** *Journal of Pharmacognosy and Phytotherapy*, *4*(4), 53-61.

Amar AR and Maysa ME (2010). **Anti-ulcer effects of cinnamon and chamomile Aqueous Extracts in Rat Models.** *The Journal of American Science*, *6*(12), 209-216.

Aparicio-Fernández X, Reynoso-Camacho R, Castaño-Tostado E, García-Gasca T, de Mejía EG, Guzmán-Maldonado SH, Elizondo G, Yousef GG, Lila MA and Loarca-Pina G (2008). **Antiradical capacity and induction of apoptosis on HeLa cells by a *Phaseolus vulgaris* extract.** *Plant foods for human nutrition*, *63*(1), 35-40.

Appleby P, Beral V, de Gonzalez AB, Colin D, Franceschi S, Goodhill A, Green J, Peto J, Plummer M and Sweetland S (2007). **Cervical cancer and hormonal contraceptives: collaborative reanalysis of individual data for 16 573 women with cervical cancer and 35 509 women without cervical cancer from 24 epidemiological studies.** *Lancet*, *370*(9599), 1609-1621.

Arthur K, Belliard JC, Hardin SB, Knecht K, Chen CS and Montgomery S (2012). **Practices, attitudes, and beliefs associated with complementary and alternative medicine (CAM) use among cancer patients.** *Integrative cancer therapies*, *11*(3), 232-242.

Atalah E, Urteaga C, Rebolledo A, Villegas RA, Medina E, and Csendes A (2001). **Diet, smoking and reproductive history as risk factor for cervical cancer**. *Revista medica de Chile*, *129*(6), 597-603.

Aziz MH, Dreckschmidt NE, and Verma AK (2008). **Plumbagin, a Medicinal Plant–Derived Naphthoquinone, Is a Novel Inhibitor of the Growth and Invasion of Hormone-Refractory Prostate Cancer.** *Cancer research*, *68*(21), 9024-9032.

Barnes PM, Bloom B and Nahin RL (2008). **Complementary and alternative medicine use among adults and children: United States, 2007.** *Natl Health Stat Report*, *10*(12),1-23.

Basu P, Dutta S, Begum R, Mittal S, Dutta PD, Bharti AC, Panda CK, Biswas J, Dey B, Talwar GP, Das BC (2013). **Clearance of Cervical Human Papillomavirus Infection by Topical Application of Curcumin and Curcumin Containing**

Polyherbal Cream: A Phase II Randomized Controlled Study. *Asian Pacific Journal of Cancer Prevention*, *14*(10), 5753-5759.

Bent S (2008). **Herbal medicine in the United States: review of efficacy, safety, and regulation.** *Journal of general internal medicine*, *23*(6), 854-859.

Berridge MJ, Lipp P and Bootman MD (2000). **The versatility and universality of calcium signalling.** *Nature reviews Molecular cell biology*, *1*(1), 11-21.

Bhutia SK, Mallick SK, Stevens SM, Prokai L, Vishwanatha JK, and Maiti TK (2008). **Induction of mitochondria-dependent apoptosis by *Abrus* agglutinin derived peptides in human cervical cancer cell.** *Toxicology in Vitro*, *22*(2), 344-351.

Bonfili L, Amici M, Cecarini V, Cuccioloni M, Tacconi R, Angeletti M, Fioretti E, Keller JN and Eleuteri AM (2009). **Wheat sprout extract-induced apoptosis in human cancer cells by proteasomes modulation.** *Biochimie*, *91*(9), 1131-1144.

Boon HS, Olatunde F and Zick SM (2007). **Trends in complementary/ alternative medicine use by breast cancer survivors: comparing survey data from 1998 and 2005.** *BMC women's health*, *7*(1), 4.

Bosch FX, Lorincz A, Munoz N, Meijer CJLM and Shah KV (2002). **The causal relation between human papillomavirus and cervical cancer.** *Journal of clinical pathology*, *55*(4), 244-265.

Brandacher G, Winkler C, Schroecksnadel K, Margreiter R and Fuchs D (2006). **Antitumoral activity of interferon-γ involved in impaired immune function in cancer patients.** *Current drug metabolism*, *7*(6), 599-612.

Bravo-Cuellar A, Ortiz-Lazareno PC, Lerma-Diaz JM, Dominguez-Rodriguez JR, Jave-Suarez LF, Aguilar-Lemarroy A, del Toro-Arreola S, de Celis-Carrillo R, Sahagun-Flores JE, de Alba-Garcia JE and Hernandez-Flores G. (2010). **Sensitization of cervix cancer cells to Adriamycin by Pentoxifylline induces an increase in apoptosis and decrease senescence.** *Molecular Cancer*, *9*(114)

Brock KE, Berry G, Mock PA, MacLennan R, Truswell AS and Brinton LA (1988). **Nutrients in diet and plasma and risk of in situ cervical cancer.** *Journal of the National Cancer Institute*, *80*(8), 580-585.

Brodská B and Holoubek A (2011) **Generation of reactive oxygen species during apoptosis induced by DNA-damaging agents and/or histone deacetylase inhibitors.** *Oxidative medicine and cellular longevity*, *2011*.

Bruni L, Barrionuevo-Rosas L, Serrano B, Brotons M, Cosano R, Munoz J, Bosch FX, de Sanjose S and Castellsague X (2014a) **ICO Information Center on HPV and Cancer (HPV information Center). Human Papillomavirus and Related Diseases in World.** *Summary Report 2014*.

Bruni L, Barrionuevo-Rosas L, Serrano B, Brotons M, Cosano R, Munoz J, Bosch FX, de Sanjose S and Castellsague X (2014b) **ICO Information Center on HPV and Cancer (HPV information Center). Human Papillomavirus and Related Diseases in India.** *Summary Report 2014*.

Buckley CH, Butler EB and Fox H (1982). **Cervical intraepithelial neoplasia.** *Journal of clinical pathology*, *35*(1), 1-13.

Burd EM (2003). **Human papillomavirus and cervical cancer.** *Clinical microbiology reviews*, *16*(1), 1-17.

Cao Z, Lin W, Huang Z, Chen X, Zhao J, Zheng L, Ye H, Liu Z, Liao L and Du J (2013). **Ethyl acetate extraction from a Chinese herbal formula, Jiedu Xiaozheng Yin, inhibits the proliferation of hepatocellular carcinoma cells via induction of G_0/G_1 phase arrest in vivo and in vitro.** *International journal of oncology*, *42*(1), 202-210.

Chae HJ, Yang SK, Kim DS, Kim HM, Chae SW, Keum KS and Kim HR (2004). **Ge-Jee-Bok-Ryung-Hwan induces apoptosis in human cervical carcinoma HeLa cells:-An endoplasmic reticulum stress pathway.** *Life sciences*, *75*(25), 2997-3016.

Chaudhary A, Sharma U, Vig AP, Singh B and Arora S (2014). **Free radical scavenging, antiproliferative activities and profiling of variations in the level of**

phytochemicals in different parts of broccoli (*Brassica oleracea* italica). *Food chemistry*, *148*, 373-380.

Chavez-Blanco A, Perez-Sanchez V, Gonzalez-Fierro A, Vela-Chavez T, Candelaria M, Cetina L,Vidal S and Dueñas-Gonzalez A (2004). **HER2 expression in cervical cancer as a potential therapeutic target.** *BMC cancer*, *4*(1), 59.

Chen HL, Han FJ, Gong YQ, Wang XX, Hou LH and Wu XK (2010). **Effects of Erhuangsan with complex prescription of traditional Chinese medicine on cells proliferation inhibitory and the expression of Bcl-2 protein in Hela cells of cervical cancer [J].** *Journal of Modern Oncology*, *11*.

Chen Z, Gu K, Zheng Y, Zheng W, Lu W and Shu XO (2008). **The use of complementary and alternative medicine among Chinese women with breast cancer.** *Journal of Alternative and Complementary Medicine, 14,* 1049–1055.

Cheng BC (1983). **A review of cinnamon.** *Zhong Cao Yao*, *14*(134), 4762-4765.

Choudhari AS, Suryavanshi SA and Kaul-Ghanekar R (2013). **The Aqueous Extract of Ficus religiosa Induces Cell Cycle Arrest in Human Cervical Cancer Cell Lines SiHa (HPV-16 Positive) and Apoptosis in HeLa (HPV-18 Positive).** *PloS one*, *8*(7), e70127.

Choudhari AS, Suryavanshi SA, Ingle H and Kaul-Ghanekar R (2011). **Evaluating the antioxidant potential of aqueous and alcoholic extracts of *Ficus religiosa* using ORAC assay and assessing their cytotoxic activity in cervical cancer cell lines.** *Biotechnol. Bioinf. Bioeng*, *1*(4), 443-450.

Choudhary GP (2006). **Evaluation of ethanolic extract of *Ficus religiosa* bark on incision and excision wounds in rats.** *Planta indica*, *2*(3), 17-9.

Choudhury D, Das A, Bhattacharya A and Chakrabarti G (2010). **Aqueous extract of ginger shows antiproliferative activity through disruption of microtubule network of cancer cells.** *Food and chemical toxicology*, *48*(10), 2872-2880.

Cornelio DB, Roesler R and Schwartsmann G (2009). **Emerging therapeutic agents for cervical cancer.** *Recent patents on anti-cancer drug discovery*, *4*(3), 196-206.

Cragg GM and Newman DJ (2005). **Plants as a source of anti-cancer agents.** *Journal of ethnopharmacology*, *100*(1), 72-79.

Cui Y, Shu XO, Gao Y, Wen W, Ruan ZX and Jin F (2004). **Use of complementary and alternative medicine by Chinese women with breast cancer.** *Breast Cancer Research and Treatment, 85,* 263–270.

Das M, Mandal S, Mallick B and Hazra J (2013). **Ethnobotany, Phytochemical and pharmacological aspect of *Cinnamomum zeylanicum* Blume.** *International Research Journal of Pharmacy*. *4:* 58-63

De Villiers EM, Fauquet C, Broker TR, Bernard HU and zur Hausen H (2004). **Classification of papillomaviruses.** *Virology*, *324*(1), 17-27.

De Vuyst H, Ndirangu G, Moodley M, Tenet V, Estambale B, Meijer CJ, Snijders PJ, Clifford G, Franceschi S (2012). **Prevalence of human papillomavirus in women with invasive cervical carcinoma by HIV status in Kenya and South Africa.** *International Journal of Cancer*, *131*(4), 949-955.

Deryugina EI and Quigley JP (2006). **Matrix metalloproteinases and tumor metastasis.** *Cancer and Metastasis Reviews*, *25*(1), 9-34.

Deshmukh T, Yadav B, Badole S, Bodhankar S, Dhaneshwar S (2007). **Antihyperglycaemic activity of alcoholic extract of *Ficus religiosa* leaves in alloxan induced diabetic mice.** *Journal of Herbal Medicine and Toxicology,* 1, 80–86.

D'Herde K and Leybaert L (1997). **Intracellular free calcium related to apoptotic cell death in quail granulosa cell sheets kept in serum-free culture.** *Cell death and differentiation*, *4*(1), 59-65.

Dickinson JA, Stankiewicz A, Popadiuk C, Pogany L, Onysko J and Miller AB (2012). **Reduced cervical cancer incidence and mortality in Canada: national data from 1932 to 2006.** *BMC Public Health*, *12*(1), 992.

Dikshit R, Gupta PC, Ramasundarahettige C, Gajalakshmi V, Aleksandrowicz L, Badwe R, Kumar R, Roy S, Suraweera W, Bray F, Mallath M, Singh PK, Sinha DN, Shet

AS, Gelband H and Jha P. (2012). **Cancer mortality in India: a nationally representative survey.** *The Lancet*, *379*(9828), 1807-1816.

Dobbelstein M and Moll U (2014). **Targeting tumour-supportive cellular machineries in anticancer drug development.** *Nature Reviews Drug Discovery*, *13*(3), 179-196.

Dragland S, Senoo H, Wake K, Holte K and Blomhoff R (2003). **Several culinary and medicinal herbs are important sources of dietary antioxidants.** *The Journal of nutrition*, *133*(5), 1286-1290.

Duchen MR (2000). **Mitochondria and calcium: from cell signalling to cell death.** *The Journal of Physiology*, *529*(1), 57-68.

Dürst M, Gissmann L, Ikenberg H and Zur Hausen H (1983). **A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions.** *Proceedings of the National Academy of Sciences*, *80*(12), 3812-3815.

Ehrenberg B1, Montana V, Wei MD, Wuskell JP and Loew LM (1988). **Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes.** *Biophysical journal*, *53*(5), 785-794.

Elmore S (2007). **Apoptosis: a review of programmed cell death.** *Toxicologic pathology*, *35*(4), 495-516.

Evan GI and Vousden KH (2001). **Proliferation, cell cycle and apoptosis in cancer.** *Nature*, *411*(6835), 342-348.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, and Parkin DM (2010). **Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.** *International journal of cancer*, *127*(12), 2893-2917.

Foley G, Alston R, Geraci M, Brabin L, Kitchener H and Birch J (2011). **Increasing rates of cervical cancer in young women in England: an analysis of national data 1982–2006.** *British journal of cancer*, *105*(1), 177-184.

Foltz V, St Pierre Y, Rozenberg S, Rossignol M, Bourgeois P, Joseph L, Adam V, Penrod JR, Clarke AE, and Fautrel B (2005). **Use of complementary and alternative therapies by patients with self-reported chronic back pain: a nationwide survey in Canada.** *Joint Bone Spine*, *72*(6), 571-577.

Frumovitz M, Querleu D, Gil-Moreno A, Morice P, Jhingran A, Munsell MF, Macapinlac HA, Leblanc E, Martinez A and Ramirez PT. (2014). **Lymphadenectomy in Locally Advanced Cervical Cancer Study (LiLACS): Phase III Clinical Trial Comparing Surgical with Radiologic Staging in Patients with Stages IB2–IVA Cervical Cancer.** *Journal of minimally invasive gynecology*, *21*(1), 3-8.

Fujiki H, Suganuma M, Kurusu M, Okabe S, Imayoshi Y, Taniguchi S and Yoshida T (2003). **New TNF-α releasing inhibitors as cancer preventive agents from traditional herbal medicine and combination cancer prevention study with EGCG and sulindac or tamoxifen.** *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *523*, 119-125.

Fulda S, Galluzzi L and Kroemer G (2010). **Targeting mitochondria for cancer therapy.** *Nature reviews Drug discovery*, *9*(6), 447-464.

Fuss IJ, Kanof ME, Smith PD and Zola H (2009). **Isolation of whole mononuclear cells from peripheral blood and cord blood.** *Current protocols in immunology*, 7-1.

Galil J (1984). ***Ficus religiosa L.*-the tree-splitter.** *Botanical journal of the Linnean Society*, *88*(3), 185-203.

Genovese C, Trani D, Caputi M and Claudio PP (2006). **Cell cycle control and beyond: emerging roles for the retinoblastoma gene family.** *Oncogene*, *25*(38), 5201-5209.

Giacinti C and Giordano A (2006). **RB and cell cycle progression.** *Oncogene*, *25*(38), 5220-5227.

Giordano S and Petrelli A (2008). **From single-to multi-target drugs in cancer therapy: when aspecificity becomes an advantage.** *Current medicinal chemistry*, *15*(5), 422-432.

Giorgi C, Agnoletto C, Bononi A, Bonora M, De Marchi E, Marchi S, Missiroli S, Patergnani S, Poletti F, Rimessi A, Suski JM, Wieckowski MR and Pinton P. (2012). **Mitochondrial calcium homeostasis as potential target for mitochondrial medicine.** *Mitochondrion*, *12*(1), 77-85.

GLOBOCAN. International Agency for Research on Cancer; (2008).

Gopu CL, AherS, Mehta H, Paradkar AR and Mahadik KR (2008**). Simultaneous determination of cinnamaldehyde, eugenol and piperine by HPTLC densitometric method.** *Phytochemical analysis*, *19*(2), 116-121.

Gulluoglu BM, Cingi A, Cakir T and Barlas A (2008). **Patients in northwestern Turkey prefer herbs as complementary medicine after breast cancer diagnosis.** *Breast Cancer, 3,* 269–273.

Halperin EC (2006). **Particle therapy and treatment of cancer.** *The lancet oncology*, *7*(8), 676-685.

Haneef J, Parvathy M, Sithul H and Sreeharshan S (2012). **Bax translocation mediated mitochondrial apoptosis and caspase dependent photosensitizing effect of Ficus religiosa on cancer cells.** *PloS one*, *7*(7), e40055.

Hannani D, Locher C, Yamazaki T, Colin-Minard V, Vetizou M, Aymeric L, Viaud S, Sanchez D, Smyth MJ, Bruhns P, Kroemer G and Zitvogel L (2013). **Contribution of humoral immune responses to the antitumor effects mediated by anthracyclines.** *Cell Death & Differentiation*, *21*(1), 50-58.

Harris P and Rees R (2000). **The prevalence of complementary and alternative medicine use among the general population: a systematic review of the literature.** *Complementary therapies in medicine*, *8*(2), 88-96.

He DM, Yuan XF and Li JB. (2012) **Effects of compound matrine capsule on cells proliferation inhibitory and cell cycle in cervical cancer cells.** *Lishizhen Med Mat Med Res, 23*, 1737-8

Hellberg D, Nilsson S, Haley NJ, Hoffman D and Wynder E (1988). **Smoking and cervical intraepithelial neoplasia: nicotine and cotinine in serum and cervical**

mucus in smokers and nonsmokers. *American journal of obstetrics and gynecology*, *158*(4), 910-913.

Helyer LK, Chin S, Chui BK, Fitzgerald B, Verma S, Rakovitch E and Clemons M (2006). **The use of complementary and alternative medicines among patients with locally advanced breast cancer–a descriptive study.** *BMC cancer*, *6*(1), 39.

Henderson JW and Donatelle RJ (2003). **Complementary and alternative medicine use by women after completion of allopathic treatment for breast cancer.** *Alternative therapies in health and medicine*, *10*(1), 52-57.

Henley SA and Dick FA (2012). The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell Div*, *7*(10).

Hernandez BY, McDuffie K, Franke AA, Killeen J and Goodman MT (2004). **Reports: plasma and dietary phytoestrogens and risk of premalignant lesions of the cervix.** *Nutrition and cancer*, *49*(2), 109-124.

Herold C, Ocker M, Ganslmayer M, Gerauer H, Hahn EG and Schuppan D (2002). **Ciprofloxacin induces apoptosis and inhibits proliferation of human colorectal carcinoma cells.** *British journal of cancer*, *86*(3), 443-448.

Hirose K, Hamajima N, Takezaki T, Kuroishi T, Kuzuya K, Sasaki S, Tokudome S, and Tajima K. (1998). **Smoking and dietary risk factors for cervical cancer at different age group in Japan.** *Journal of epidemiology/Japan Epidemiological Association*, *8*(1), 6-14.

Huang D, Ou B, Hampsch-Woodill M, Flanagan JA and Deemer EK (2002). **Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer.** *Journal of Agricultural and Food Chemistry*, *50*(7), 1815-1821.

Huang WC, Hsu RM, Chi LM, Leu YL, Chang YS and Yu JS (2007). **Selective downregulation of EGF receptor and downstream MAPK pathway in human cancer cell lines by active components partially purified from the seeds of *Livistona chinensis* R. Brown.** *Cancer letters*, *248*(1), 137-146.

Huh K, Zhou X, Hayakawa H, Cho JY, Libermann TA, Jin J, Harper JW and Munger K. (2007). **Human papillomavirus type 16 E7 oncoprotein associates with the cullin 2 ubiquitin ligase complex, which contributes to degradation of the retinoblastoma tumor suppressor.** *Journal of virology*, *81*(18), 9737-9747.

Husain A, Virmani OP, Popli SP, Misra LN, Gupta MM, Srivastava GN, Abraham Z and Singh AK (1992). **Dictionary of Indian Medicinal Plants**, CIMAP, Lucknow, India. 546

IARC, "Globocan 2012," http://globocan.iarc.fr/Pages/fact sheets cancer.aspx.

Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D (2011). **Global cancer statistics.** *CA: a cancer journal for clinicians*, *61*(2), 69-90.

Jones DL and Münger K (1997). **Analysis of the p53-mediated G1 growth arrest pathway in cells expressing the human papillomavirus type 16 E7 oncoprotein.** *Journal of virology*, *71*(4), 2905-2912.

Kaddour-Djebbar I, Lakshmikanthan V, Shirley RB, Ma Y, Lewis RW and Kumar MV (2006). **Therapeutic advantage of combining calcium channel blockers and TRAIL in prostate cancer.** *Molecular cancer therapeutics*, *5*(8), 1958-1966.

Kamei T, Kumano H, Iwata K, Nariai Y and Matsumoto T (2000). **The effect of a traditional Chinese prescription for a case of lung carcinoma.** *The Journal of Alternative and Complementary Medicine*, *6*(6), 557-559.

Kamiyama H, Takano S, Ishikawa E, Tsuboi K and Matsumura A (2005). **Anti-angiogenic and immunomodulatory effect of the herbal medicine" Juzen-taiho-to" on malignant glioma.** *Biological and Pharmaceutical Bulletin*, *28*(11), 2111-2116.

Kaul R, Mukherjee S, Ahmed F, Bhat MK, Chhipa R, Galande S and Chattopadhyay S (2003). **Direct interaction with and activation of p53 by SMAR1 retards cell-cycle progression at G2/M phase and delays tumor growth in mice.** *International journal of cancer*, *103*(5), 606-615.

Khan A, Safdar M, Khan MMA, Khattak KN and Anderson RA (2003). **Cinnamon improves glucose and lipids of people with type 2 diabetes.** *Diabetes care*, *26*(12), 3215-3218.

Khandelwal KR (2005) Practical Pharmacognosy. Nirali Prakashan 149–160.

Kim H, Choi JA, Park GS and Kim JH (2012). **BLT2 up-regulates interleukin-8 production and promotes the invasiveness of breast cancer cells.** *PloS one*, *7*(11), e49186.

Kim H, Kim H, Mosaddik A, Gyawali R, Ahn KS and Cho SK (2012). **Induction of apoptosis by ethanolic extract of mango peel and comparative analysis of the chemical constitutes of mango peel and flesh.** *Food Chemistry*, *133*(2), 416-422.

Kim H, Moon JY, Mosaddik A and Cho SK (2010). **Induction of apoptosis in human cervical carcinoma HeLa cells by polymethoxylated flavone-rich *Citrus grandis* Osbeck (Dangyuja) leaf extract.** *Food and Chemical Toxicology*, *48*(8), 2435-2442.

Kim HG, Song H, Yoon DH, Song BW, Park SM, Sung GH, Cho JY, Park HI, Choi S, Song WO, Hwang KC and Kim TW (2010). ***Cordyceps pruinosa* extracts induce apoptosis of HeLa cells by a caspase dependent pathway.** *Journal of ethnopharmacology*, *128*(2), 342-351.

Kim SH, Hyun SH and Choung SY (2006). **Anti-diabetic effect of cinnamon extract on blood glucose in db/db mice.** *Journal of ethnopharmacology*, *104*(1), 119-123.

Kim W, Khil LY, Clark R, Bok SH, Kim EE, Lee S, Jun HS and Yoon JW. (2006). **Naphthalenemethyl ester derivative of dihydroxyhydrocinnamic acid, a component of cinnamon, increases glucose disposal by enhancing translocation of glucose transporter 4.** *Diabetologia*, *49*(10), 2437-2448.

Kirana H, Agrawal SS and Srinivasan BP (2009). **Aqueous extract of *Ficus religiosa Linn.* reduces oxidative stress in experimentally induced type 2 diabetic rats.** *Indian journal of experimental biology*, *47*(10), 822.

Kirana H, Jali MV and Srinivasan BP (2011). **The study of aqueous extract of Ficus religiosa Linn. on cytokine TNF-α in type 2 diabetic rats.** *Pharmacognosy research*, *3*(1), 30.

Kirtikar KR and Basu BD (1993). **Indian Medicinal Plants**. second ed. *Periodical experts book agency*, New Delhi, India, *(3)* 2317–2319

Kirwan JM, Symonds P, Green JA, Tierney J, Collingwood M and Williams CJ (2003). **A systematic review of acute and late toxicity of concomitant chemoradiation for cervical cancer.** *Radiotherapy and Oncology*, *68*(3), 217-226.

Kochetkov DV, Il'inskaia GV, Komarov PG, Strom E, Agapova LS, Ivanov AV, Budanov AV, Frolova EI and Chumakov PM. (2006).**Transcriptional inhibition of human papilloma virus in cervical carcinoma cells reactivates functions of the tumor suppressor p53**. *Molekuliarnaia biologiia*, *41*(3), 515-523.

Koppikar SJ, Choudhari AS, Suryavanshi SA, Kumari S, Chattopadhyay S and Kaul-Ghanekar R (2010). **Aqueous cinnamon extract (ACE-c) from the bark of *Cinnamomum cassia* causes apoptosis in human cervical cancer cell line (SiHa) through loss of mitochondrial membrane potential.** *BMC cancer*, *10*(1), 210.

Korbakis D and Scorilas A (2012). **Quantitative expression analysis of the apoptosis-related genes BCL2, BAX and BCL2L12 in gastric adenocarcinoma cells following treatment with the anticancer drugs cisplatin, etoposide and taxol.** *Tumor Biology*, *33*(3), 865-875.

Kremser T, Evans A, Moore A, Luxford K, Begbie S, Bensoussan A, Marigliani R, and Zorbas H (2008). **Use of complementary therapies by Australian women with breast cancer.** *Breast Journal*, *17*, 387-394.

Kumar H, Goswami M, Yadav S, and Rao CV (2011). **Evaluation of In-Vitro Antioxidant Activity in *Ficus religiosa (L)* Leaves.** *International Journal of Research in Pharmacy & Science*, *1*(2).

Kumar, BN, Dhurandhar M, Aggarwal B, Anant S, Daniel K, Deng G, ... and White JD. (2013). **Proceedings of the Indo-US bilateral workshop on accelerating**

botanicals/biologics agent development research for cancer chemoprevention, treatment, and survival.

Kwon HJ, Bae SY, Kim KH, Han CH, Cho SH, Nam SW, Choi YS and Kim BW (2007). **Induction of apoptosis in HeLa cells by ethanolic extract of *Corallina pilulifera*.** *Food chemistry*, *104*(1), 196-201.

Kwon HJ, Hong YK, Kim KH, Han CH, Cho SH, Choi JS and Kim BW (2006). **Methanolic extract of *Pterocarpus santalinus* induces apoptosis in HeLa cells.** *Journal of ethnopharmacology*, *105*(1), 229-234.

Kwon HK, Jeon WK, Hwang JS, Lee CG, So JS, Park JA, Ko BS and Im SH (2009). **Cinnamon extract suppresses tumor progression by modulating angiogenesis and the effector function of CD8^{+}T cells.** *Cancer letters*, *278*(2), 174-182.

Landoni F, Maneo A, Colombo A, Placa F, Milani R, Perego P, Favini G, Ferri L and Mangioni C (1997). **Randomised study of radical surgery versus radiotherapy for stage Ib-IIa cervical cancer.** *The Lancet*, *350*(9077), 535-540.

Lauerova L, Dusek L, Simickova M, Kocák I, Vagundová M, Zaloudík J, and Kovarik J. (2001). **Malignant melanoma associates with Th1/Th2 imbalance that coincides with disease progression and immunotherapy response.** *Neoplasma*, *49*(3), 159-166.

Lee BJ, Kim YJ, Cho DH, Sohn NW and Kang H (2011). **Immunomodulatory effect of water extract of cinnamon on anti-CD3-induced cytokine responses and p38, JNK, ERK1/2, and STAT4 activation.** *Immunopharmacology and immunotoxicology*, *33*(4), 714-722.

Lee CW, Hong DH, Han SB, Park SH, Kim HK, Kwon BM, Kim HM (1999). **Inhibition of human tumor growth by 2'-hydroxy- and 2'-benzoyloxycinnamaldehydes.** *Planta Medica*. 65263-266.

Lee D, Kwon JH, Kim EH, Kim ES and Choi KY (2010). **HMGB2 stabilizes p53 by interfering with E6/E6AP-mediated p53 degradation in human papillomavirus-positive HeLa cells.** *Cancer letters*, *292*(1), 125-132.

Lee SH, Lee SY, Son DJ, Lee H, Yoo HS, Song S, Oh KW, Han DC, Kwon BM, and Hong JT (2005). **Inhibitory effect of 2′-hydroxycinnamaldehyde on nitric oxide production through inhibition of NF-κB activation in RAW 264.7 cells.** *Biochemical pharmacology*, *69*(5), 791-799.

Lehn H, Villa LL, Marziona F, Hilgarth M, Hillemans HG and Sauer G (1988). **Physical state and biological activity of human papillomavirus genomes in precancerous lesions of the female genital tract.** *The Journal of general virology*, *69*, 187-196.

Lehtinen M, Ault KA, Lyytikainen E, Dillner J, Garland SM, Ferris DG, Koutsky LA, Sings HL, Lu S, Haupt RM and Paavonen J. (2011). **Chlamydia trachomatis infection and risk of cervical intraepithelial neoplasia.** *Sexually transmitted infections*, *87*(5), 372-376.

Lengacher CA, Bennett, MP, Kip KE, Gonzalez L, Jacobson P and Cox CE (2006). **Relief of symptoms, side effects, and psychological distress through use of complementary and alternative medicine in women with breast cancer.** *Oncology Nursing Forum*, *33*, 97–104

Levine AJ, Perry ME, Chang A, Silver A, Dittmer D, Wu M and Welsh D (1994). **The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumorigenesis.** *British journal of cancer*, *69*(3), 409.

Li GL, Jiang W, Xia Q, Chen SH, Ge XR, Gui SQ and Xu CJ (2010). **HPV E6 down-regulation and apoptosis induction of human cervical cancer cells by a novel lipid-soluble extract (PE) from *Pinellia pedatisecta* Schott *in vitro*.** *Journal of ethnopharmacology*, *132*(1), 56-64.

Li H, Wang LJ, Qiu GF, Yu JQ, Liang SC and Hu XM (2007). **Apoptosis of Hela cells induced by extract from *Cremanthodium humile*.** *Food and chemical toxicology*, *45*(10), 2040-2046.

Li J, Li Q, Feng T and Li K (2008). **Aqueous extract of *Solanum nigrum* inhibit growth of cervical carcinoma (U14) via modulating immune response of tumor bearing mice and inducing apoptosis of tumor cells.** *Fitoterapia*, *79*(7), 548-556.

Li J, Sun GZ, Lin HS, Pei YX, Qi X, An C, Yu J, Hua BJ (2008). **The herb medicine formula "Yang Wei Kang Liu" improves the survival of late stage gastric cancer patients and induces the apoptosis of human gastric cancer cell line through Fas/Fas ligand and Bax/Bcl-2 pathways.** *International immunopharmacology*, *8*(9), 1196-1206.

Li X, Yang G, Li X, Zhang Y, Yang J, Chang J, Sun X, Zhou X, Guo Y, Xu Y, Liu J, Bensoussan A (2013). **Traditional Chinese medicine in cancer care: A review of controlled clinical studies published in Chinese.** *PloS one*, *8*(4), e60338.

Liotta LA (1986). **Tumor invasion and metastases-role of the extracellular matrix: Rhoads Memorial Award lecture.** *Cancer Res*, *46*(1), 1-7.

Liu SH and Cheng YC (2012b). **Old formula, new Rx: the journey of PHY906 as cancer adjuvant therapy.** *Journal of ethnopharmacology*, *140*(3), 614-623.

Liu X, Zhao M, Wu K, Chai X, Yu H, Tao Z and Wang J (2012a). **Immunomodulatory and anticancer activities of phenolics from emblica fruit *Phyllanthus emblica L.*** *Food Chemistry*, *131*(2), 685-690.

Li-Weber M (2013). **Targeting apoptosis pathways in cancer by Chinese medicine.** *Cancer letters*, *332*(2), 304-312.

Ly JD, Grubb DR, and Lawen A (2003). **The mitochondrial membrane potential (Δψm) in apoptosis; an update.** *Apoptosis*, *8*(2), 115-128.

Ma H, Lai F, Xie H, Wang J and Wang H (2008). **Involvement of the Bcl-2 Family Members in Pinus massoniana Bark Extract induced Apoptosis in HeLa Cells.** *Phytotherapy Research*, *22*(11), 1472-1476.

Malik F, Kumar A, Bhushan S, Mondhe DM, Pal HC, Sharma R, Khajuria A, Singh S, Singh G, Saxena AK, Suri KA, Qazi GN, Singh J (2009). **Immune modulation and apoptosis induction: Two sides of antitumoural activity of a standardised herbal formulation of *Withania somnifera*.** *European Journal of Cancer*, *45*(8), 1494-1509.

Malo A, Krüger B, Seyhun E, Schäfer C, Hoffmann RT, Göke B and Kubisch CH (2010). **Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, trypsin activation, and acinar cell apoptosis while increasing secretion in rat pancreatic acini.** *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *299*(4), G877-G886.

Mao JJ, Farrar JT, Xie SX, Bowman MA and Armstrong K (2007). **Use of complementary and alternative medicine and prayer among a national sample of cancer survivors compared to other populations without cancer.** *Complementary Therapies in Medicine*, *15*(1), 21-29.

Marchiole P, Tigaud JD, Costantini S, Mammoliti S, Buenerd A, Moran E and Mathevet P (2011). **Neoadjuvant chemotherapy and vaginal radical trachelectomy for fertility-sparing treatment in women affected by cervical cancer (FIGO stage IB–IIA1).** *Gynecologic oncology*, *122*(3), 484-490.

Matan N, Rimkeeree H, Mawson AJ, Chompreeda P, Haruthaithanasan V and Parker M (2006). **Antimicrobial activity of cinnamon and clove oils under modified atmosphere conditions.** *International journal of food microbiology*,*107*(2), 180-185.

McFarland B, Bigelow D, Zani B, Newsom J and Kaplan M (2002). **Complementary and alternative medicine use in Canada and the United States.** *American Journal of Public Health*, *92*(10), 1616-1618.

McFarland GB (1944). **Thai-English Dictionary.** *Stanford University press,* Stanford, California, 601.

Meeta LD, Agarwal N, Vaze N, Shah R and Malik S (2013). **Clinical practice guidelines on menopause: An executive summary and recommendations.** *Journal of mid life health*, *4*(2), 77.

Meijerman I, Beijnen JH and Schellens JH (2006). **Herb-drug interactions in oncology: focus on mechanisms of induction.** *The Oncologist*, *11*(7), 742-752.

Meissner JD (1999). **Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines.** *Journal of general virology*, *80*(7), 1725-1733.

Millar WJ (2001). **Patterns of use--alternative health care practitioners.** *Health Reports*, *13*(1), 9-21.

Mitra AB, Murty VVVS, Pratap M, Sodhani P and Chaganti RSK (1994). **ERBB2 (HER2/neu) oncogene is frequently amplified in squamous cell carcinoma of the uterine cervix.** *Cancer research*, *54*(3), 637-639.

Molassiotis A, Scott JA, Kearney N, Pud D, Magri M, Selvekerova S, Bruyns I, Fernadez-Ortega P, Panteli V, Margulies A, Gudmundsdottir G, Milovics L, Ozden G, Platin N, Patiraki E (2006). **Complementary and alternative medicine use in breast cancer patients in Europe.** *Supportive Care in Cancer, 14,* 260–267.

Montazeri A, Sajadian A, Ebrahimi M and Akbari, ME (2005). **Depression and the use of complementary medicine among breast cancer patients.** *Supportive Care in Cancer, 13,* 339–342.

Moretti S, Chiarugi A, Semplici F, Salvi A, De Giorgi V, Fabbri P and Mazzoli S (2001). **Serum imbalance of cytokines in melanoma patients.** *Melanoma research*, *11*(4), 395-399.

Mukakalisa I, Bindler R, Allen C and Dotson J (2014). **Cervical Cancer in Developing Countries: Effective Screening and Preventive Strategies: With an Application in Rwanda.** *Health care for women international*, (just-accepted), 00-00.

Muller PA, Vousden KH and Norman JC (2011). **p53 and its mutants in tumor cell migration and invasion.** *The Journal of cell biology*, *192*(2), 209-218.

Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ (2003). **Epidemiologic classification of human papillomavirus types associated with cervical cancer.** *New England Journal of Medicine*, *348*(6), 518-527.

Nair R and Chanda S. (2007). **Antibacterial activities of some medicinal plants of the western region of India.** *Turkish Journal of Biology*, *31*(4), 231-236.

Nambotin SB, Tomimaru Y, Merle P, Wands JR and Kim M (2012). **Functional consequences of WNT3/Frizzled7-mediated signaling in non-transformed hepatic cells.** *Oncogenesis*, *1*(10), e31.

National Center for Complementary and Alternative Medicine. (2009). **What is CAM?** Retrieved from http://nccam.nih.gov/health/whatiscam/overview.htm

Navo MA1, Phan J, Vaughan C, Palmer JL, Michaud L, Jones KL, Bodurka DC, Basen-Engquist K, Hortobagyi GN, Kavanagh JJ, Smith JA. (2004). **An assessment of the utilization of complementary and alternative medication in women with gynecologic or breast malignancies.** *Journal of Clinical Oncology, 22,* 671–677

Niculescu AB, Chen X, Smeets M, Hengst L, Prives C and Reed SI (1998). **Effects of $p21^{Cip1/Waf1}$ at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication.** *Molecular and cellular biology*, *18*(1), 629-643.

O'Garra A, Barrat FJ, Castro AG, Vicari A and Hawrylowicz C (2008). **Strategies for use of IL-10 or its antagonists in human disease.** *Immunological reviews*, *223*(1), 114-131.

Oubrahim H Stadtman ER and Chock PB (2001). **Mitochondria play no roles in Mn (II)-induced apoptosis in HeLa cells.** *Proceedings of the National Academy of Sciences*, *98*(17), 9505-9510.

Overall CM and López-Otín C (2002). **Strategies for MMP inhibition in cancer: innovations for the post-trial era.** *Nature Reviews Cancer*, *2*(9), 657-672.

Owens B, Jackson M and Berndt A (2009). **Complementary therapy used by Hispanic women during treatment for breast cancer.** *Journal of Holistic Nursing*, *27*, 167-176.

Panchawat S and Sisodia SS (2010). **Comparison of free radical scavenging activity of Ficus religiosa Linn. stem bark extracts prepared by different methods of extraction.** *Journal of Herbal Medicine and Toxicology*, *4*(2), 163-166.

Panda H (2005). **Herbs Cultivation and Medicinal Uses**. second ed. *National Institute of Industrial Research*, Delhi, India, 302-303

Pandey G and Madhuri S (2009). **Some medicinal plants as natural anticancer agents.** *Pharmacognosy Reviews*, *3*(6), 259.

Paramita S, Soewarto S, Widodo MAA and Sumitro SB (2010). **High parity and hormonal contraception use as risk factors for cervical cancer in East Kalimantan.** *Medical Journal of Indonesia*, *19*(4), 268-72.

Park J (2005). **Use of alternative health care.** *Health Reports*, *16*(2), 39-42.

Park TW, Fujiwara H and Wright TC (1995). **Molecular biology of cervical cancer and its precursors.** *Cancer*, *76*(S10), 1902-1913.

Pecorelli S (2009) **Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium.** *International Journal of Gynecology and Obstetrics*, 105:103-104

Pellikainen JM, Ropponen KM, Kataja VV, Kellokoski JK, Eskelinen MJ and Kosma V M (2004). **Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in breast cancer with a special reference to activator protein-2, HER2, and prognosis.** *Clinical Cancer Research*, *10*(22), 7621-7628.

Peng B, Hu Q, Liu X, Wang L, Chang Q, Li J, Tang J, Wang N, Wang Y (2009). **Duchesnea phenolic fraction inhibits in vitro and in vivo growth of cervical cancer through induction of apoptosis and cell cycle arrest.** *Experimental Biology and Medicine*, *234*(1), 74-83.

Pourmorad F, Hosseinimehr SJ and Shahabimajd N (2006). **Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants.** *African journal of biotechnology*, *5*(11).

Prehn JH, Bindokas VP, Marcuccilli CJ, Krajewski S, Reed JC and Miller RJ (1994). **Regulation of neuronal Bcl2 protein expression and calcium homeostasis by transforming growth factor type beta confers wide-ranging protection on rat hippocampal neurons.** *Proceedings of the National Academy of Sciences*, *91*(26), 12599-12603.

Preuss HG, Echard B, Polansky MM and Anderson R (2006). **Whole cinnamon and aqueous extracts ameliorate sucrose-induced blood pressure elevations in spontaneously hypertensive rats.** *Journal of the American College of Nutrition*, *25*(2), 144-150.

Pullaiah T. (2006). *Encyclopaedia of world medicinal plants* (Vol. 1). Daya books. 958-959

Qin B, Nagasaki M, Ren M, Bajotto G, Oshida Y and Sato Y (2003). **Cinnamon extract (traditional herb) potentiates in vivo insulin-regulated glucose utilization via enhancing insulin signaling in rats.** *Diabetes research and clinical practice*, *62*(3), 139-148.

Quan H, Lai D, Johnson D, Verhoef M and Musto R (2008). **Complementary and alternative medicine use among Chinese and white Canadians.** *Canadian Family Physician*, *54*(11), 1563-1569.

Rajkumar T, Franceschi S, Vaccarella S, Gajalakshmi V, Sharmila A, Snijders PJ, Muñoz N, Meijer CJ, Herrero R. (2003). **Role of paan chewing and dietary habits in cervical carcinoma in Chennai, India.** *British journal of cancer*, *88*(9), 1388-1393.

Rakovitch E, Pigno JP, Chartier C, Ezer M, Verma S, Dranitsaris G and Clemons M (2005). **Complementary and alternative medicine use is associated with an increased perception of breast cancer risk and death.** *Breast Cancer Research and Treatment*, *90*, 139-148.

Ramogola-Masire D, McGrath CM, Barnhart KT, Friedman HM and Zetola NM (2011). **Subtype distribution of human papillomavirus in HIV-infected women with cervical intraepithelial neoplasia stages 2 and 3 in Botswana.** *International*

journal of gynecological pathology: official journal of the International Society of Gynecological Pathologists, *30*(6), 591.

Rangaswami H, Bulbule A and Kundu GC (2004). **Nuclear factor-inducing kinase plays a crucial role in osteopontin-induced MAPK/IκBα kinase-dependent nuclear factor κB-mediated promatrix metalloproteinase-9 activation.** *Journal of Biological Chemistry*, *279*(37), 38921-38935.

Rathee P, Rathee S, Rathee D, Rathee D and Kalia AN (2010). **In Vitro Antioxidant Studies and Total Phenolic Content of Ficus Religiosa Fruits Extract.** *Pharmacologyonline, 2:*737-44.

Ratnasooriya WD, Jayakody JRAC and Dharmasiri MG (1998). **An aqueous extract of trunk bark of *Ficus religiosa* has anxiolytic activity.** *Medical science research, 26,* 817-819.

Ravindran PN, Nirmal-Babu K and Shylaja M (Eds.) (2003). **Cinnamon and cassia: the genus Cinnamomum.** CRC press.

Rejiya CS, Cibin TR and Abraham A (2009). **Leaves of *Cassia tora* as a novel cancer therapeutic–An *in vitro* study.** *Toxicology in vitro*, *23*(6), 1034-1038.

Ricci MS and Zong WX (2006). **Chemotherapeutic approaches for targeting cell death pathways.** *The oncologist*, *11*(4), 342-357.

Rivlin N, Brosh R, Oren M and Rotter V (2011**). Mutations in the p53 tumor suppressor gene important milestones at the various steps of tumorigenesis.** *Genes and cancer*, *2*(4), 466-474.

Roth MA and Kobayashi KM (2008). **The use of complementary and alternative medicine among Chinese Canadians: results from a national survey.** *Journal of immigrant and minority health*, *10*(6), 517-528.

Rubin P, Constine LS, Marks LB (2014). **ALERT-Adverse Late Effects of Cancer Treatment.** *Medical Radiology. Radiation Oncology, 2,* 551-552.

Sa G and Das T (2008). **Anti cancer effects of curcumin: cycle of life and death.** *Cell Division, 3*(1), 14.

Salido M, Gonzalez JL and Vilches J (2007). **Loss of mitochondrial membrane potential is inhibited by bombesin in etoposide-induced apoptosis in PC-3 prostate carcinoma cells.** *Molecular cancer therapeutics, 6*(4), 1292-1299.

Samdup DZ, Smith RG and Song SI (2006**). The use of complementary and alternative medicine in children with chronic medical conditions.** *American journal of physical medicine and rehabilitation, 85*(10), 842-846.

Sánchez-Alcázar JA, Schneider E, Martínez MA, Carmona P, Hernández-Muñoz I, Siles E, De La Torre P, Ruiz-Cabello J, García I, Solis-Herruzo JA. (2000) **Tumor necrosis factor-alpha increases the steady-state reduction of cytochrome b of the mitochondrial respiratory chain in metabolically inhibited L929 cells.** *Journal of Biochemistry* 275: 13353-13361.

Saraç N and Şen B (2014). **Antioxidant, mutagenic, antimutagenic activities, and phenolic compounds of *Liquidambar orientalis* Mill. var. *orientalis*.** *Industrial Crops and Products, 53*, 60-64.

Sasson IM, Haley NJ, Hoffmann D, Wynder EL, Hellberg D and Nilsson S (1985). **Cigarette smoking and neoplasia of the uterine cervix: smoke constituents in cervical mucus.** *The New England journal of medicine, 312*(5), 315-316.

Scheffner M, Münger K, Byrne JC and Howley PM (1991). **The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines.** *Proceedings of the National Academy of Sciences, 88*(13), 5523-5527.

Schoene NW, Kelly MA, Polansky MM and Anderson RA (2005). **Water-soluble polymeric polyphenols from cinnamon inhibit proliferation and alter cell cycle distribution patterns of hematologic tumor cell lines.** *Cancer letters, 230*(1), 134-140.

Sen S, Chakraborty R and De B (2011). **Challenges and opportunities in the advancement of herbal medicine: India's position and role in a global context.** *Journal of Herbal Medicine*, *1*(3), 67-75.

Senanayake UM, Lee TH and Wills RB (1978). **Volatile constituents of cinnamon (Cinnamomum zeylanicum) oils.** *Journal of agricultural and food chemistry*, *26*(4), 822-824.

Shafi G, Munshi A, Hasan TN, Alshatwi AA, Jyothy A and Lei DK (2009). **Induction of apoptosis in HeLa cells by chloroform fraction of seed extracts of Nigella sativa.** *Cancer Cell Int*, *9*, 29.

Shukla S, Bharti AC, Hussain S, Mahata S, Hedau S, Kailash U, Kashyap V, Bhambhani S, Roy M, Batra S, Talwar GP, Das BC. (2009). **Elimination of high-risk human papillomavirus type HPV16 infection by 'Praneem' polyherbal tablet in women with early cervical intraepithelial lesions.** *Journal of cancer research and clinical oncology*, *135*(12), 1701-1709.

Sikström B, Hellberg D, Nilsson S and Mårdh PA (1995). **Smoking, alcohol, sexual behaviour and drug use in women with cervical human papillomavirus infection.** *Archives of gynecology and obstetrics*, *256*(3), 131-137.

Singh D and Goel RK (2009). **Anticonvulsant effect of *Ficus religiosa*: Role of serotonergic pathways.** *Journal of ethnopharmacology*, *123*(2), 330-334.

Singh D, Singh B and Goel RK (2011). **Traditional uses, phytochemistry and pharmacology of *Ficus religiosa*: A review.** *Journal of ethnopharmacology*, *134*(3), 565-583.

Singh G, Maurya S, DeLampasona MP and Catalan CA (2007). **A comparison of chemical, antioxidant and antimicrobial studies of cinnamon leaf and bark volatile oils, oleoresins and their constituents.** *Food and Chemical Toxicology*, *45*(9), 1650-1661.

Singh MP and Panda H (2005). *Medicinal herbs with their formulations*. Daya Books.404-405

Singh R, Koppikar SJ, Paul P, Gilda S, Paradkar AR and Kaul-Ghanekar R (2009). **Comparative analysis of cytotoxic effect of aqueous cinnamon extract from Cinnamomum zeylanicum bark with commercial cinnamaldehyde on various cell lines.** *Pharmaceutical Biology*, *47*(12), 1174-1179.

Sklar C (2005). **Maintenance of ovarian function and risk of premature menopause related to cancer treatment.** *JNCI Monographs*, *2005*(34), 25-27.

Smith JS, Herrero R, Bosetti C, Muñoz N, Bosch FX, Eluf-Neto J, Castellsagué X, Meijer CJ, Van den Brule AJ, Franceschi S and Ashley R (2002). **Herpes simplex virus-2 as a human papillomavirus cofactor in the etiology of invasive cervical cancer.** *Journal of the National Cancer Institute*, *94*(21), 1604-1613.

Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R and Clifford GM (2007). **Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: A meta-analysis update.** *International journal of cancer*, *121*(3), 621-632.

Smyth MJ, Godfrey DI, Trapani JA (2001) **A fresh look at tumor immunosurveillance and immunotherapy.** *Nat. Immunol.* 2, 293-299

Solomon D and Nayar R. (Eds.) (2004). *The Bethesda System for reporting cervical cytology: definitions, criteria, and explanatory notes*. Springer.

Spies L, Koekemoer TC, Sowemimo AA, Goosen ED and Van de Venter M (2013). **Caspase-dependent apoptosis is induced by *Artemisia afra* Jacq. ex Willd in a mitochondria-dependent manner after G_2/M arrest.** *South African Journal of Botany*, *84*, 104-109.

Sreelekshmi R, Latha PG, Arafat MM, Shyamal S, Shine VJ, Anuja GI, Suja RS and Rajasekharan S (2007). **Anti-inflammatory, analgesic and anti-lipid peroxidation studies on stem bark of *Ficus religiosa Linn*.** *Natural product radiance*, *6*(5), 377-381.

Sun A, Chia JS, Chiang CP, Hsuen SP, Du JL, Wu, CW and Wang WB (2005). **The chinese herbal medicine Tien-Hsien liquid inhibits cell growth and induces**

apoptosis in a wide variety of human cancer cells. *Journal of Alternative and Complementary Medicine*, *11*(2), 245-256.

Talwar GP, Dar SA, Rai MK, Reddy KV, Mitra D, Kulkarni SV, Doncel GF, Buck CB, Schiller JT, Muralidhar S, Bala M, Agrawal SS, Bansal K, Verma JK (2008). **A novel polyherbal microbicide with inhibitory effect on bacterial, fungal and viral genital pathogens.** *International journal of antimicrobial agents*, *32*(2), 180-185.

Tavakkol-Afshari J, Brook A and Mousavi SH (2008). **Study of cytotoxic and apoptogenic properties of saffron extract in human cancer cell lines.** *Food and Chemical Toxicology*, *46*(11), 3443-3447.

Tayarani-Najaran Z, Mousavi SH, Asili J and Emami SA (2010). **Growth-inhibitory effect of *Scutellaria lindbergii* in human cancer cell lines.** *Food and chemical toxicology*, *48*(2), 599-604.

The Ayurvedic Pharmacopoeia of India, (2001). *The Controller of Publication, Delhi, India*, *1*(1).

Tung YT, Chua MT, Wang SY and Chang ST (2008). **Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (*Cinnamomum osmophloeum*) twigs.** *Bioresource technology*, *99*(9), 3908-3913.

Vaccarella S, Herrero R, Dai M, Snijders PJ, Meijer CJ, Thomas JO, Hoang Anh PT, Ferreccio C, Matos E, Posso H, de Sanjosé S, Shin HR, Sukvirach S, Lazcano-Ponce E, Ronco G, Rajkumar R, Qiao YL, Muñoz N, Franceschi S (2006). **Reproductive factors, oral contraceptive use, and human papillomavirus infection: pooled analysis of the IARC HPV prevalence surveys.** *Cancer Epidemiology Biomarkers and Prevention*, *15*(11), 2148-2153.

Vaccarella S, Herrero R, Snijders PJ, Dai M, Thomas JO, Hieu NT, Ferreccio C, Matos E, Posso H, de Sanjosé S, Shin HR, Sukvirach S, Lazcano-Ponce E, Muñoz N, Meijer CJ, Franceschi S (2008). **Smoking and human papillomavirus infection: pooled analysis of the International Agency for Research on Cancer HPV Prevalence Surveys.** *International journal of epidemiology*, *37*(3), 536-546.

Van der Weg F and Streuli RA (2003). **Use of alternative medicine by patients with cancer in a rural area of Switzerland.** *Swiss Medical Weekly*, *133*, 233-240

Vardi A, Formiggini F, Casotti R, De Martino A, Ribalet F, Miralto A and Bowler C (2006). **A stress surveillance system based on calcium and nitric oxide in marine diatoms.** *PLoS biology*, *4*(3), e60.

Vaz AF, Pinto-Neto AM, Conde DM, Costa-Paiva L, Morais SS and Esteves SB (2008). **Quality of life and acute toxicity of radiotherapy in women with gynecologic cancer: a prospective longitudinal study.** *Archives of gynecology and obstetrics*, *278*(3), 215-223.

Vicus D, Sutradhar R, Lu Y, Elit L, Kupets R and Paszat L (2014). **The association between cervical cancer screening and mortality from cervical cancer: A population based case–control study.** *Gynecologic oncology*. *133*(2), 167-171

Vinutha B, Prashanth D, Salma K, Sreeja SL, Pratiti D, Padmaja R, Radhika S, Amit A, Venkateshwarlu K, Deepak M. (2007). **Screening of selected Indian medicinal plants for acetylcholinesterase inhibitory activity.** *Journal of ethnopharmacology*, *109*(2), 359-363.

Vousden KH (2000). **p53: death star.** *Cell*, *103*(5), 691-694.

Wade CR, Jackson PG and Van Rij AM (1985). **Quantitation of malondialdehyde (MDA) in plasma, by ion-pairing reverse phase high performance liquid chromatography.** *Biochemical medicine*, *33*(3), 291-296.

Wanchai A, Armer JM and Stewart BR (2010). **Complementary and alternative medicine use among women with breast cancer: a systematic review.** *Clinical Journal of Oncology Nursing*, *14*(4), E45-E55.

Wang XF, Du J, Zhang TL, Zhou QM, Lu YY, Zhang H and Su SB (2013). **Inhibitory effects of PC-SPESII herbal extract on human breast cancer metastasis.** *Evidence-Based Complementary and Alternative Medicine*, *2013*.

Wang YH, Avula B, Nanayakkara ND, Zhao J and Khan I A (2013). **Cassia cinnamon as a source of coumarin in cinnamon-flavored food and food supplements in the United States.** *Journal of agricultural and food chemistry*,*61*(18), 4470-4476.

Weigent DA, Stanton GJ and Johnson HM (1983). **Interleukin 2 enhances natural killer cell activity through induction of gamma interferon.** *Infection and immunity*, *41*(3), 992-997.

Wiles J and Rosenberg MW (2001). **'Gentle caring experience': Seeking alternative health care in Canada.** *Health and place*, *7*(3), 209-224.

Woods Ignatoski KM, Dziubinski ML, Ammerman C and Ethier SP (2005). **Cooperative Interactions of HER-2, HPV-16 Oncoproteins in the Malignant Transformation of Human Mammary Epithelial Cells.** *Neoplasia*, *7*(8), 788-798.

Wu DC, Li S, Yang DQ and Cui YY (2011). **Effects of *Pinus massoniana* bark extract on the adhesion and migration capabilities of HeLa cells.** *Fitoterapia*, *82*(8), 1202-1205.

Wu DY (1981) *Chung I Tsa Chin* 22(1): 34-5

Yang X, Jin G, Nakao Y, Rahimtula M, Pater MM and Pater A (1996). **Malignant transformation of HPV 16-immortalized human endocervical cells by cigarette smoke condensate and characterization of multistage carcinogenesis.** *International journal of cancer*, *65*(3), 338-344.

Yao Z, and Shulan Z (2008). **Inhibition effect of Guizhi-Fuling-decoction on the invasion of human cervical cancer.** *Journal of ethnopharmacology*, *120*(1), 25-35.

Yoon MJ, Lee HJ, Kim JH and Kim DK (2006). **Extracellular ATP induces apoptotic signaling in human monocyte leukemic cells, HL-60 and F-36P.** *Archives of pharmacal research*, *29*(11), 1032-1041.

Yoshinouchi M, Hongo A, Nakamura K, Kodama J, Itoh S, Sakai H and Kudo T (1999). **Analysis by multiplex PCR of the physical status of human papillomavirus type 16 DNA in cervical cancers.** *Journal of clinical microbiology*, *37*(11), 3514-3517.

Yu D, Wang SS, Dulski KM, Tsai CM, Nicolson, GL and Hung MC (1994). **c-erbB-2/neu overexpression enhances metastatic potential of human lung cancer cells by induction of metastasis-associated properties.** *Cancer research*, *54*(12), 3260-3266.

Yu XY, Zeng CQ, Gao S, et al (2012) **The study on apoptosis of human cervical cancer HeLa cell induced by *Phryma leptostachya* L. var. asiatica Hara extract and related molecular mechanism.** *Cell Mol Immunol, 28*, 608-10

Zeng YW, Liu XZ, Lv ZC and Peng YH (2012). **Effects of *Ficus hirta*Vahl.(Wuzhimaotao) extracts on growth inhibition of HeLa cells.** *Experimental and Toxicologic Pathology*, *64*(7), 743-749.

Zhang XP, Liu F and Wang W (2011). **Two-phase dynamics of p53 in the DNA damage response.** *Proceedings of the National Academy of Sciences*, *108*(22), 8990-8995.

Zhang Z1, Knobloch TJ, Seamon LG, Stoner GD, Cohn DE, Paskett ED, Fowler JM, Weghorst CM. (2011). **A black raspberry extract inhibits proliferation and regulates apoptosis in cervical cancer cells.** *Gynecologic oncology*, *123*(2), 401-406.

Zhao Y, Cao X, Zheng Y, Tang J, Cai W, Wang H, Gao Y, Wang Y. (2012). **Relationship between cervical disease and infection with human papillomavirus types 16 and 18, and herpes simplex virus 1 and 2.** *Journal of medical virology*, *84*(12), 1920-1927.

Zheng ZM, and Baker CC. (2006). **Papillomavirus genome structure, expression, and post-transcriptional regulation.** *Frontiers in bioscience: a journal and virtual library*, *11*, 2286.

Zhou R, Tardivel A, Thorens B, Choi I and Tschopp J (2010). **Thioredoxin-interacting protein links oxidative stress to inflammasome activation.** *Nature immunology*, *11*(2), 136-140.

Zoratti M and Szabò I (1995). **The mitochondrial permeability transition.** *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes*, *1241*(2), 139-176.

Publications (5)

Research Articles (4)

1. **Choudhari Amit S.**, Suryavanshi Snehal A., and Kaul-Ghanekar Ruchika. "The Aqueous Extract of *Ficus religiosa* Induces Cell Cycle Arrest in Human Cervical Cancer Cell Lines SiHa (HPV-16 Positive) and Apoptosis in HeLa (HPV-18 Positive)." ***PloS One*.** 2013; 8.7 e70127.

2. **Choudhari Amit S.**, Suryavanshi Snehal A., Ingle Harshad., and Kaul-Ghanekar Ruchika. "Evaluating the antioxidant potential of aqueous and alcoholic extracts of *Ficus religiosa* using ORAC assay and assessing their cytotoxic activity in cervical cancer cell lines." ***Biotechnol. Bioinf. Bioeng*.** 2011; 1(4), 443-450.

3. **Choudhari Amit S***., Koppikar Soumya J*., Suryavanshi Snehal A., Kumari Shweta, Chattopadhyay Samit, and Kaul-Ghanekar Ruchika. "Aqueous cinnamon extract (ACE-c) from the bark of Cinnamomum cassia causes apoptosis in human cervical cancer cell line (SiHa) through loss of mitochondrial membrane potential." ***BMC cancer.*** 2010; 10(1): 210. *(* equal contribution)*

4. **Choudhari Amit S.**, Pandita Savita., Suryavanshi Snehal A., and Kaul-Ghanekar Ruchika. "Herbal formulation FC posses anticancer and immunomodulatory activity." ***Planta Medica.*** 2014 (*Communicated*)

Book Chapter (1)

1. Kaul-Ghanekar Ruchika, **Choudhari Amit S.**, Pandita Savita. "Biomarker for early detection of cervical cancer" in ***Noninvasive Molecular Markers in Gynecologic Cancers***. CRC press, Taylor and Francis group. 2014 (*In press*).

Awards (2)

1. **Senior Research Fellowship** was awarded to the project entitled **"Analyzing the Chemopreventive Potential of Novel Herbal Composition (FC) in Cervical Cancer and Studying the Underlying Molecular Mechanisms governing its Anticancer Activity"** by Council of Scientific & Industrial Research (**CSIR**), India. 2012-2014

2. **Best poster presentation award** was awarded to the paper titled **"Aqueous extract of Ficus religiosa bark induce apoptosis, cell cycle arrest and inhibits migration and invasion of cervical cancer cells"** at 9th "National Research Scholars Meet in Life Science" held on December 19-20, 2013 in **Advanced Centre for Treatment, Research and Education in Cancer (ACTREC)**, Navi-Mumbai.

Paper presented in Meetings and Conferences (5)

1. **Choudhari Amit S**., Suryavanshi Snehal A., and Kaul-Ghanekar Ruchika. "Aqueous extract of Ficus religiosa bark induce apoptosis, cell cycle arrest, and inhibits migration and invasion of cervical cancer cells" at **5th International Conference on Translational Cancer Research: Multi- Targeted Approach to Treatment of Cancer.** February 6-9, 2014. Vighanbhavan, New Delhi. ***(Poster presentation)***

2. **Choudhari Amit S**., Suryavanshi Snehal A., and Kaul-Ghanekar Ruchika. "Aqueous extract of Ficus religiosa bark induce apoptosis, cell cycle arrest and inhibits migration and invasion of cervical cancer cells" at **9th National Research Scholars Meet in Life Science.** December 19-20, 2013. ACTREC, Navi-Mumbai. ***(Poster presentation)***

3. **Choudhari Amit S**., Suryavanshi Snehal A., and Kaul-Ghanekar Ruchika. "Effects of aqueous extract of *Ficus religiosa* on apoptosis, cell cycle arrest, migration and invasion of cervical cancer cells" at **9th DAE-BRNS Life Sciences symposium on Current Advances in Immunobiology and Cancer**. November 28-30, 2013. BARC, Mumbai. ***(Poster presentation)***

4. **Choudhari Amit S**., Suryavanshi Snehal A., and Kaul-Ghanekar Ruchika. "Evaluating the antioxidant potential of aqueous and alcoholic extracts of *Ficus religiosa* and assessing their cytotoxic activity in cervical cancer cell lines" in **2nd National Conference on Biotechnology, Bioinformatics and Bioengineering** held on February 24-25, 2012. Kolhapur. ***(Oral presentation)***

5. **Choudhari Amit S**., Koppikar Soumya J., Suryavanshi Snehal A., Kumari Shweta, Chattopadhyay Samit, and Kaul-Ghanekar Ruchika. "Cinnamon induce apoptosis nand regulates HER-2 expression in SiHa cells" at **International Conference on Recent Advances in Free Radical Research, Natural Products, Antioxidants and Radioprotectors in Health and 9th Annual Meeting of The Society of Free Radical Research India.** January 11-13, 2010. Hyderabad. (***Poster presentation)***