ANALYZING THE CHEMOPREVENTIVE POTENTIAL OF HERBAL FORMULATION (HC9) IN BREAST CANCER AND ELUCIDATING ITS MECHANISMS

A thesis submitted to

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In Biotechnology

By

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Under the guidance of

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March 2015

Certificate of Guide

This is to certify that the work incorporated in the thesis entitled **"Analyzing the chemopreventive potential of herbal formulation (HC9) in breast cancer and elucidating its mechanisms"** submitted by **Ms. Snehal Suryavanshi** 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 during the period from **August 2010** to **March 2015** under my direct supervision/ guidance.

Place : Pune

Date : / /

Dr. Ruchika Kaul-Ghanekar

(Supervisor/Research Guide)

Certificate

This is to certify that the work incorporated in the thesis entitled **"Analyzing the chemopreventive potential of herbal formulation (HC9) in breast cancer and elucidating its mechanisms"** 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 **Ms. Snehal Suryavanshi** 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.

Place : Pune

Date : / /

Director,
Prof. Akhilesh Mishra
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I declare that the thesis entitled **"Analyzing the chemopreventive potential of herbal formulation (HC9) in breast cancer and elucidating its mechanisms"** 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 during the period from **August 2010** to **March 2015** 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 material obtained from other sources has been duly acknowledged in the thesis.

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Signature of the Candidate

Ms. Snehal Arjunrao Suryavanshi
(Ph.D candidate)

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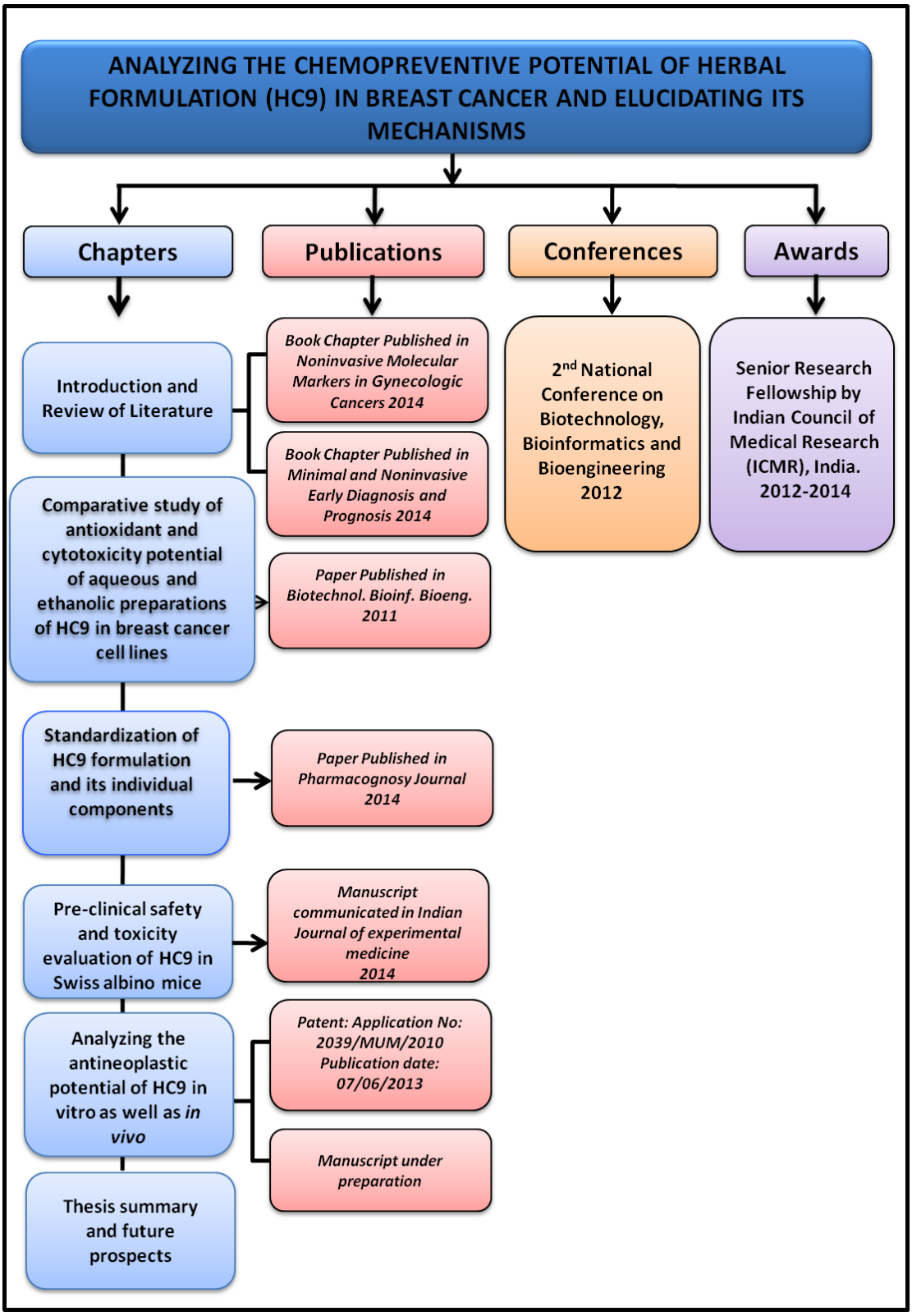
Snehal Arjunrao Suryavanshi

Dedicated to my.....

Family..

THESIS AT GLANCE



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List of Abbreviations

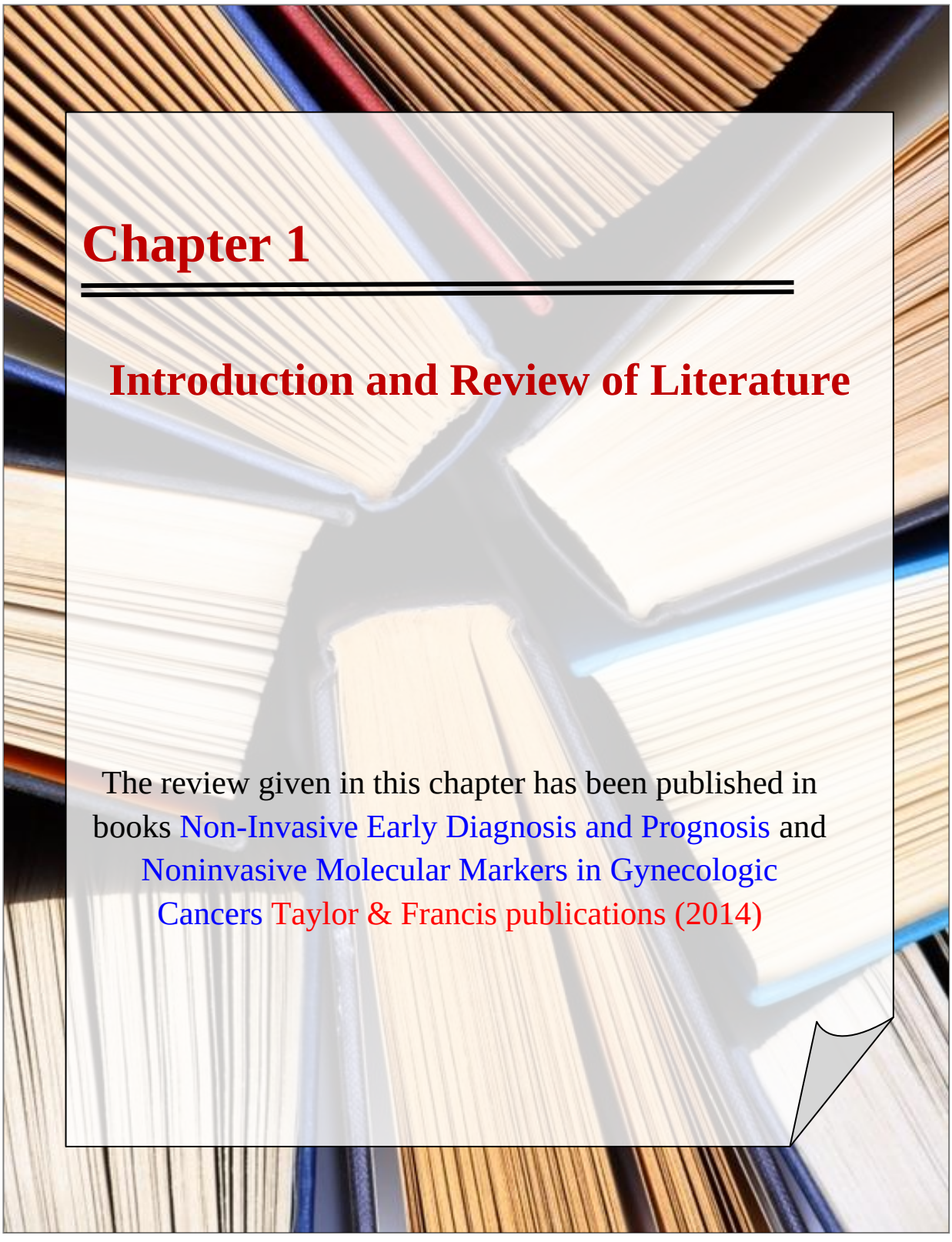
Δy_m	Mitochondrial Membrane Potential
AA	Ascorbic Acid
AAPH	2,2'-azobis-2-methyl-propanimidamide
ACTREC	Advanced Centre for Treatment, Research and Education in Cancer
ADR	Adriamycin
AJCC	American Joint Committee On Cancer
ALB	Albumin
ALP	Alkaline Phosphatase
ALP	Anti-Lipid Peroxidation
ASR	Age-Standardized incidence Rate
AUC	Area Under Curve
B	Basinophil Count
BAX	BCL2 Associated X protein
BCL-2	B-Cell Lymphoma 2
BHT	Butylated Hydroxyl Toluene
BMI	Body Mass Index
BRCA1	Breast cancer type 1 susceptibility protein
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAM	Complementary and Alternative Medicine
COX-2	Cyclooxegenase 2
CREA	Serum Creatinine
CT	Chemotherapy
CUX	CCAAT-displacement protein/cut homeobox (CDP/Cux)
Cy 3	Cyanine 3
DAF-FM	4-Amino-5 Methylamino-2,7 difluorofluorescein
DAPI	4,6-diamidino-2-phenylindole
D-BIL	Direct Bilirubin
DCF-DA	2,7-dichlorofluorescin diacetate
DCIS	Ductal Carcinoma In Situ

DMEM	Dulbecco's Modified Eagles Medium
DMF	Dimethylformamide
dNTP	Deoxynucleotide triphophates
DPPH	1-Diphenyl-2-Picryl Hydrazyl
DTT	Dithiothreitol
E	Eosinophil Count
ECM	Extracellular Matrix
EGF 3	Epidermal Growth Factor-3
ELISA	Enzyme Linked Immunosorbent Assay
EMEA	European Agency for the Evaluation of Medicinal Products
ER	Estrogen
EtOAc	Ethyl Acetate
FA	Formic Acid
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCCP	Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone
FCR	Folin-Ciocalteu's Phenol Reagent
$FeCl_3$	Ferric Chloride
$FeSO_4$	Ferrous Sulphate
FITC	Fluorescein isothiocyanate
FLUO 3/AM	Fluo-3-acetoxymethyl
GAA	Glacial Acetic Acid
Glu	Glucose
Hb	Hemoglobin
HBSS	Hank's Balance Salt Solution
HC9	Polyherbal composition of nine herbs
$HC9_{aq}$	Aqueous extract of HC9
$HC9_{et}$	Ethanolic extract of HC9
HEPES	Hydroxyethyl piperazinethanesulfonic acid
HER2	Epidermal Growth Factor Receptor 2
Hif1-α	Hypoxia Inducing Factor 1 -Alpha
HM	Herbal Medicine
HPTLC	High Performance Thin Layer Chromatography

HRP	Horse Raddish Peroxidase
HRT	Hormone Replacement Therapy
HSIL	High-grade Intraepithelial Lesion
IBC	Inflammatory Breast Cancer
IDC	Invasive Ductal Carcinoma
IDRI	Indian Drug Research Institute
IFN-γ	Interferon-gamma
IL	Interleukin
ILC	Invasive Lobular Carcinoma
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl enzamidazolocarbocyanin iodide
K	Potassium
$K_3Fe[CN]_6$	Potassium Ferricyanide
KCl	Potassium Chloride
L	Lymphocyte Count
LCIS	Lobular Carcinoma In Situ
LR-HPV	Low Risk Human Papilloma Virus
LSIL	Low-grade Squamous Intraepithelial Lesion
M	Monocyte Count
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MeOH	Methanol
MHT	Menopausal Hormone Therapy
MMP-2	Matrix Metalloproteinase-2
MMP-9	Matrix Metalloproteinase-9
MMuLV	Moloney Murine Leukemia Virus
MPCC	Medicinal Plants Conservation Center
MRI	Magnetic Resonance Imaging
MTT	4,5-dimethylthiazol-2-yl-2,5-diphenylthiazolium bromide
NA	Sodium
n-but	N-Butanol
NCCAM	National Center for Complementary and Alternative Medicine

NCCS	National Centre for Cell Science
NEDD	Napthyl Ethylene Diamine Dihydrochloride
NF-kB	Nuclear Factor kappa-B
NK	Natural Killer
NO	Nitric Oxide
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
ORAC	Oxygen Radical Absorbance Capacity
P	Platelet Counts
p16	P^{16ink4}
p21	$P21^{waf1/Cip1}$
PARP	Poly ADP-Ribose Polymerase
PBMC	Peripheral Blood Monocyte Cells
PBS	Phosphate Buffered Saline
PCNA	Poliferating Cell Nuclear Antigen
PCV	Packed Cell Volume
PHFs	polyherbal formulations
PHT	Post-Menopausal Hormone Therapy
PMSF	Phenylmethanesulfonyl fluoride
PR	Progestrone
Rb	Retinoblastoma Tumor Suppressor Protein
RNOS	Reactive Nitrogen and Oxygen Species
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Radiotherapy
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S.G.	Specific Gravity
SATB1	Special AT-Rich Sequence Binding Protein 1
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SES	Socioeconomic Status
SGOT	Serum Glutamate Oxaloacetete Trasaminase
SGPT	Serum Glutamate Pyruvate Transaminase

SMAR-1	Scaffold/Matrix Attachment Region Binding Protein 1
SNP	Sodium Nitroprusside
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
T-BIL	Total Bilirubin
TCA	Trichloroacetic Acid
TCOL	Total Cholesterol
TG	Triglycerides
TIMP	Tissue Inhibitor of Metalloproteinase
TP	Total Protein
UREA	Blood Urea
USP	United States Pharmacopeia
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization



Chapter 1

Introduction and Review of Literature

The review given in this chapter has been published in books Non-Invasive Early Diagnosis and Prognosis and Noninvasive Molecular Markers in Gynecologic Cancers Taylor & Francis publications (2014)

SECTION 1

An Overview of Breast Cancer

1.1.Background of breast cancer

Breast cancer is a major health burden in women worldwide (Ferlay J et al., 2010, National NCI program database, Patnaik JL *et al*., 2011). It is a leading cause of cancer related death for women aged between 35 and 55 years worldwide (Parkin et al., 2005). Heterogeneity, aggressive behavior and multi-factorial etiology are some of the important factors that impose a great clinical challenge in the management of breast cancer (Jensen A *et al*., 2008). Significant advances in the prevention, diagnosis and management of breast cancer have been made in recent years (Wiechmann L *et al.,* 2008; Harnett A et al., 2009). However, breast cancer remains a complex disease process and invites attention for further advances in scientific knowledge and clinical care to improve the lives of patients (Lemieux J et al., 2008).

1.1.2. Epidemiology of breast cancer in world

Breast cancer is the most common malignancy observed in women in both highly developed and developing countries and comprises around 18% of all the female cancers (Patnaik JL et al., 2011) (Figure 1.1). The incidence rates of breast cancer have tremendously increased in the last two decades (American Cancer Society, 2012; Coughlin SS et al., 2009). Incidence and mortality rates vary internationally by more than 5-folds (Key TJ et al 2001). The number of cases have rapidly increased from 641,000 cases in 1980 to 1,643,000 cases in 2010, with an annual rate of increase of around 3.1% (Forouzanfar MH et al., 2011). The incidence rates are higher in Western and Northern Europe, Australia/New Zealand and North America; moderate in South America, the Caribbean and Northern Africa; and lower in sub-Saharan Africa and Asia (Jemal A *et al.,* 2011) (Table 1.1). The age-standardized incidence is highest in North America and lowest in central Africa with

around 99.4 and 16.5 cases per 100,000 cases respectively (Boyle P et al., 2010). However, incidence rates are rising rapidly in most of the low-and middle-income countries (LMCs) compared to the developed countries where incidence rates are already high (Boyle P et al 2010). The mortality rate due to breast cancer has been estimated to be around 1,30,000 per year in females (Kohrmann A *et al*, 2009). This indicates that a disease once called as 'a disease of the western world' has been termed as a 'global health challenge'. Hence the commitment to cure it should also be a global effort (Formenti SC et al 2012).

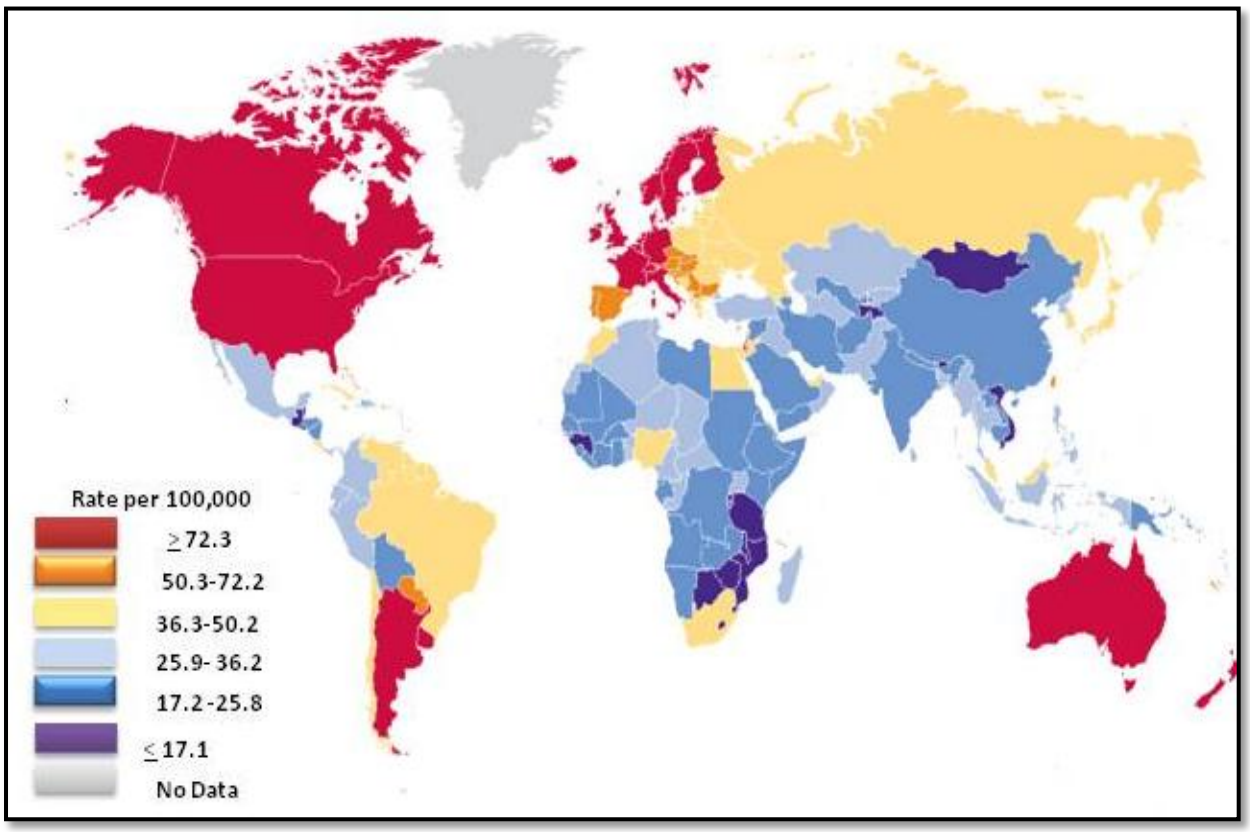


Figure 1.1. International epidemiological variation in breast cancer cases

Source: GLOBOCAN 2008

Table 1.1: World key statistics of breast cancer (Source: GLOBOCAN 2008**)**

Regions	Incidence			Mortality		
	Cases (1000)	* ASR per (100,000)	*Cum. Risk (%) (Age 0–74)	Cases (1000)	*ASR per (100,000)	*Cum. Risk (%) (Age 0–74)
World	1383.5	39	4.1	458.4	12.5	1.3
More developed regions	692.2	66.4	7.1	189.5	15.3	1.7
Less developed regions	691.3	27.3	2.8	269	10.8	1.2
Eastern Africa	17.9	19.3	2.1	10	11.4	1.3
Middle Africa	8.3	21.3	2.1	4.7	13.1	1.4
Northern Africa	28	32.7	3.2	14.6	17.8	1.8
Southern Africa	9	38.1	4.2	4.5	19.3	2.1
Western Africa	29.4	31.8	3.4	16.3	19	2.1
Caribbean	9	39.1	4.3	3.4	14.2	1.6
Central America	17.5	26	2.8	6.5	9.6	1
South America	88.4	44.3	4.8	27.1	13.2	1.4
Northern America	205.5	76.7	8.4	45.6	14.8	1.6
Eastern Asia	240.3	25.3	2.6	61.7	6.3	0.7
South-Eastern Asia	87	31	3.2	36.8	13.4	1.4
South-Central Asia	173	24	2.5	82.6	12	1.3

Continued…`

Regions	Incidence			Mortality		
	Cases (1000)	* ASR per (100,000)	*Cum. Risk (%) (Age 0–74)	Cases (1000)	*ASR per (100,000)	*Cum. Risk (%) (Age 0–74)
Western Asia	28.5	32.5	3.4	12.3	14.3	1.5
Central and Eastern Europe	114.6	45.3	5	47.5	17	1.9
Northern Europe	69.5	84	9	18.3	17.8	1.9
Southern Europe	91.3	69	7.4	25.6	15.3	1.7
Western Europe	149.4	90	9.6	37.3	17.5	1.9
Australia/ New Zealand	16.1	85.5	9.4	3.4	15.4	1.7
Melanesia	0.6	22.8	2.4	0.3	13.2	1.4
Micronesia/ Polynesia	0.3	58	6.1	0.1	13.2	1.5
India	115	22.9	-	53	11.1	-

1.1.3. Epidemiology of breast cancer in India

Breast cancer ranks at first position among Indian women (Globacan 2012). It accounts for around 7% of global cancer burden and one-fifth of all the cancers in India (Figure 1.2) (Khokhar A 2012). There is a remarkable variation in the incidence and mortality rates of breast cancer observed in metropolitan, urban and rural Indian women (Agarwal G et al 2008). It is the most common cancer in urban females and ranks second in the rural population. Incidence rates are higher in metropolitan areas, modest in urban areas and minimal in rural areas. There is a three-fold rise in incidence rate in metropolitan women compared to the rural population (Agarwal G et al 2008). It is estimated that around 90,000 of the total cancer cases and 50,000 of the total cancer related deaths occurred in women due to the disease every year in India (Saxena S et al 2005). The number of cases have rapidly increased from 80,000 in 2005 to 1,22,000 new cases in 2011and it would reach up to 1,41,000 cases by 2016 (Badwe RA et al 2011). The age-standardized incidence rates (ASR) range from around 6.2 to 39.5 per 100,000 Indian women, which is much lower than in developed countries (Boyle P et al 2010; Datta K et al 2012). ASR varies from region, ethnicity as well as religion (Agarwal G et al 2008). Breast cancer burden is lower in the Indian women below 40 years of age; moderate in women between 40 and 50 years of age; and highest in women older than 50 year age (Chauhan A et al 2011). Many features of urbanized lifestyle are responsible for this rapid increase in the incidence of breast cancer (Takiar R et al 2010).

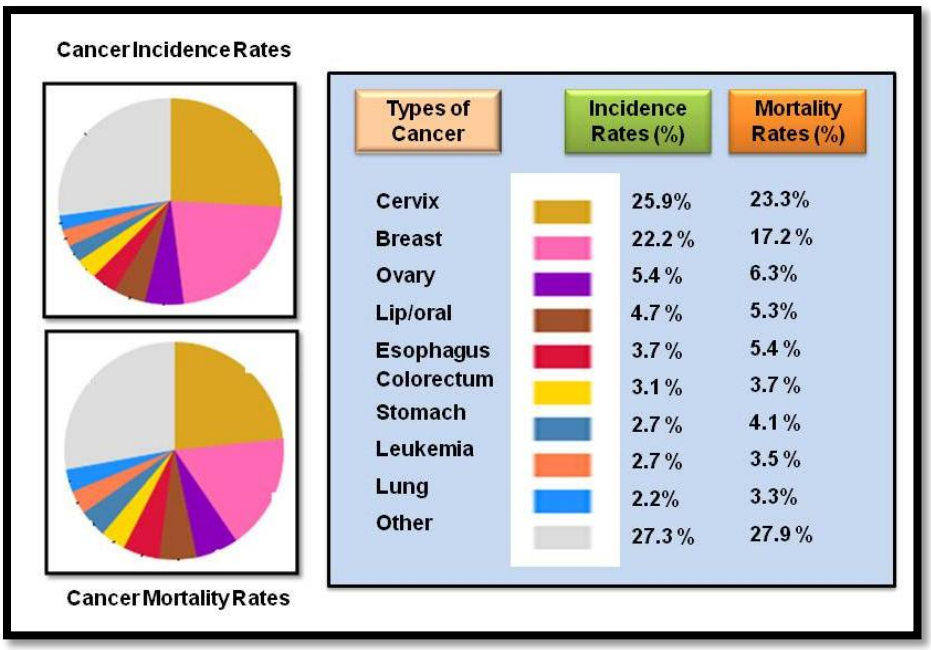


Figure 1.2. Epidemiology of breast cancer in India. (Source: Current Scenario of Breast Cancer: An Overview. Suryavanshi S et al.,2013).

1.1.4. Etiology of the breast cancer

Breast cancer has multi-factorial etiology (Jensen A *et al*., 2008). The most important modifiable and non-modifiable risk factors include age, race, ethnicity, gender, family history as well as environmental, hormonal, dietary, lifestyle and genetic factors (Figure 1.3) (MacMahon B *et al.,* 2006). Older age has been linked to the cancer development wherein 1 out of 8 invasive breast cancers are found in women younger than 45, while about 2 out of 3 invasive breast cancers are found in women around 55 years of age or older (Harrison PA *et al.,* 2010). Socioeconomic status (SES) has also been found to determine the risk of breast cancer. Unlike other cancers, the risk of breast cancer development has been shown to be positively associated with the higher SES (Inumaru LE *et al.,* 2012). Around 5-10% of breast cancer cases have been attributed to genetic mutations (Stoppa-Lyonnet D *et al.,* 2009). For example, inherited mutations in BRCA1 and BRCA2 genes are the most

common hereditary cause of breast cancer (Pijpe A *et al.*, 2012). Obesity is also considered to be one of the main risk factors and is positively associated with postmenopausal women (Sinicrope FA *et al.,* 2010) as it may raise the estrogen levels. Exposure to radiations and mutagenic agents (Ronckers CM *et al.*, 2005), use of oral contraceptives, post-menopausal hormone therapy (PHT), hormone replacement therapy (HRT) and menopausal hormone therapy (MHT) are some of the reasons that may be responsible for the rise in breast cancer incidence rates (Norman SA *et al.,* 2003). Besides these, alcohol consumption and cigarette smoking are other life style factors that may also increase the cancer risk (Chen WY *et al.,* 2011). All these factors may disturb the cellular signaling pathways resulting into altered molecular mechanisms leading to carcinogenesis (Nguyen PL. *et al.*, 2008; Marotta LL *et al.*, 2011).

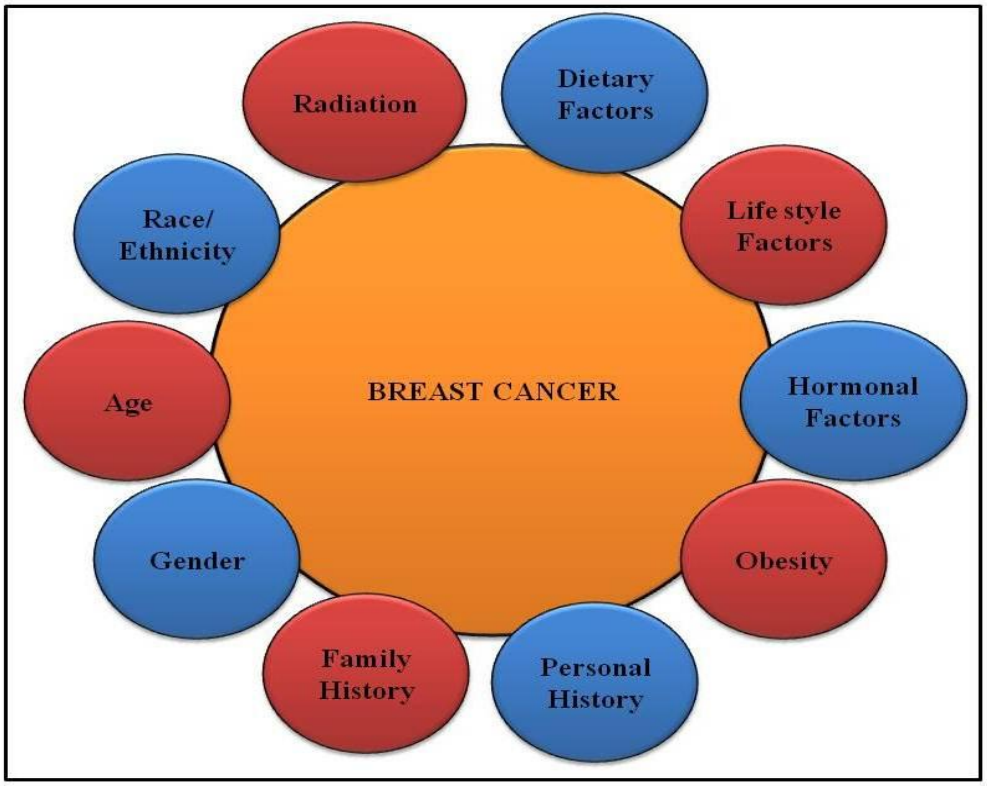


Figure 1.3. Probable Risk factors for breast cancer development. (Source: Cancer Biomarkers: Minimal and Noninvasive Early Diagnosis and Prognosis, Suryavanshi S et al., 2014).

1.1.5. Types of breast cancer

Around 95% of the breast carcinomas begin in the epithelial cells of the breast whereas very rare cases of breast sarcomas are observed in connective tissues such as muscle tissue, fat tissue, or blood vessels (Richie RC et al 2003). Breast cancer may be invasive or non-invasive depending upon the type and the stage of the disease (Souzaki M *et al.*, 2011) (Figure 1.4). It has been divided into ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), non-invasive lobular carcinoma (lobular carcinoma in situ) and invasive lobular carcinoma (ILC) (Muggerud AA *et al.*, 2010; Hanby AM *et al.*, 2008). These in situ carcinomas remain confined at a place, with no invasion of the underlying basement membrane into the surrounding breast tissue

(Suryadevara A *et al.*, 2010). Such type of localized and confined malignancy has negligible potential for metastases (Muggerud AA *et al.*, 2010).

When the ductal or lobular malignancy breaks all the way through the wall of the duct or lobule into the fatty tissue of the breast and invades to other parts of the body, the malignancy is considered as invasive (or infiltrating) ductal or lobular carcinoma (Suryadevara A *et al.*, 2010). The potential for metastases is very high in invasive disease that ultimately leads to the mortality (Richie RC *et al.*, 2003). Besides the above, there are additional types that include inflammatory breast cancer (IBC), Phyllodes tumor, angiosarcoma, Paget's disease of the nipple and triple-negative breast cancer (Figure 1.5) (Yang R *et al.*, 2009, Glazebrook KN *et al.*, 2008, Dalberg K *et al*., 2008).

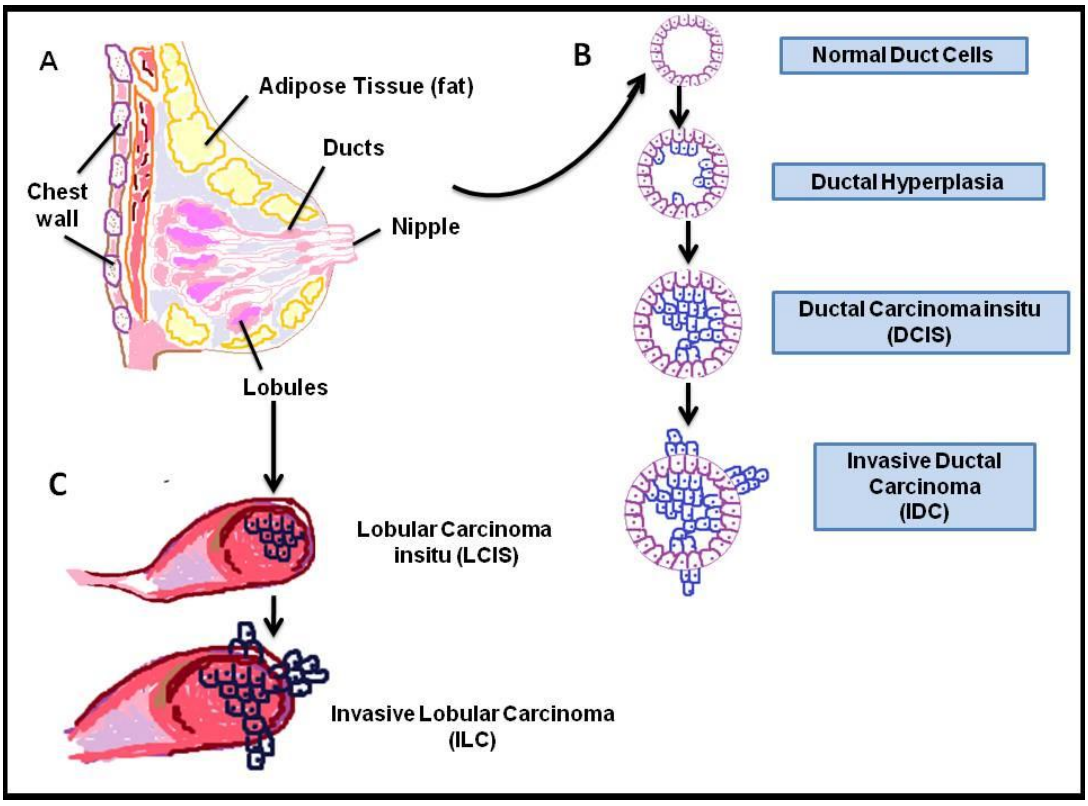


Figure 1.4. Anatomy of normal breast along with different types of breast cancer: (A) Anatomy of normal breast. *Anatomy of breast showing ducts, lobules, nipple, adipose tissue and chest wall* ***(B) Development of Ductal carcinoma****: Hyperplasia is a pre-cancerous*

condition that describes an accumulation of abnormal cells in a normal breast duct and is associated with an increased risk of developing cancer. In atypical hyperplasia, cells keep dividing and become more abnormal, then the condition may be reclassified as noninvasive breast cancer [ductal carcinoma in situ (DCIS)]. When the abnormal cells tend to outgrow and start migrating to the nearby organs, the condition may be classified as invasive ductal carcinoma (IDC). ***(C) Development of Lobular carcinoma:*** *If the cells start proliferating abnormally and tend to accumulate inside the lobules, but do not spread to the other tissues, the condition is known as lobular carcinoma in situ (LCIS). If LCIS cells tend to outgrow and metastasize to the nearby organs, then the condition is known as invasive lobular carcinoma (ILC).* (Source: Cancer Biomarkers: Minimal and Noninvasive Early Diagnosis and Prognosis, Suryavanshi S et al., 2014).

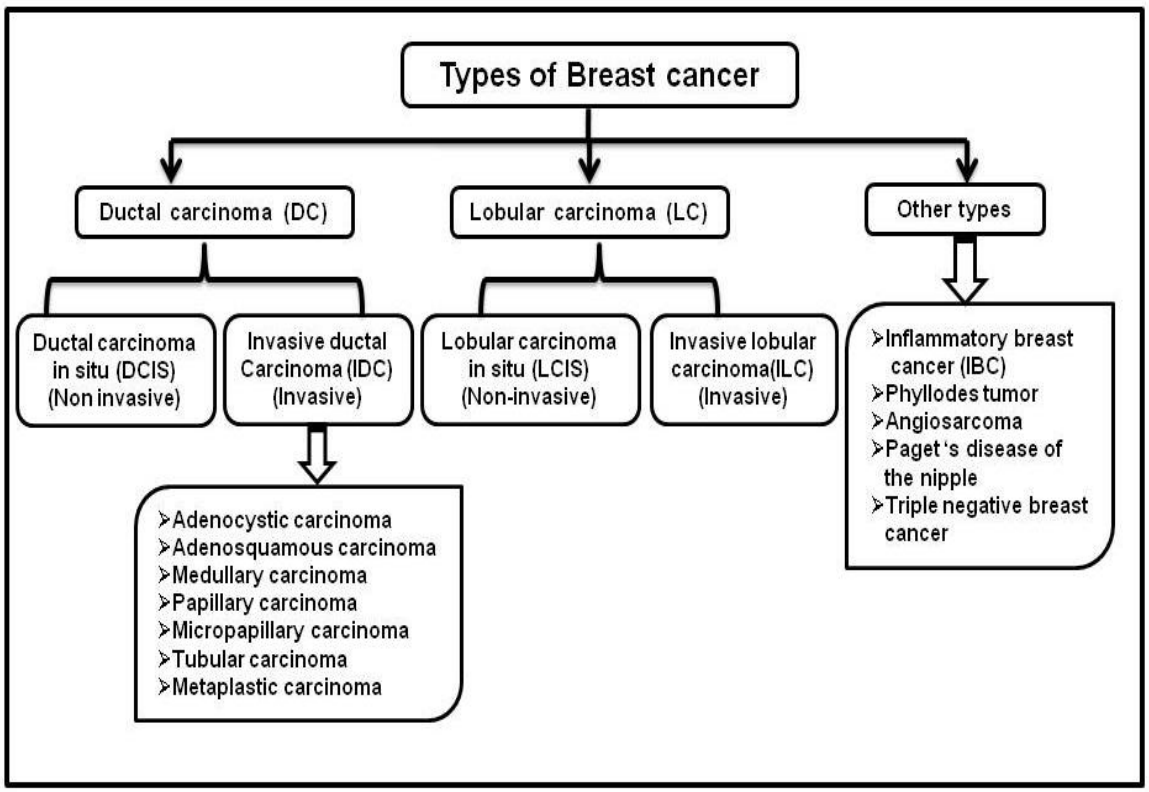


Figure 1.5. Types of breast cancer*: Breast cancer is broadly classified into two types such as ductal carcinoma (DC) and lobular carcinoma (LC). DC is divided into ductal carcinoma in situ (DCIS) (non-invasive) and invasive ductal carcinoma (IDC), the latter being divided into adenocystic, adenosquamous, medullary, mucinous, papillary, micropapillary, tubular and metaplastic carcinoma. LC is further grouped into non-invasive or lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC). However, there are some other types that include inflammatory breast cancer (IBC), Phyllodes tumor, Angiosarcoma, Paget's disease*

of the nipple and triple-negative breast cancer. (Source: Cancer Biomarkers: Minimal and Noninvasive Early Diagnosis and Prognosis, Suryavanshi S et al., 2014).

1.1.6. Breast cancer TNM staging

The staging of any cancer describes upon the extent of its spread in the body and helps in better prognosis and treatment, thereby increasing the chances of survival in the patients. Stages can be decided by the tumor size (less than 2 cm, or between 2-5 cm or more than 5 cm), lymph node involvement as well as by invasive or non-invasiveness of the tumor (Abeloff MD *et al.*, 2008).

The American Joint Committee on Cancer (AJCC) has designated TNM system ('T' stands for tumor; 'N' for node and 'M' for metastasis) for staging the cancer and has categorized the breast tumors into stage 0, I, IIA, IIB, IIIA, IIIB and IV (Table 1.2) (Singletary SE *et al.*, 2002). Stage 0 is non-invasive whereas I-IV are invasive stages of the breast cancer. Table 1.2 describes different stages along with 5 year survival of breast cancer patients.

Table 1.2: Breast cancer stages (Source: Cancer Biomarkers: Minimal and Noninvasive Early Diagnosis and Prognosis, Suryavanshi S et al., 2014).

Stage	TNM	Description	5-year Survival
0	Tis N0 M0	No tumor in regional lymph nodes, no distant metastases	99%
I	T1 N0 M0	Tumor is less than or equal to 2 centimeters, no spread of tumor to regional lymph nodes, no distant metastases	92%
IIA	T0 N1 M0	Tumor is smaller than 2 cm with micrometastases in 1 to 3 axillary lymph nodes, no distant metastases	82%
	T1 N1 M0	Tumor is less than or equal to 2 centimeters, metastases to movable ipsilateral nodes, no distant metastases	
	T2 N0 M0	Tumor is between 2 and 5 centimeters, no tumor is regional lymph nodes, no distant metastases	

IIB	T2 N1 M0	Tumor is between 2 and 5 centimeters, metastases to movable ipsilateral nodes, no distant metastases	65%
	T3 N0 M0	Tumor is over 5 centimeters, no tumor is regional lymph nodes, no distant metastases	
IIIA	T0 N2 M0	Tumor is less than or equal to 2 centimeters, metastases to 4 to 9 axillary lymph nodes, no distant metastases	47%
	T1 N2 M0	Tumor is between 2 and 5 centimeters, metastases to 4 to 9 axillary lymph nodes, no distant metastases	
	T2 N2 M0	Tumor is over 5 centimeters, metastases to movable ipsilateral nodes, no distant metastases	
	T3N2M0	Tumor is over 5 centimeters, metastases to 4 to 9 axillary lymph nodes, no distant metastases	
IIIB	T4 Any N M0	Tumor of any size growing into the chest wall or skin, any nodal involvement, no distant metastases	44%
	Any T N3 M0	Any primary tumor involvement, metastases to more than 10 axillary lymph nodes, no distant metastases	
IV	Any T Any N M1	Any primary tumor involvement, any nodal involvement, distant metastases	14%

1.1.7. Signs and symptoms of breast cancer

There are typically no signs or symptoms observed in breast cancer patients when the tumor is very small at an early stage (www.nationalbreastcancer.org). Patients with large tumors have painless, hard mass with the irregular edges. Most of the breast cancers can be tender, soft, or rounded and in some cases they may be painful. Other possible less common signs and symptoms of the breast cancer include swelling of all or part of a breast, skin irritation or dimpling, redness, scaliness, or thickening, distortion, tenderness of the breast, nipple pain, nipple retraction, discharge and ulceration (Hussain AN et al 2006). Thus, screening mammography is recommended for every symptomatic or non-symptomatic woman who is at more risk for developing breast cancer (Barlow WE et al 2002; Montazeri A et al 2008).

1.1.8. Current methods for early diagnosis, prognosis and therapy

Early detection has become easy with advances in screening techniques that include routine mammography programs and/or palpation (either self-examination or by physician or nurse practitioner), digital mammography, sonogram, thermography, transillumination, xeromammograpy, CT scan, magnetic resonance imaging (MRI), ultrasound imaging, radionuclide imaging, positron emission tomography (PET-CT), 99mTc-sestamibi scintimammography, electrical impedance tomography (EIT), biopsy as well as genetic testing (Lehtimaki T et al., 2011; Vaughan CL et al., 2012). Genetic testing is recommended for women having a strong family history of breast or ovarian cancer. (Nelson HD et al., 2005; Palma M et al., 2006; Mackay J et al., 2010). Breast cancers detected by screening mammography have more favourable prognostic characteristics than cancers detected by other methods (Kobeissi L et al., 2011; McCann J et al., 2002). These techniques enhance the radiologist's ability to detect cancer and assess the disease extent, which is crucial in treatment planning and staging (Prasad SN et al 2007; LingLing Pan et al 2010). Despite the available screening facilities, diagnosis of the breast cancer remains inadequate due to the low sensitivity/specificity, relative complexity and high cost-to-benefit ratio (Prasad SN et al., 2007; Heiko Schoder et al., 2007).

During the past few years, biomarkers have gained significant importance in the diagnosis of many diseases. Prognostic and predictive biomarkers such as ER, PR, HER2, p53, BRCA1/2 as well as many others are currently being used for the early diagnosis of breast cancer (Cianfrocca M et al., 2004). Table 1.3 enlists various imaging methods that are being used in the breast cancer detection along with their sensitivity, specificity as well as advantages and disadvantages.

Table 1.3: List of imaging tools along with their sensitivity, specificity as well as advantages and disadvantages (Source: Cancer Biomarkers: Minimal and Noninvasive Early Diagnosis and Prognosis, Suryavanshi S et al., 2014).

Imaging tools	Sensitivity	Specificity	Advantages	Disadvantages	References
Screening mammography by X-ray	90%	75%	Mortalityreduction; improved treatment of early disease; low cost; minimal discomfort	Radiation risk and other risks; risk of false alarm	Gotzsche PC *et al.* 2011
Ultra-sound by sound waves	89 %	78 %	Ultrasound pictures may show whether a lump is solid or filled with fluid; useful for detecting cancer in women at higher risk	Associated with false positive and false negative results; not widely available or routinely used	Ingram I *et al.* 2012
CT. Scan by Computerized Tomography	-	-	Best way to image internal mammary nodes and to evaluate the chest and axilla after mastectomy	Radiation exposure in CT scan can accumulate and can increase the risk of developing cancer; risk of allergic reaction due to dye administration	Pettrigrew P *et al.* 2009
PET Scan by radioactive material	75%	92.3%	Diagnose disease even before the structural changes are visible; provides both an anatomical and functional view of the suspected cells	It does not reliably detect tumors smaller than 5–10 mm	Stephan KD 2010
MRI by magnetic fields	90%	72%	MRI is more sensitive; identifies the primary site of cancer in the breast; high sensitivity; non-invasive; usually painless medical test	False positive results; more expensive than mammography	Houssami *et al.* 2009

1.1.9. Treatment strategy for breast cancer

There are several treatment options available to cure or improve the survival and quality of the patients diagnosed with breast cancer that includes surgery, chemotherapy, radiation therapy, hormonal therapy and targeted therapies (Perez EA et al., 2011; Howard JH et al., 2012) (Figure 1.6). The most appropriate treatment is given to the patient either alone or in combination depending upon the woman's risk profile and stage of the disease. It is based on the tumor size, location, involvement of lymph nodes and whether or not the tumor has spread to the surrounding tissue or distant organs (Naeim A et al., 2010). Although these treatments are very effective but they are associated with side effects (Azim HA et al., 2011). Table 1.4 describes various treatment options available for breast cancer along with their advantages and side effects.

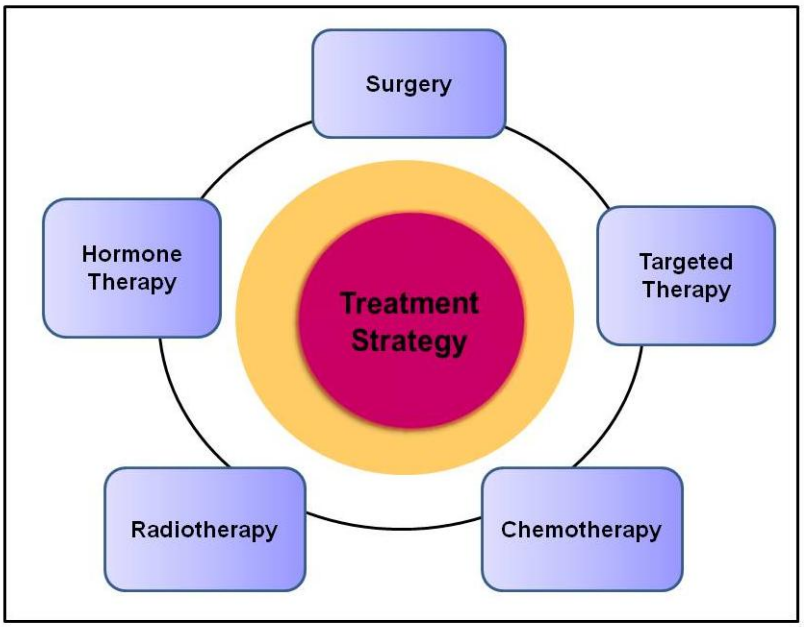


Figure 1.6. Treatment strategies for breast cancer. (Source: Current Scenario of Breast Cancer: An Overview, Suryavanshi S et al.,2013).

Table 1.4: Different treatment options for breast cancer with its advantages and side effects (Source: Cancer Biomarkers: Minimal and Noninvasive Early Diagnosis and Prognosis, Suryavanshi S et al., 2014)

Treatments	Sub types	Description	Side effects
Surgery	Lumpectomy Quadrantectomy Mastectomy	Removal of the lump in the breast and some of the tissue around it or whole breast along with some of the lymph nodes under the arm during the surgery.	Pain, temporary swelling, tenderness and hard scar tissue that forms in the surgical site, post-surgical pain, wound infection, hematoma (buildup of blood in the wound), as well as seroma (buildup of clear fluid in the wound)
Radiation therapy	External beam radiotherapy Brachytherapy	This treatment uses high-energy x-rays to kill cancer cells and shrink tumors	Swelling and heaviness in the breast, sunburn-like skin changes in the treated area and fatigue
Chemotherapy	Adjuvant chemotherapy Neoadjuvant chemotherapy	Chemotherapy is the use of special drugs to damage or kill cancer cells	Hair loss , mouth sores , loss of appetite, nausea and vomiting ,low blood cell counts , menstrual changes, neuropathy, heart damage, increased risk of leukemia, increased chance of infections, bleeding, fatigue
Hormone therapy	-	It is used to block hormones in the body that might helpcancers grow	Fatigue, hot flashes, vaginal dryness or discharge, and mood swings
Targeted therapy	Inhibition of ER/HER2/PARP/ILGF/PI3K/Akt/mTOR signaling pathway	Targeted therapy uses drugs to identify and attack specific markers on cancer cells	Mouth sores, diarrhea, nausea, fatigue, low blood counts, shortness of breath, cough, increased levels of blood lipids and blood sugars, liver problems or a decrease in heart function, fetal harm

SECTION 2

Complementary and Alternative therapies

1.2.1. Complementary and Alternative therapies for breast cancer

The use of Complementary and Alternative Medicines (CAM) is an extremely promising approach to block the development of cancer (Wang HH et al 2012). It is becoming increasingly popular among the breast cancer patients throughout the world (Baum M et al 2006). It has been estimated that the use of CAM in cancer patients varied from 7-64% with higher use (47-83%) in breast cancer patients (Helyer et al., 2006). The objectives of CAM therapies are diverse that include the reduction of toxicity and other side-effects associated with the chemotherapy; improvement of cancer-related symptoms; improving the survival and quality of life of the patients and modulating the immune system. Such therapies help in rapid recovery as well as improvement in general and emotional health of the patients (Bell RM et al., 2009; Wanchai A et al., 2010; Wang HH et al., 2012; Puataweepong P et al 2012).

According to the National Center for Complementary and Alternative Medicine (NCCAM), there are five treatment approaches utilized in CAM therapies that include whole medical systems; mind-body medicine; biologically based practices; manipulative and body-based practices; and energy medicine (NCCAM, 2011) (Table 1.5).

Table 1.5: Various domains of the Complementary and Alternative medicine (CAM) therapies (Source: Current Scenario of Breast Cancer: An Overview, Suryavanshi S et al., 2013).

Various domains of CAM	Description	Therapies used
Whole medical systems	These include culturally based healing traditions such as traditional Chinese medicine and Ayurveda as well as western medical systems such as Homeopathy and Naturopathy	Herbs, massage, yoga and acupuncture
Mind-body medicine	These therapies are based on the spiritual healing that involve the mind's capacity to influence the body's functions	Prayer, hypnotherapy, meditation, imagery, visualization, music therapy, and art therapy.
Biologically based practices	These therapies include nutritional factors that alter the body mechanisms and pathways	Dietary supplements, vitamins, and herbal medicines
Manipulative and body-based practices	These therapies are used for the relaxation of the body	Massage therapy as well as chiropractic and osteopathic manipulation
Energy medicines	These therapies are based on the energy fields that surround and permeate the body	Reiki and Qigong

1.2.2. Herbs and herbal formulations as anticancer drugs

Among various CAM therapies, a great emphasis has been given towards the research on traditional and herbal medicines as main sources of chemopreventive drugs (Stefania N et al 2009). Recently, the medicinal and economic benefits of these plants have been increasingly accepted in both developing and industrialized nations (Tan W et al 2011). About 60% of currently used anti-cancer drugs are derived from natural sources such as plants, marine organisms and microorganisms (Wang S et al.,

2009; Bin Liu et al., 2010; Baeshen NA et al., 2012). A wide variety of naturally occurring substances from plant food have been shown to offer protection from carcinogenic exposure. Various phytochemicals have been identified in fruits, vegetables, spices, and grains that exhibit chemopreventive potential (Kinghorn AD et al 2009; Tan W et al 2011). Plants have played a significant role as a source of effective anticancer agents (Jain R et al 2010; Pandey G et al 2009). Herbal drugs and their active ingredients have efficiency to regulate the molecular mechanisms and various signaling pathways involved in carcinogenesis (Cragg GM et al 2005). Therefore, compounds derived from the herbs and herbal formulations are of considerable interest to oncologists. The active phytochemicals that have been isolated from the plants include curcumin (turmeric), resveratrol (red grapes, pea-nuts and berries), genistein (soybean), diallyl sulfide (allium),S-allyl cysteine (allium), allicin (garlic), lycopene (tomato), capsaicin (red chilli), diosgenin (fenugreek), 6-gingerol (ginger), ellagic acid (pomegranate), ursolic acid (apple, pears, prunes), silymarin (milk thistle), anethol (anise, camphor, and fennel), catechins (green tea), eugenol (cloves), indole-3-carbinol (cruciferous vegetables), limonene (citrus fruits), beta carotene (carrots), and dietary fiber. These phytocompounds have the potential to inhibit various cell signaling pathways and regulate the molecular mechanisms such as oxidation, inflammation, apoptosis, cell proliferation, cell cycle, invasion, metastasis and angiogenesis (Gosslau A et al 2004; Shishodia S et al 2004; Kim GY et al 2004; Lee SH et al 2005; Aggarwal BB 2006; Yazdanparast R et al 2007; Lemieux Jet al 2008; Anand P et al 2008; Tan W et al 2011; Elkady AI et al 2012).

Herbal remedies with either single herb or polyherbal formulations are playing prime role in the healthcare system because of their wide biological activities, easy accessibility, cost effectiveness and safety (Gatkal S et al., 2012; Osemene KP et al.,

2011; Bent S et al., 2008). The polyherbal formulations yield better outcomes than single herbs, as it takes advantage of synergy and interaction between numerous phytochemicals present in the component herbs (Mukherjee PK et al, 2011). Since herbal medicines are not toxic to the normal cells at therapeutic doses, they provide a new ray of hope for cancer patients (Sagar SM et al., 2005).

SECTION 3

Structure and Organization of Thesis

1.3.1. Rationale of using polyherbal composition, HC9, for studying its anticancer activity in breast cancer cell lines

"Stanyashodhana Kashaya" (SSK) is an Ayurvedic formulation that has been prescribed for treatment of various disorders of mammary gland, specifically in detoxification of breast milk and lactation-related problems (Charak Samhita, Chapter 22). It is a balanced mixture of 10 different medicinal plants that include *Picrorhiza kurroa* (Kutaki), *Cyperus rotundus* (Musta), *Zingiber officinale* (Sunthi), *Cedrus deodara (*Devdar), *Tinospora cordifolia* (Gulvel), *Holarrhena antidysenterica* (Indrajav), *Swertia chirata* (Kadechirai), *Cyclea peltata* (Patha), *Hemidesmus indicus* (Anantmul) and *Marsdenia tenacissima* (Murva). Based on SSK, we prepared a polyherbal formulation made up of only nine different medicinal plants because *Marsdenia tenacissima* (Murva), the tenth plant material, which is found only in Himalayan region, was not available. The other remaining plant materials were readily available. Since each component of HC9 has known anticancer activity, we hypothesized that HC9 as a whole formulation would exhibit anticancer activity against breast cancer. In the present work, we have standardized HC9 formulation, assessed its quality, evaluated its safety in rodents and studied its antineoplastic potential in breast cancer cell lines by elucidating the underlying mechanisms. We have also evaluated anticancer activity of HC9 *in vivo* in mouse melanoma C57BL/6 model.

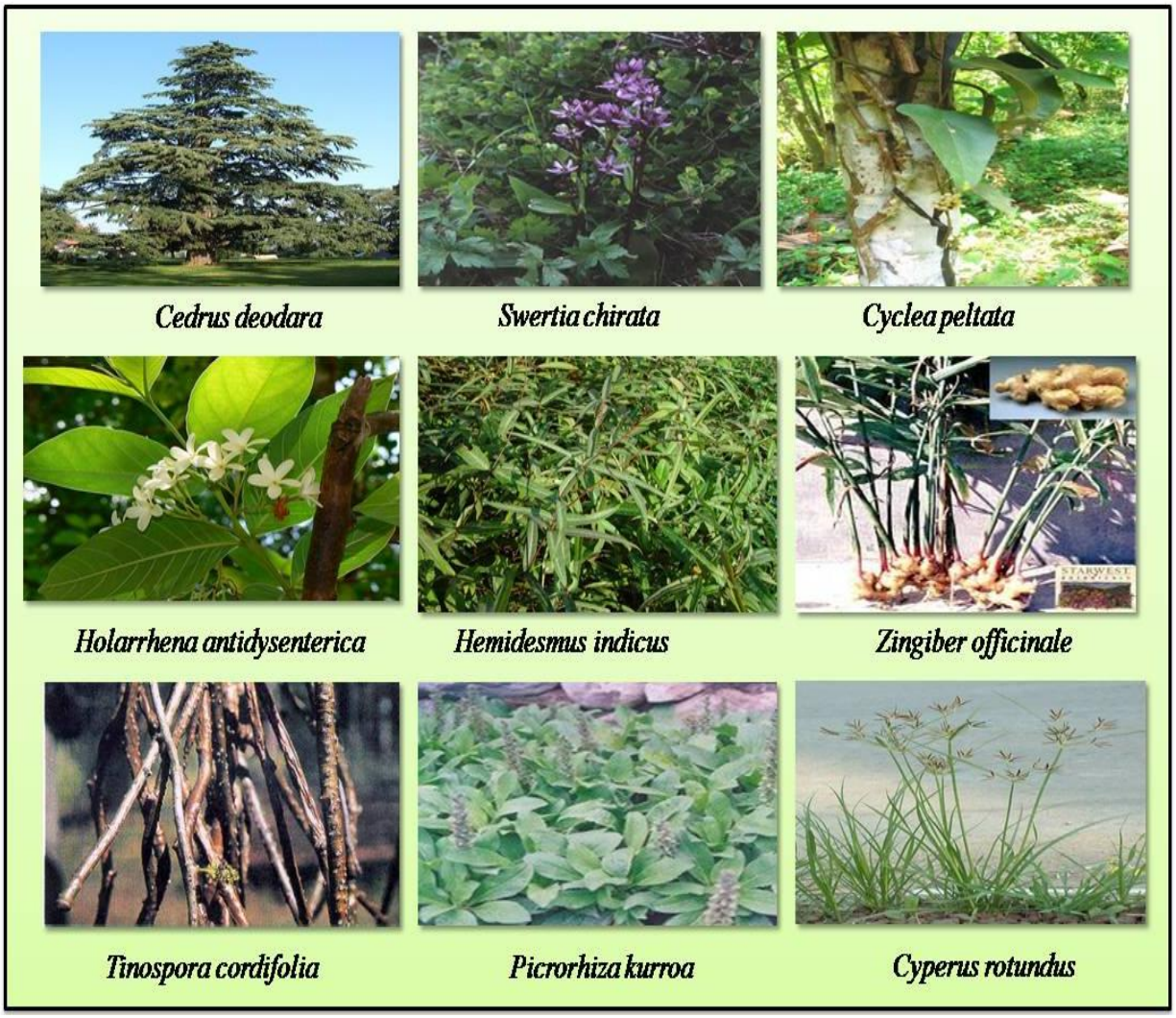


Figure 1.7: Medicinal plants present in HC9

Table 1.6: Reported activity of individual plants in HC9

Common names	Botanical names	Family	Parts used	Reported anticancer activity	Medicinal properties
Kutaki	*Picrorhiza kurroa*	Plantaginaceae	Root	Breast and skin cancer. Protect against Adrynomycin induced cardiomyopathy	Antioxidant, immunomodulatory, antibacterial, antiperiodic, hepatoprotective, antiasthamatic, gastrointestinal, anti-urinary activity
Nagarmotha	*Cyperus rotundus*	Cyperaceae	Rhizome	Gastric cancer, lymphoma, leukemia, cytotoxic and apoptotic role	Appetizer, antioxidant, immunomodulatory, anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, high estrogens reducer, breast pain inhibitory activity
Sunthi	*Zingiber officinale*	Zingiberaceae	Bark	Breast, cervical, colon, lung, ovary, prostate cancer. Protect against Doxorubicin induced acute nephrotoxicity	Antioxidant, appetizer, anti-inflammatory, antiangiogenic, cardiotonic, antiplatelet, antihepatotoxic, antifungal activity
Devdar	*Cedrus deodara*	Pinaceae	Root	Breast, cervical, skin, leukemia, colon, lung and ovary cancer	Anti-inflammatory, immunomodulator, anti-ulcer, anti-fungal, anti-arthritic, anti-allergic, anti-oxidant activity

Gulvel	*Tinospora cordifolia*	Menispermaceae	Stem	Prostate, liver, skin and breast cancer. Protect against chemo induced leucopenia	Immunomodulator, antioxidant, anti-inflammatory, anti-stress, gastrointestinal and hepatoprotection, anti-allergic activity
Indrajav	*Holarrhena antidysenterica*	Apocynaceae	Seed	Anticancer	Appetizer, anti-diabetic, diarrhea, anti-oxidant activity
Kadechirai	*Swertia chirata*	Gentianaceae	Whole plant	Skin cancer	Blood purifier, appetizer, Antioxidant, , anti-inflammatory, immunomodulator, anti-hepatotoxic, antidiabetic, antimicrobial activity
Patha	*Cissampelos pareira*	Menispermaceae	Root	Lung, leukemia, lymphoma	Antioxidant, immunomodulator, anti-inflamatory activity, skin diseases, gastric ulcers, cardiac and abdominal pain reducer
Anantmul	*Hemidesmus indicus*	Apocynaceae	Root	Hepatocancer	Blood purifier, appetizer, antioxidant , anti-inflammatory, anti-microbial, anti-hepatotoxic activity and used to treat kidney, urinary and skin diseases

1.3.2. Thesis Flow and its Organization

The aim of the thesis is to provide scientific evidence regarding the quality, efficacy and safety of polyherbal formulation HC9 and to strengthen its significant potential in chemoprevention and treatment of breast cancer.

The entire thesis is organized into five chapters and the contents of each chapter have been summarized as follows:

Chapter 1: Introduction and Review of Literature

Chapter1 encompasses the introductory remarks on general background, global as well as Indian scenario of breast cancer followed by highlighting the etiology, signs and symptoms, screening, diagnosis and treatment options for breast cancer. It includes the importance of Complementary and Alternative Medicine (CAM) with a special emphasis on herbal medicine in treatment of breast cancer

Chapter 2: Comparative study of antioxidant and cytotoxicity potential of aqueous and ethanolic preparations of HC9 in breast cancer cell lines

Chapter 2 describes a comparative analysis of antioxidant potential of aqueous and ethanolic extracts of HC9 by performing various antioxidant assays. It also describes a comparative analysis of cytotoxicity potential of aqueous ($HC9_{aq}$) and ethanolic ($HC9_{et}$) extracts by analyzing the effect of the extracts on cell viability of breast cancer cell lines, MCF-7 and MDA MB-231, and non-cancerous cell line HEK-293. Further, the chapter discusses about the difference in the effect of HC9 versus its individual components on cell viability of breast cancer (MCF-7 and MDA MB-231) and non-cancerous cell lines (HEK-293, HaCaT and MCF-10A).

Chapter 3: Standardization of HC9 and its individual plant materials

Chapter 3 includes standardization of HC9 and its components by analyzing their qualitative and quantitative HPTLC profiles using standard marker compounds.

Chapter 4: Pre-clinical toxicity evaluation of HC9 in Swiss albino mice

Chapter 4 describes toxicity evaluation of HC9 in Swiss albino mice by performing acute and sub-acute toxicity studies. The chapter presents analysis of hematological, biochemical and histopathological parameters in treated and untreated mice.

Chapter 5: Analyzing the antineoplastic potential of HC9 in vitro as well as *in vivo*

This chapter provides valuable insights towards the anti-cancer activity of HC9 against breast cancer cell lines and analyzes the underlying molecular mechanisms governing its anticancer activity. The chapter also discusses the anticancer effect of HC9 on melanoma induced tumors in C57BL/6 mice.

Finally, bibliography has been provided which lists down previously published research articles that were invaluable during conception and progress of the current study. Manuscripts arising out of the research work embodied in this thesis have also been enlisted.

Chapter 2

Comparative study of antioxidant and cytotoxicity potential of aqueous and ethanolic preparations of HC9

The research work given in this chapter has been published in Biotechnology, Bioinformatics and Bioengineering (2011)

SECTION 1

Comparative study of antioxidant potential of aqueous and ethanolic preparations of HC9

Abstract

In this chapter, antioxidant potential of aqueous ($HC9_{aq}$) and ethanolic ($HC9_{et}$) extracts of HC9 was analyzed for its free radical scavenging activity, ferric reducing power, anti-lipid peroxidation (ALP) and total polyphenol content. Our results demonstrated that $HC9_{et}$ exhibited significant DPPH radical scavenging activity with IC_{50} value of 162.41 µg/ml compared to $HC9_{aq}$ having IC_{50} value of 172.89 µg/ml. Both the extracts showed a concentration-dependent increase in ferric reducing power activity. However, $HC9_{et}$ exhibited slightly higher activity than $HC9_{aq}$. The total polyphenol content of $HC9_{aq}$ and $HC9_{et}$ were 144.06 and 193.34 mg/g, respectively, equivalent to gallic acid concentration. $HC9_{et}$ exhibited significant anti-lipid peroxidation (ALP) activity in a dose-dependent manner than $HC9_{aq}$ with IC_{50} values of 66.7 and 84.7 µg/ml, respectively.

2.1.1 Introduction

Oxidative stress generates free radical species and is one of the major causes of many chronic and degenerative diseases including cancer (Nikam S et al., 2009; Vogiatzi G et al., 2009; Pashkow FJ et al., 2011; Park HS et al., 2009; Klaunig JE et al., 2010; Texel SJ et al., 2011; Mishra OP et al., 2012; Li Xet al., 2010). These free radicals cause membrane lipid peroxidation resulting into decrease in membrane fluidity, loss of enzyme receptor activity and damage to membrane proteins leading to cell inactivation (Dean RT et al., 1993).

Antioxidants have been known to play an important role in alleviating the deleterious effects induced by free radicals by blocking the initiation or propagation of oxidizing chain reactions (Waris G et al., 2006). Both synthetic and natural antioxidants have tremendous potency to prevent free radical formation. However, the former have often been found to be unsafe or toxic in the long term. Thus, the global interest has currently shifted towards the use of natural antioxidants, mostly present in herbs, for health benefits (Miguel MG et al., 2010). According to the World Health Organization, around 80% of the world's population is resorting towards traditional medicines (Gurib-Fakim A, 2006). An inverse correlation between consumption of fruits, vegetables, spices, cereals and the risk of cancer development suggest that antioxidants present in the plants act as effective agents for the cancer prevention (Liu RH, 2004). Polyphenols and flavonoids are the major antioxidant compounds present in different plants. They function as anticancer agents through induction of cellular defense systems including the detoxifying and antioxidant enzymes as well as through inhibition of cell proliferation and inflammatory pathways leading to cell cycle arrest and/or apoptosis (Pandey KB et al., 2009).

In the present study, we have evaluated the antioxidant potential of aqueous and ethanolic preparation of HC9. The comparison of $HC9_{aq}$ and $HC9_{et}$ was carried out to know the significant activity of HC9 by performing free radical scavenging, ferric reducing power activity and anti-lipid peroxidation (ALP) activity along with estimation of total polyphenol content of both the extracts.

2.1.2. Materials and methods

2.1.2.1. Chemicals and Reagents

All the chemicals used were of analytical grade. 1, 1-Diphenyl-2-picryl hydrazyl (DPPH), ascorbic acid was obtained from Sigma Chemicals, USA. Napthyl ethylene diamine dihydrochloride (NEDD) was obtained from from SISCO Research Laboratories Pvt. Ltd, Mumbai, India. Folin-Ciocalteu's phenol reagent (FCR) was obtained from SD Fine-Chemicals Limited, Mumbai. Sodium nitroprusside (SNP), sulphanilamide, phosphoric acid, ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$), trichloroacetic acid (TCA), thiobarbituric acid (TBA), potassium chloride (KCl), potassium ferricyanide ($K_3Fe[CN]_6$), Tris hydrochloride buffer were obtained from Qualigens.

2.1.2.2. Plant material and extraction

The whole/parts of all nine component herbs of HC9 were purchased from Shri Shailya Medi-Pharms (Solapur, Maharashtra, India). The individual bulk herb samples were stored in air-tight containers and kept in air-conditioned environment until further use. The samples were authenticated and validated macroscopically and microscopically in Department of Botany, Agharkar Research Institute (ARI), Pune (Maharashtra, India). Voucher specimens of herbs have been deposited at the

Department of Botany, Agharkar Research Institute and Herbaria of Medicinal Plant Conservation Centre (MPCC), Pune (Table 2.1).

Table 2.1. Composition of polyherbal formulation (HC9)

Plant materials in HC9	Family	Parts used	Voucher specimen number
Picrorhiza kurroa	Plantaginaceae	Root	R-120
Cyperus rotundus	Cyperaceae	Rhizome	R-121
Zingiber officinale	Zingiberaceae	Bark	R-122
Cedrus deodara	Pinaceae	Root	S/B-096
Tinospora cordifolia	Menispermaceae	Stem	S/B-097
Holarrhena antidysenterica	Apocynaceae	Seed	S-119
Swertia chirata	Gentianaceae	Whole plant	WP-078
Cissampelos pareira	Menispermaceae	Root	MPCC 290
Hemidesmus indicus	Apocynaceae	Root	MPCC 2354

All nine plant materials of HC9 were washed, dried and fine powdered separately. For the preparation of ethanolic extract, equal parts of each powdered plant material of HC9 were mixed in 1:1 ratio and subjected to soxhlet extraction method using ethanol. The resulting extracts were centrifuged at 13000 rpm for 15 min to remove the particulate matter. The supernatants were filter-sterilized using Swiney filter (pore size, 0.45 µm) and the resultant filtrates were stored in aliquots at -80°C until further use.

The aqueous extract was prepared according to Ayurvedic Pharmacopia of India (API) wherein the powdered mixture containing equal parts of each plant material was added to 144 ml water (1:16 ratio of plant material: water) in hot water

extractor. The extract was centrifuged at 13000 rpm for 15 min, filter-sterilized using Swiney filter (pore size, 0.45 µm) and the resultant filtrate was stored in aliquots at -80°C until use.

2.1.2.3. Free Radical Scavenging Activity by DPPH Method

The radical scavenging efficiency of both aqueous ($HC9_{aq}$) and ethanolic ($HC9_{et}$) extracts of HC9 on the DPPH radical was estimated using the method of Brand-Williams et al. with slight modifications (Brand-Williams W et al., 1995). A freshly prepared methanolic DPPH solution (33 mg in 1 L of methanol) was mixed with various concentrations of the extract (10, 20, 40, 80 and 160 µg/ml) in the ratio of 5:1, respectively. The contents were vigorously mixed, incubated at room temperature in the dark for 15 minutes and the absorbance was measured at 517 nm using a Perkin Emler UV-VS Spectrophotometer. Methanolic DPPH solution along with solvent without extract served as a control. All the experiments were carried out in triplicates and repeated at least three times at different time points. The free radical scavenging capacity (RSC) of the tested compounds was expressed as percentage of DPPH elimination and was calculated according to the following equation:

% RSC = Absorbance of Control - Absorbance of Extract)/ Absorbance of Control × 100.

2.1.2.4. Reducing Power Assay

The standard spectrophotometric method (Oyaizu M, 1986) was used for the measurement of reducing power potential of both aqueous ($HC9_{aq}$) and ethanolic ($HC9_{et}$) extracts of HC9. Various concentrations of both the extracts (10, 20, 40, 80 and 160 µg/ml) in 2.5 ml of phosphate buffer (pH 6.6) were mixed with 2.5 ml potassium ferricyanide (1%). The mixture was incubated at 50° C for 15 min and

around 2.5 ml TCA (10%) was added to it followed by centrifugation at 3000 g for 10 min. A portion of the supernatant (2.5 ml) was taken to which 2.5 ml of water and 0.5 ml of $FeCl_3$ (0.1%) were added. Absorbance was measured spectrophotometrically at 700 nm. Ascorbic acid was used as a positive control.

2.1.2.5. Anti-lipid Peroxidation Activity

Anti-lipid peroxidation (ALP) potential was determined using goat liver homogenate and thiobarbituric acid-malondialdehyde (TBA-MDA) (Wade J et al., 1985). Animal tissue (liver) was perfused with 0.15 M KCl. Perfused tissue was homogenized in ice cold 0.15 M KCl (10% w/v) using mortar and pestle. Different concentrations (10-160 µg/ml) of aqueous ($HC9_{aq}$) and ethanolic ($HC9_{et}$) extracts of HC9 were prepared to which 0.5 ml of homogenate and 1 ml of 0.15 M KCl were added. Lipid peroxidation was induced by adding 0.1 ml ice cold solution of 1mM $FeCl_3$ and the whole mixture was incubated at 37^0C for 30 min. In order to stop the reaction, 0.25 N HCl containing 15% trichloroacetic acid (TCA), 0.38% thiobarbituric acid (TBA) and 0.2ml 0.05% butylated hydroxyl toluene (BHT) was added. The reaction mixtures were heated at 80^0C for 60 min, cooled at room temperature and centrifuged at 6000 rpm for 15 min. Supernatant was collected and measured at 532 nm. ALP % was calculated using the formula:

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

2.1.2.6. Total Phenolic Content

The total phenolic content of aqueous ($HC9_{aq}$) and ethanolic ($HC9_{et}$) extracts of HC9 was determined by using Folin-Ciocalteu method (Pourmorad F et al., 2006). Aliquots

(0.1 ml) of both the extracts were transferred into the test tubes and their volumes adjusted up to 0.5 ml with distilled water. Around 5 ml of 10 times diluted Folin-Ciocalteu reagent and 4 mL of 1M sodium carbonate were added in each tube. The mixture was vortexed for 10 seconds and incubated for 15 min at 37°C for color development. The absorbance was measured at 765 nm using a Perkin Emler UV-VIS spectrophotometer. Samples of the extract were evaluated at a final concentration of 1 mg/ml.

2.1.2.7. Statistical analysis

All the assays were performed in triplicates and repeated at least three times at different time points and the data was presented as mean ± SD. Statistical analysis was done by SigmaStat 3.5 program (Systat Software, Inc.) using 1-way ANOVA. The α level used for comparisons was $\alpha = 0.05$.

2.1.3. Results

2.1.3.1. HC9 exhibited significant free radical scavenging activity

The free radical scavenging activity of both the aqueous and ethanolic extracts of HC9 formulation was evaluated by DPPH assay. Both the extracts exhibited significant free radical scavenging activity in a concentration dependent manner (Figure 2.1). At 160 µg/ml concentration, $HC9_{aq}$ and $HC9_{et}$ showed ~46.3 and 49.3% ($p \leq 0.0001$) DPPH free radical scavenging activity, respectively. The corresponding IC_{50} values of the $HC9_{aq,}$ $HC9_{et}$ as well the standard ascorbic acid were 172.89, 162.41 and 16.005 µg /ml, respectively.

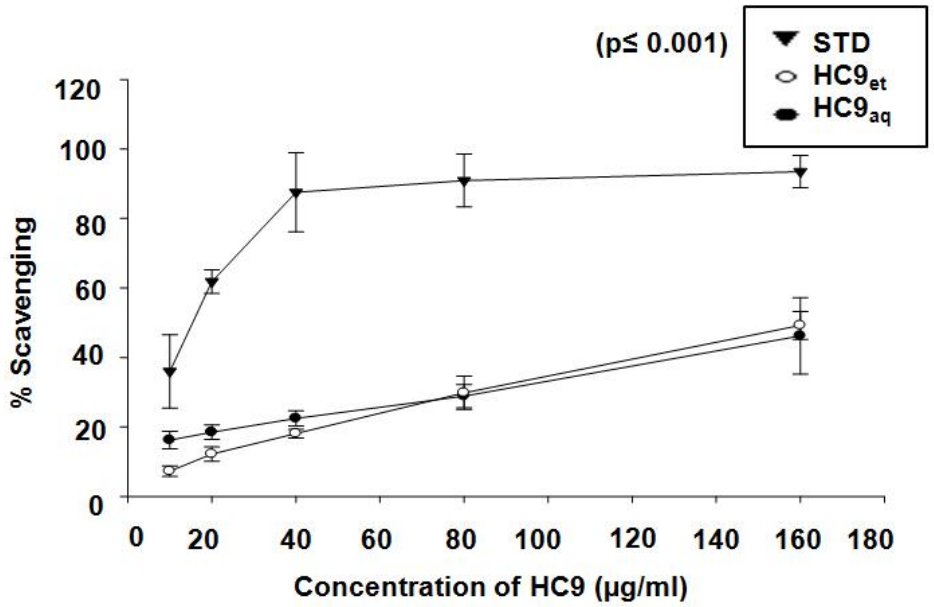


Figure 2.1: Free Radical Scavenging activity of $HC9_{aq}$ and $HC9_{et}$. *The graph represents the free radical scavenging activity of $HC9_{aq}$ and $HC9_{et}$ as measured by DPPH assay at 517 nm.*

2.1.3.2. Dose- dependent increase in reducing power potential of HC9

To determine the reducing power property of aqueous and ethanolic HC9, different concentrations of both the extracts were incubated with potassium ferricyanide [$K_3Fe(CN)_6$] in presence of phosphate buffer. It was observed that the reducing potential of both $HC9_{aq}$ and $HC9_{et}$ increased in a dose-dependent manner (Figure 2.2).

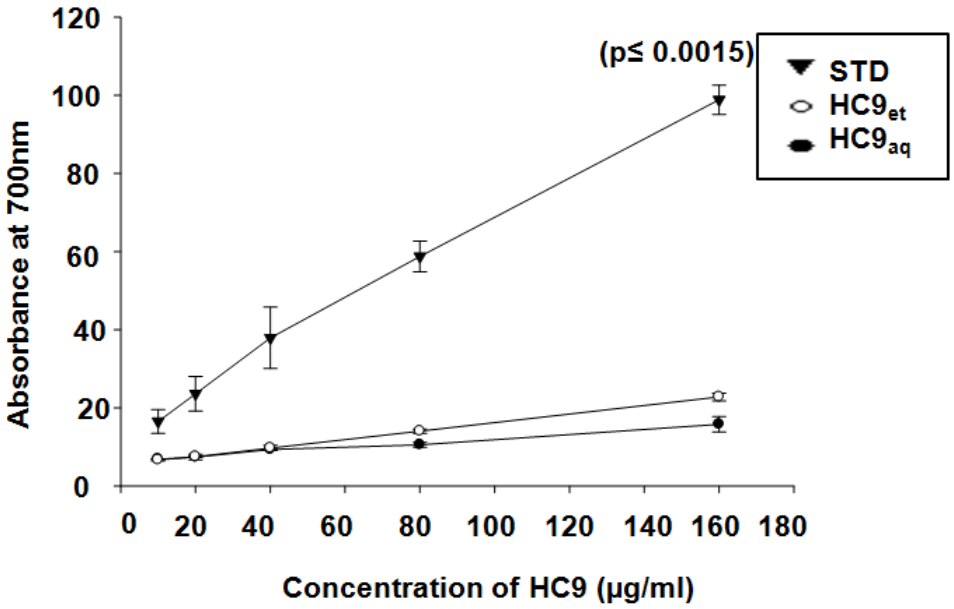


Figure 2.2: Reducing Power Activity of $HC9_{aq}$ and $HC9_{et}$ *The graph represent Reducing Power Activity of $HC9_{aq}$ and $HC9_{et}$ as measured by ferric reducing assay at 700 nm.*

2.1.3.3. HC9 inhibited the lipid peroxidation

We used modified TBARS method to see the ALP activity of $HC9_{aq}$ and $HC9_{et}$. Animal tissue (liver) homogenate was mixed with different concentrations of $HC9_{aq}$ and $HC9_{et}$ and lipid peroxidation was induced with $FeCl_3$. It was observed that both the extracts have significant ALP activity which increased in a dose-dependent manner (Figure 2.3). At 160 µg/ml concentration, $HC9_{aq}$ and $HC9_{et}$ were found to exhibit ~78.8 and 90.5% inhibition of lipid peroxidation, respectively. The corresponding IC_{50} values of the $HC9_{aq}$ and $HC9_{et}$ were 84.7 and 66.7µg/ml, respectively ($p \leq 0.0431$).

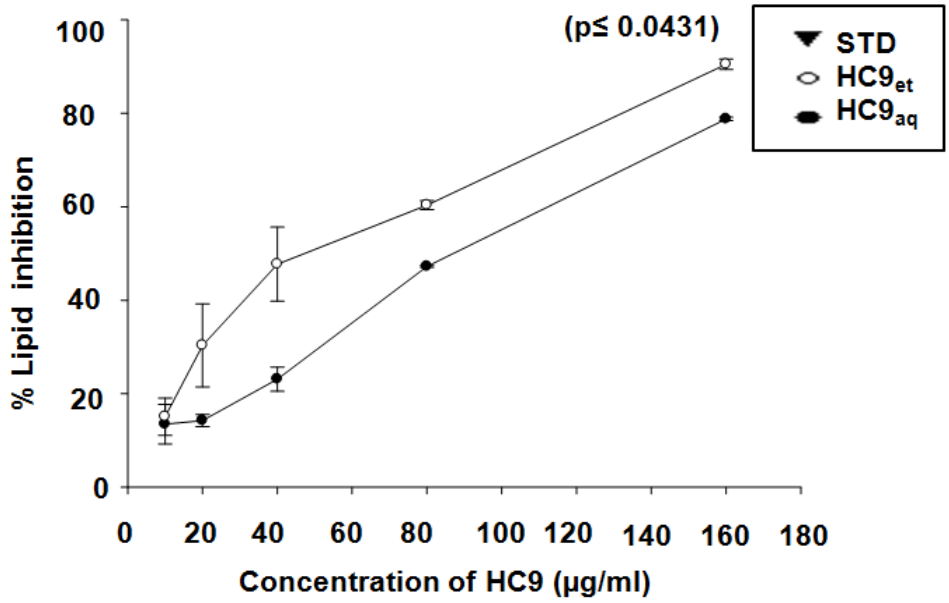


Figure 2.3: Anti-Lipid Peroxidation activity of $HC9_{aq}$ and $HC9_{et}$. *The graph represents anti-lipid peroxidation activity of $HC9_{aq}$ and $HC9_{et}$ as measured by TBARs method at 532 nm.*

2.1.3.4. Phenolic Content in HC9

Polyphenols are the major plant compounds possessing antioxidant activity. Folin-Ciocalteu method was performed to determine the total phenol content of both the extracts. Different concentrations of standard gallic acid were prepared and mixed with diluted Folin-Ciocalteu reagent along with sodium carbonate. Total Phenol Content was determined in comparison with standard gallic acid and the result was expressed in terms of mg/g of extract (Figure 2.4). The total polyphenol content of $HC9_{aq}$ and $HC9_{et}$ were found to be ~144.06 and 193.34 mg/g, respectively.

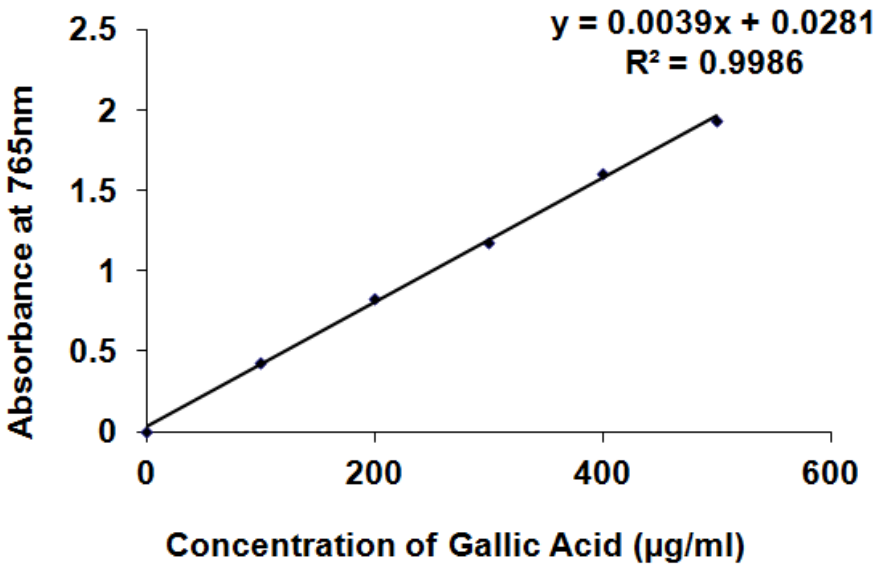


Figure 2.4: Standard gallic acid calibration curve. *Grahical representation of standard gallic acid calibration curve as measured by Folin-Ciocalteu method at 765 nm*

2.1.4. Discussion

The main objective of the present study was to evaluate the antioxidant potential of aqueous and ethanolic extracts of HC9. Antioxidants have been defined as substances that, when present at low concentrations, delay or prevent oxidative damage process due to the presence of reactive oxygen species by blocking the initiation or propagation of oxidizing chain reactions (van der Loo B et al., 2008). It has long been recognized that naturally occurring substances in higher plants have antioxidant activity.

The DPPH radical has been widely used to investigate the scavenging activities of several natural compounds including phenolic compounds (Valko M et al., 2007), or crude mixtures such as ethanolic or water extracts of plants. Antioxidants scavenge DPPH radical through the donation of a hydrogen atom, forming the reduced DPPH-H (Dean RT et al., 1993). After reduction, the color changes from purple to yellow and reduction of DPPH radicals can be observed by the

decrease in absorbance at 517 nm. Both aqueous and ethanolic preparations of HC9 showed scavenging activity in a concentration dependent manner. The results of the DPPH free radical scavenging assay suggest that components within HC9 extract are capable of scavenging free radicals via electron or hydrogen donating mechanisms and thus should be potent enough to prevent the initiation of deleterious free radical mediated chain reactions.

Reducing power of extract is one of the supporting feature by which antioxidant activity of extract can be determined. For the measurement of the reducing ability, ''Fe^{3+}– Fe^{2+} transformation'' in the presence of aqueous and ethanolic preparation was observed. The reducing properties are generally associated with the presence of reductones (Lim SD et al., 2005) which break down the chain reaction by hydrogen donation resulting into antioxidant effect. HC9 was found to possesses significant antioxidant activity that was comparable with ascorbic acid.

Lipid peroxidation (LPO) is a cellular process that commonly takes place under normal physiological conditions. Under excessive oxidative stress, the level of LPO becomes very high. Lipid peroxidation arises from the reaction of free radicals with lipids and is considered an important feature of cell injury leading to deterioration of cellular constituents including lipids, proteins and nucleic acids. HC9 extract exhibited significant anti-lipid peroxidation activity. This may be due to significant free radical scavenging which eventually contributed to anti-lipid peroxidation activity of the extract.

In addition, the antioxidant activity may be due to phenolic compounds present in the extract. The role of phenolics as scavengers of free radicals has been widely reported as well as accepted (Komali AS et al., 1999; Moller JKS et al., 1999).

It has been suggested that intake of food rich in polyphenolic compounds could inhibit cancer significantly (Wang S et al., 2011).

Slightly higher antioxidant potential of ethanolic extract was observed than aqueous extract. The higher antioxidant activity of the ethanolic extracts indicates that the formulation may be most effective when prepared in ethanol. Our study reveals that both the preparation has significant antioxidant effects and can be evaluated for its anticancer activity.

Before evaluating its anticancer mechanism, it's necessary to define the dose and its effect on cancerous and normal cell line. Thus, in next section of this chapter, we have evaluated the cytotoxic potential of $HC9_{aq}$ and $HC9_{et}$. Further, we have also compared the cytotoxic activity of $HC9_{et}$ with respect to its individual components.

SECTION 2

Effect of ethanolic and aqueous preparations of HC9 and its individual components on cell viability of breast cancer cell lines

Abstract

A thorough comparative analysis of cytotoxic activity of $HC9_{aq}$ and $HC9_{et}$ was performed in breast cancerous (MCF-7 and MDA MB-231) and non-cancerous (HEK-293) cell lines. The results showed that $HC9_{et}$ decreased cell viability of breast cancer cell lines at lower doses compared to $HC9_{aq}$. We then compared the cytotoxic activity of $HC9_{et}$ with its individual components. Results from MTT assay demonstrated that at 160 µg/ml concentration, HC9 reduced the viability of both MCF-7 and MDA MB -231 with no effect on non-cancerous cell lines, MCF-10A, HaCaT and HEK-293. In contrast, at this concentration, the individual components of HC9 decreased viability of not only of cancerous cells, but also non-cancerous cells. These results suggested that the HC9 formulation was safe for non-cancerous cells and could be explored for its anticancer activity in breast cancer.

2.2.1. Introduction

Comparative cytotoxicity study of $HC9_{aq}$ and $HC9_{et}$ was carried out in breast cancer and non-cancerous cell lines, by MTT dye uptake to know whether the aqueous or ethanolic extract of HC9 has significant cytotoxicity potential against breast cancer.

We also compared the cytotoxic activity of $HC9_{et}$ with its individual components in breast cancer and non-cancerous cell lines to evaluate whether the individual components of HC9 were more active than the whole formulation.

2.2.2. Materials and methods

2.2.2.1. Chemicals and reagents

Tissue culture plastic ware was purchased from BD Biosciences (CA, USA) and Axygen Scientific Inc (CA, USA). Dulbecco's Modified Eagles Medium (DMEM), penicillin and streptomycin were obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2.2.2. Cell lines

The human breast carcinoma cell lines, MCF-7 and MDA MB-231 and non-cancerous transformed cell lines, HEK-293 (Human Embryonic kidney) and HaCaT (Human Keratinocyte) used in the study were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2mM L-glutamine supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin. The cells were incubated in a humidified 5% CO2 incubator at 37°C. Non-tumorigenic normal mammary epithelial cell lineMCF-10A was a kind gift from Dr.

Milind Vaidya (ACTREC, Mumbai). The cells were grown in DMEM:Ham's F12 (1:1) containing 2 mM L-glutamine supplemented with 10% fetal bovine serum, 100 U/ml of penicillin-streptomycin, 10 µg/ml insulin, 20 ng/ml EGF and 0.5 mg/ml hydrocortisone. The cells were incubated in a humidified 5% CO2 incubator at 37°C (Yaacob NK et al., 2010).

2.2.2.3. Cytotoxic Assay

Cytotoxicity of nine component herbs of HC9 and the whole formulation was determined in the cancerous and non-cancerous cell lines by MTT dye uptake (Deshpande RA et al., 2013). Briefly, MCF-7, MDA-MB-231, HEK-293, HaCaT and MCF-10A cells were seeded at 1 x 10^5/ml density in 96-well plates. Next day, the cells were incubated with various concentrations of HC9 and ethanolic extracts of individual herbs (0-160 µg/ml) for 24 h and incubated in 5% CO_2 incubator at 37°C. Next day, 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 OD 570-630 nm. The percentage viability was calculated as:

% Viability = [OD of treated cells/ OD of control cells] x 100

2.2.2.4. Statistical analysis

IC_{50} values were calculated by using Kyplot software. All the assays were performed in triplicates and repeated at least three times at different time points. The data has been presented as IC_{50} values and mean ± SD.

2.2.3. Results

2.2.3.1. HC9 significantly reduced viability of breast cancer cells

Cytotoxic activity of $HC9_{aq}$ and $HC9_{et}$ was evaluated on human breast cancer cell lines and non-cancerous cells. Both types of cells treated with $HC9_{aq}$ exhibited 100% viability up to 160 µg/ml concentration (Figure 2.5), thereby showing that it had no effect on their viability. However, $HC9_{et}$ reduced viability of both MCF7 and MDA MB231 at 80 and 160µg/ml without any effect on HEK 293 (Figure 2.6). Thus, besides antioxidant potential, $HC9_{et}$ exhibited significant cytotoxic activity in both the breast cancer cell lines and appeared to exhibit potent activity than the aqueous extract.

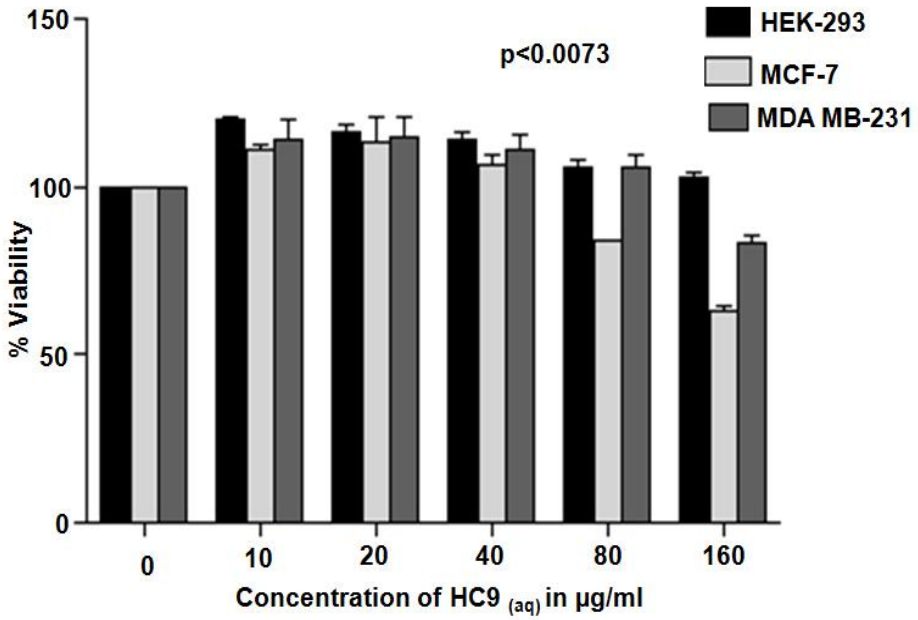


Figure 2.5: Effect of $HC9_{aq}$ on cell viability of MCF-7, MDA-MB-231 and HEK293 cells. *Cytotoxicity was measured by MTT dye uptake at OD 570-630nm.The data represents mean ± SD of three independent experiments.*

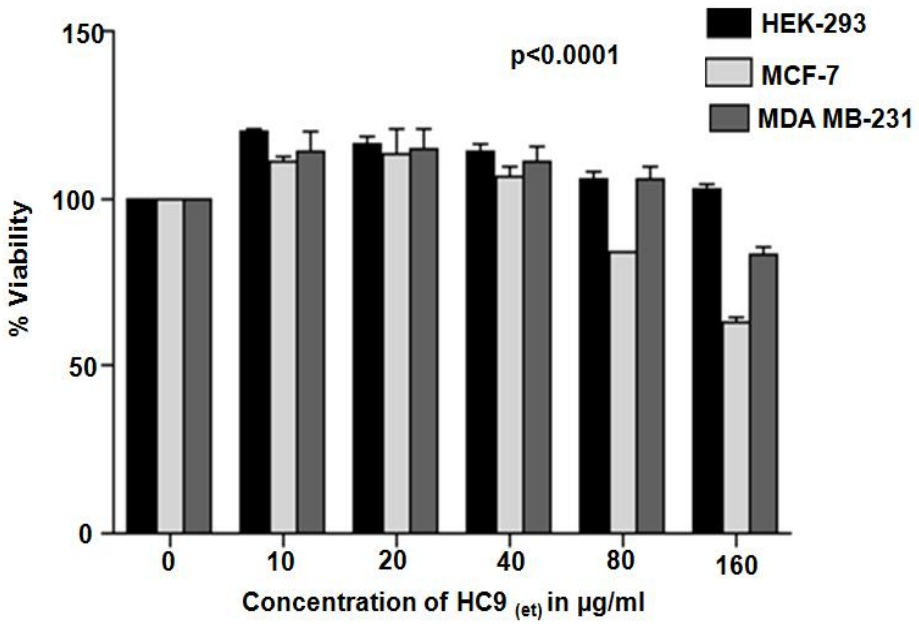


Figure 2.6: Effect of $HC9_{et}$ on cell viability of MCF-7, MDA-MB-231 and HEK293 cells. *Cytotoxicity was measured by MTT dye uptake at OD 570-630nm.The data represents mean ± SD of three independent experiments.*

2.2.3.2. Effect of HC9 and its individual component on cell viability

We wanted to compare the cytotoxic activity of $HC9_{et}$ with its component plant materials to know whether the individual components were more active than the whole formulation. Thus, effect on cell viability was evaluated in MCF-7 and MDA MB-231 and non-cancerous cell lines, MCF-10A, HaCaT and HEK-293.

The results showed that IC_{50} value of HC9 in MCF-7 was lower (150.29 µg/ml) than individual plant extracts except for *S. chirata* showing IC_{50} value of 109.35µg/ml (Table 2.2). However, *S. chirata* was cytotoxic to non-cancerous cells at lower concentrations with IC_{50} values of 65.87, 24.63 and 71.10 µg/ml in MCF-10A, HaCaT and HEK-293, respectively (Table 2.2).

In MDA MB-231, IC_{50} value of HC9 was lower (184.50 µg/ml) than individual plant extracts except for *Z. officinale*, *C. deodara,* and *H. indicus* showing IC_{50} values of 176.38, 158.62 and 130.88 µg/ml, respectively. However, these herbs

were cytotoxic to the non-cancerous cells and showed IC_{50} values of 166.67, 84.33 and 217.23 µg/ml, respectively in MCF-10A; 62.36, 58.15, 107.19 µg/ml, respectively in HaCaT and 48.71, 50.64 and 105.61, respectively in HEK-293 (Table 2.2). Interestingly, HC9 showed higher IC_{50} values in MCF-10A (>640 µg/ml), HaCaT (>640 µg/ml) and HEK-293 (586.10 µg/ml) compared to the component herbs (Table 2.2). HC9 was non-cytotoxic up to 160 µg/ml concentration in non-cancerous cell lines and exhibited significant cytotoxicity in MCF-7 and MDA MB-231 at the same concentration (Table 2.3 to Table 2.7). These results suggested that the standardized HC9 formulation was safe to non-cancerous cells and exhibited significant anticancer potential for breast cancer cells compared to the component herbs.

Table 2.2: IC_{50} values of HC9 and individual plant materials in breast cancer and non-cancerous cell lines

Plant materials in HC9	IC_{50} (µg/ml)				
	MCF-10A	**HaCaT**	**HEK-293**	**MCF-7**	**MDA MB-231**
P.kurroa	351.55	497.61	336.92	320.76	374.60
C. rotundus	193.01	131.42	144.23	180.65	331.64
Z. officinale	166.67	62.36	48.71	186.29	176.38
C. deodara	84.33	58.15	50.64	157.50	158.62
T.cordifolia	102.00	211.78	162.47	384.95	471.15
H. antidysenterica	299.95	457.38	188.82	>640	>640
S. chirata	65.87	24.63	71.10	109.35	233.95
C. pareira	89.07	137.04	72.59	291.84	492.92
H. indicus	217.23	107.19	105.61	182.99	130.88
HC9	>640	>640	586.10	150.29	184.50

Table 2.3: Percentage viability in MCF-10A treated with ethanolic extracts of individual plant materials and HC9

Conc. (µg/ml)	*P. kurroa*	*C. rotundus*	*Z. officinale*	*C. deodara*	*T.cordifolia*	*H. antidysenterica*	*S. chirata*	*C. pareira*	*H. indicus*	HC9
0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0
10	100.63±3.58	109.16±3.13	103.01±2.77	110.82±9.17	106.02±1.24	124.95±5.94	104.78±6.6	102.22±2.67	108.72±3.73	126.26±5.65
20	103.66±3.90	105.00±5.18	102.78±3.27	104.52±2.32	103.6±0.97	113.50±6.66	98.78±7.85	93.17±1.68	100.30±1.55	123.69±8.55
40	94.14±6.00	96.63±6.47	97.21±3.83	91.47±1.09	103.07±0.80	102.91±4.81	88.15±5.76	83.19±0.02	97.24±2.22	113.10±4.59
80	91.05±3.26	86.71±8.49	91.52±4.26	82.54±4.26	86.20±5.60	95.91±5.80	58.68±5.01	59.70±2.93	92.93±4.40	109.33±3.99
160	90.98±1.96	74.52±8.24	76.27±3.72	18.98±4.15	29.99±5.76	91.41±4.72	8.58±2.01	47.14±2.51	81.07±3.27	100.35±0.28
320	63.71±1.91	31.73±1.75	12.79±2.99	1.81±0.38	4.20±0.57	70.94±2.87	5.56±1.52	2.39±.06	42.85±1.70	78.06±1.75
640	11.91±2.40	6.86±0.54	1.27±0.29	0.70±0.15	0.81±0.20	3.69±1.02	0.67±0.49	2.16±.06	4.76±0.27	74.00±0.84

Table 2.4: Percentage viability in HaCaT treated with ethanolic extracts of individual plant materials and HC9

Conc. (µg/ml)	*P. kurroa*	*C. Rotundus*	*Z. officinale*	*C. deodara*	*T. cordifolia*	*H. antidysenterica*	*S. Chirata*	*C. Pareira*	*H. indicus*	HC9
0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0
10	93.51±5.42	90.58±2.19	90.39±6.85	87.89±12.10	90.11±4.42	116.61±5.32	87.96±4.98	89.86±7.30	73.57±10.0	119.41±5.97
20	86.69±5.80	87.09±2.98	91.78±3.47	94.41±7.98	89.43±2.92	105.47±7.09	66.75±3.56	85.56±3.89	73.94±5.99	118.75±5.96
40	83.58±5.41	84.93±9.38	81.85±9.43	54.97±9.52	85.19±3.87	95.29±2.90	45.57±8.01	77.80±6.78	71.50±7.62	114.02±6.68
80	78.28±3.20	72.82±4.93	37.09±2.65	48.36±7.28	71.67±3.73	84.04±1.60	3.25±1.10	71.06±7.72	60.97±8.01	113.96±4.93
160	72.0±2.43	32.71±1.75	14.58±5.02	17.21±0.04	55.01±2.08	86.11±1.91	2.55±2.30	38.39±7.06	30.72±10.76	112.92±4.40
320	68.57±3.64	11.23±0.93	8.28±1.96	9.75±0.70	34.95±2.50	81.11±11.24	3.51±1.37	14.57±0.61	17.39±0.76	108.03±6.21
640	47.25±1.34	3.09±1.09	4.41±0.63	2.88±0.24	21.02±6.25	27.58±0.81	4.39±2.15	9.40±3.30	8.78±0.17	83.24±5.16

Table 2.5: Percentage viability in HEK-293 treated with ethanolic extracts of individual plant materials and HC9

Conc. (µg/ml)	*P. kurroa*	*C. rotundus*	*Z.officinale*	*C. deodara*	*T.cordifolia*	*H.antidysenterica*	*S. chirata*	*C. pareira*	*H. indicus*	HC9
0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0
10	112.42±1.63	107.98±1.87	110.50±1.79	101.07±1.65	102.84±4.72	99.37±3.28	115.28±6.84	101.57±3.04	103.13±4.55	142.57±2.34
20	110.36±2.43	102.81±3.32	100.55±0.38	88.30±2.19	99.60±0.27	101.07±3.58	128.78±1.87	99.17±5.48	84.98±0.35	134.16±3.04
40	104.18±2.90	93.67±7.58	86.23±0.29	79.12±6.05	93.38±6.12	84.73±7.34	85.82±3.25	74.88±1.35	72.84±6.15	128.69±1.63
80	91.66±2.32	69.88±4.09	18.80±2.09	29.65±2.74	58.21±4.11	82.29±3.41	28.96±1.78	24.67±7.10	51.57±4.53	105.24±3.68
160	84.71±1.49	67.32±14.49	3.27±2.66	7.00±2.24	52.68±3.17	73.78±8.21	32.48±1.14	23.54±18.02	48.71±5.17	104.49±3.84
320	47.86±0.37	16.49±0.45	11.20±0.06	12.10±0.36	40.97±2.98	15.29±0.15	11.02±0.93	13.67±0.61	38.86±7.06	72.03±1.61
640	47.36±0.39	13.83±0.46	10.71±0.45	10.13±0.26	12.78±1.11	12.24±0.23	8.81±0.14	7.75±0.59	20.40±0.27	37.87±0.94

Table 2.6: Percentage viability in MCF-7 treated with ethanolic extracts of individual plant materials and HC9

Conc. (µg/ml)	*P. kurroa*	*C. rotundus*	*Z.officinale*	*C. deodara*	*T.cordifolia*	*H.antidysenterica*	*S. chirata*	*C. pareira*	*H. indicus*	HC9
0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0	100.00±0.0
10	112.42±1.63	107.98±1.87	110.50±1.79	101.07±1.65	102.84±4.72	99.37±3.28	115.28±6.84	101.57±3.04	103.13±4.55	142.57±2.34
20	110.36±2.43	102.81±3.32	100.55±0.38	88.30±2.19	99.60±0.27	101.07±3.58	128.78±1.87	99.17±5.48	84.98±0.35	134.16±3.04
40	104.18±2.90	93.67±7.58	86.23±0.29	79.12±6.05	93.38±6.12	84.73±7.34	85.82±3.25	74.88±1.35	72.84±6.15	128.69±1.63
80	91.66±2.32	69.88±4.09	18.80±2.09	29.65±2.74	58.21±4.11	82.29±3.41	28.96±1.78	24.67±7.10	51.57±4.53	105.24±3.68
160	84.71±1.49	67.32±14.49	3.27±2.66	7.00±2.24	52.68±3.17	73.78±8.21	32.48±1.14	23.54±18.02	48.71±5.17	104.49±3.84
320	47.86±0.37	16.49±0.45	11.20±0.06	12.10±0.36	40.97±2.98	15.29±0.15	11.02±0.93	13.67±0.61	38.86±7.06	72.03±1.61
640	47.36±0.39	13.83±0.46	10.71±0.45	10.13±0.26	12.78±1.11	12.24±0.23	8.81±0.14	7.75±0.59	20.40±0.27	37.87±0.94

Table 2.7: Percentage viability in MDA MB-231 treated with ethanolic extracts of individual plant materials and HC9

Conc. (µg/ml)	*P.kurroa*	*C.rotundus*	*Z.officinale*	*C. deodara*	*T.cordifolia*	*H.antidysenterica*	*S. chirata*	*C. pareira*	*H. indicus*	HC9
0	100.00± 0.0	100.00± 0.0	100.00± 0.0	100.00± 0.0	100.00± 0.0	100.00± 0.0	100.00± 0.0	100.00± 0.0	100.00± 0.0	100.00± 0.0
10	138.59±2.23	106.05±2.15	125.25±4.32	130.03±5.76	108.5±1.53	128.28±6.87	128.16±7.05	111.22±8.71	129.76±0.95	129.15±5.41
20	123.76±6.37	101.08±1.50	113.13±1.30	112.21±1.94	105.93±2.62	118.61±3.74	122.46±3.51	106.29±2.20	119.53±1.89	127.92±0.82
40	117.54±4.73	100.21±6.60	101.461±2.5	100.03±2.43	95.66±2.28	113.77±0.30	119.59±4.33	102.50±1.62	102.47±8.66	115.20±7.86
80	85.43±13.34	78.68±9.54	73.27±2.12	68.56±6.88	88.08±10.9	108.07±2.58	112.14±1.73	79.87±5.85	69.06±9.30	104.07±1.16
160	76.96±5.64	72.45±2.72	41.75±0.85	59.22±4.66	79.87±4.31	103.12±1.46	103.64±4.88	72.28±1.61	37.14±4.04	59.54±3.27
320	72.00±1.79	39.03±4.25	25.37±6.82	3.25±2.55	40.97±5.58	100.13±0.64	29.08±1.50	13.13±2.39	51.24±9.86	34.0±1.15
640	47.55±0.92	10.65±0.11	3.22±1.23	0.97±0.14	15.81±3.47	23.11±0.99	4.60±2.01	7.60±0.16	13.33±1.37	8.0±0.35

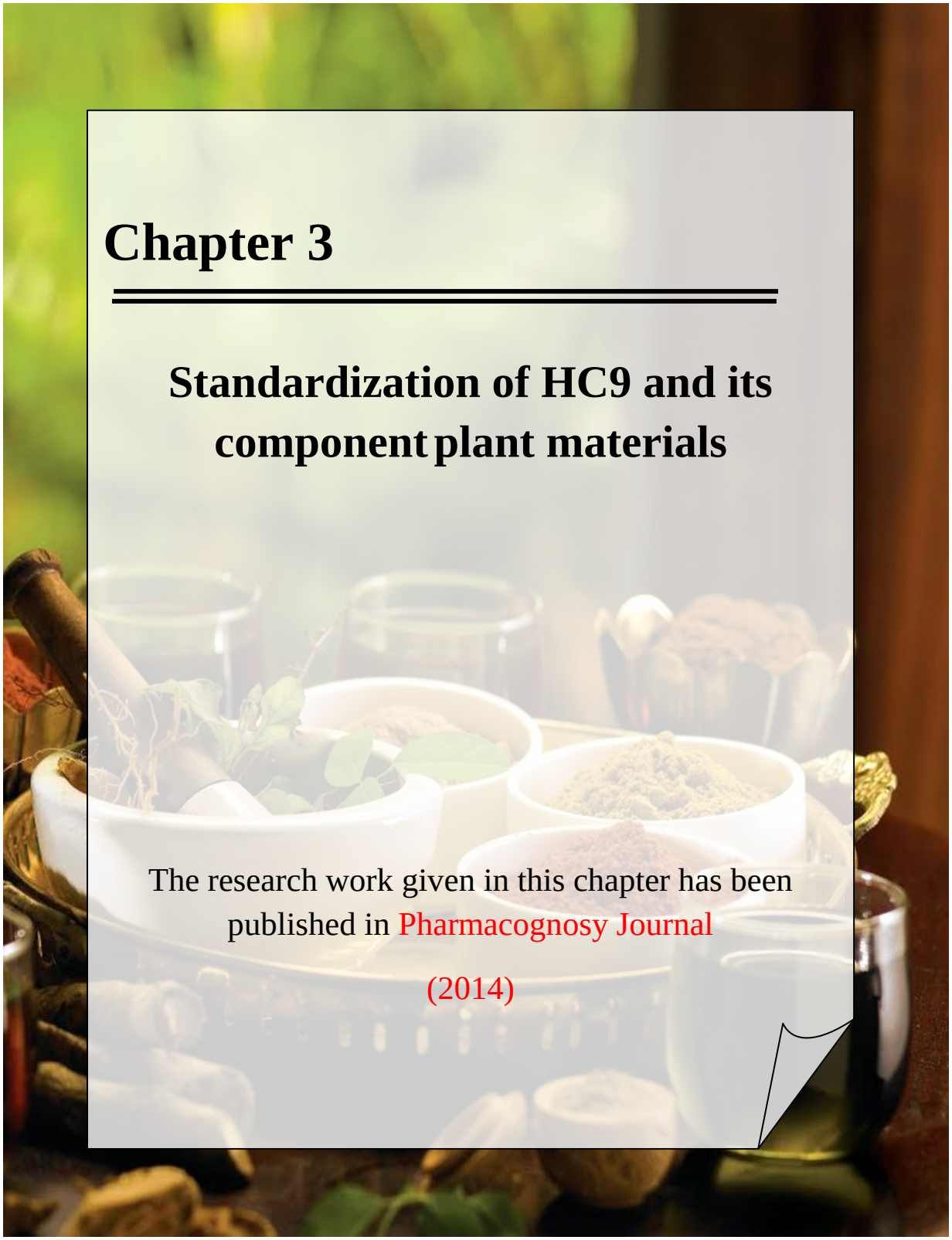
Discussion

Poly-herbal preparations are often used by traditional medical practitioners for the treatment of cancer (Kelloff GJ et al., 2000). Therefore, the current study was carried out to evaluate comparative cytotoxicity potential of aqueous ($HC9_{aq}$) and ethanolic ($HC9_{et}$) extracts. $HC9_{et}$ exhibited significant cytotoxic activity in both the breast cancer cell lines without affecting normal cell line and seemed to be more potent than the aqueous extract ($HC9_{aq}$).

Moreover, comparative studies of cytotoxicity potential of $HC9_{et}$ formulation *(Note: henceforth $HC9_{et}$ would be referred to as HC9)* with its component plant materials was carried to know whether the individual components of HC9 were more active than the whole formulation. HC9 exhibited comparable activity with individual components towards breast cancer cells, without affecting the normal cells. HC9 was more effective at lower concentrations in breast cancer cells and safe to normal cells than the individual components. Phytochemicals present in the plants have strong antioxidant and antiproliferative activities (Liu RH, 2004). Thus a combination of nine different plant materials in HC9 could have provided a synergistic effect of phytochemicals in terms of enhanced efficacy at lower dose. Moreover, each individual plant material used in the combination has been reported to have one or the other type of anticancer activity, hence, a combination of all these plants in HC9 could also have resulted into synergistic activity to reduce the viability of breast cancer cells with no cytotoxicity in non-cancerous cells. Thus HC9 could be a potent drug candidate in breast cancer and needed to be explored in detail for its antineoplastic potential.

Before evaluating anticancer mechanism of HC9, it was necessary to standardize the formulation with respect to standard marker compounds present in the

component plant materials. Thus, next chapter focus on standardization of HC9 formulation.



Chapter 3

Standardization of HC9 and its component plant materials

The research work given in this chapter has been published in Pharmacognosy Journal

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Abstract

In the present study, standardization of HC9 was carried out on the basis of botanical validation, organoleptic characters, physico-chemical and preliminary phytochemical parameters to establish its quality. Physico-chemical results revealed that HC9 contained 7.24% total ash content, 9.52 % of alcohol-soluble extractive, 0.801 specific gravity, 0.50 g/ml bulk density and exhibited 7.18% loss on drying. Phytochemical results revealed the presence of alkaloids, carbohydrates, flavanoids, saponins, tannins and phenolic compounds, and absence of terpenoids. Qualitative and quantitative HPTLC analysis of component plant materials and HC9 was done by using specific biochemical markers. The individual components of HC9 and the formulation showed the presence of marker compounds such as picroside-I, nootkatone, 6-gingerol, matairesinol, swertiamarin, berberine, connesine and 2-hydroxy-4-methoxybenzaldehyde.

3.1. Introduction

The complexity of polyherbal formulations impose a greater challenge in establishment of their quality, efficacy and safety, compared to single herbal counterparts (Kunle OF et al., 2012; Mukherjee PK et al., 2011; Choudhary N et al., 2011). Thus, it becomes important to standardize the herbal drugs by various parameters and sophisticated techniques to ensure their quality (Calixto JB et al., 2000; Barnes J et al., 2003; Bandaranayake WM et al., 2006). Various regulatory bodies such as World Health Organization (WHO), European Agency for the Evaluation of Medicinal Products (EMEA), United States Pharmacopeia (USP), and Department of AYUSH, Government of India, have provided the standardization guidelines for development of herbal preparations (Simha VKR et al., 2008; Chaudhary A et al., 2011).

In the present chapter, we have standardized each plant component of HC9 and the composite formulation by HPTLC analysis with respect to their marker compounds present in the component plant materials. We have also evaluated the physicochemical and phytochemical parameters of HC9.

3.2. Materials and methods

3.2.1. Chemicals and reagents

All other common solvents were procured from Qualigen Fine Chemicals (Mumbai, India) and HPTLC grade solvents were purchased from Merck (Mumbai, India). Reference marker compounds for High Performance Thin Layer Chromatography (HPTLC) analysis were obtained from the Natural Remedies Pvt Ltd (Bangalore, Karnataka-India) and Sigma-Aldrich (St. Louis, MO, USA). The solvents used for

high-performance thin-layer chromatography (HPTLC) analysis were obtained from MERCK (Mumbai, India).

3.2.2. Collection, identification and authentication of plant materials

The whole/parts of all nine component herbs of HC9 were purchased from Shri Shailya Medi-Pharms (Solapur, Maharashtra, India). The individual bulk herb samples were stored in air-tight containers and kept in air-conditioned environment until further use. The samples were authenticated and validated macroscopically and microscopically in Department of Botany, Agharkar Research Institute (ARI), Pune (Maharashtra, India). Voucher specimens of herbs have been deposited at the Department of Botany, Agharkar Research Institute and Herbaria of Medicinal Plant Conservation Centre (MPCC), Pune (Table 3.1).

3.2.3. Extract preparation

Ethanolic extracts of individual herbs as well as HC9 was prepared as described in chapter 2 section 2.1.2.2

3.2.4. Organoleptic evaluation of HC9

The organoleptic characters of the powdered HC9 were evaluated by appearance, size, shape, color, texture, odor and taste according to the guidelines of Indian Pharmacopoeia (Madhav NVS et al., 2011).

3.2.5. Determination of physicochemical parameters of HC9

Physico-chemical parameters such as total ash content, total viable count, loss on drying of extract, ethanol-extractable matter in the mixture of air-dried powder

material, determination of pH, bulk density as well as specific gravity of HC9 extract were carried out at Indian Drug Research Institute (IDRI), Pune (Maharashtra, India) according to the prescribed standard methods in Indian Pharmacopoeia (Madhav NVS et al., 2011).

3.2.6. Preliminary phytochemical analysis of HC9

The preliminary phytochemical analysis of HC9 was done by Indian Drug Research Institute (IDRI), Pune (Maharashtra, India). The extract was screened to detect the presence of secondary metabolites such as alkaloids, carbohydrates, flavanoids, saponins, terpenoids, tannins and phenolic compounds (Madhav NVS et al., 2011).

3.2.7. HPTLC finger printing profile

Identity of individual herbs and HC9 formulation was confirmed by detecting the presence of marker compounds such as picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde in *P. kurroa***,** *C. rotundus, Z.officinale***,** *C. deodara***,** *T. cordifolia***,** *H. antidysenterica***,** *S. chirata***,** *C. pareira*and *H. indicus,* respectively. Stocks and working solutions of different marker compounds were prepared in respective diluents (Table 3.2). Standard (marker compounds) and samples (HC9 and nine individual herbs) were applied onto a thin layer chromatography (TLC) plate, using an automatic TLC sampler (Linomat 5) as described previously (Singh et al., 2009).

Table 3.1: Solvents of marker compounds along with their concentrations

Marker compounds	Solvents	Stock solution (mg/ml)	Working solution (mg/ml)
Picroside-I	Methanol	2	0.2
Nootkatone	Methanol	10	0.1
6-Gingerol	Methanol	10	2
Matairesinol	Methanol	1	0.1
Berberine	Methanol	2	0.02
Conesine	Methanol	1	1
Swertiamarin	Methanol	2.3	0.02
2-hydroxy-4-methoxybenzaldehyde	n-Hexane	10	0.2

In brief, the samples (standards and test samples) were spotted as bands (8 mm width) with a Camag (Muttenz, Switzerland) Hemilton microlitre syringe onto a pre-coated aluminum-backed silica gel 60F-254 plate (20 × 10 cm; layer thickness 250 μm; Merck, Darmstadt, Germany) using a Camag high-performance thin-layer chromatography (HPTLC) system equipped with an automatic TLC sampler (Linomat 5), TLC scanner 3, and integrated software Win-Cats version 4. A constant application rate (0.1μl/s) was employed and the space between the two bands was 6 mm. The respective working solutions of standards (Table 3.1) were applied to the TLC plate along with the test solution. Linear ascending development was carried out in 20 cm X 10 cm twin trough glass chamber pre-saturated with the respective mobile phase. The optimum chamber saturation time for the mobile phase was 20 min at room temperature. The chromatoplates were developed up to 80 mm under chamber saturation conditions to get good resolution of phytochemical contents. Subsequent to development, TLC plates were dried in a current of air with the help of an air-dryer to evaporate solvents from the plates. The plates were examined using A Camag model

III TLC scanner with CATS 4.0 integration software. Densitometric scanning was performed in the appropriate absorbance mode with a slit dimension of 6 × 0. 45 mm and a scanning speed of 10 mm/s. A deuterium lamp was used as source of radiation. The amount of marker compounds present in HC9 was determined from the calibration curve obtained by plotting the concentration of standard against the peak area of test samples.

3.3. Results

3.3.1. Organoleptic evaluation

The powdered HC9 was evaluated for its organoleptic properties. The results revealed that HC9 was dark green in color with characteristic odor, bitter taste and fine texture. These parameters form the basic criteria for selecting a raw drug. Fine texture of powdered HC9 indicated the smoothness and surface uniformity that forms the primary character to assess the quality of a herbal drug (Chandel HS et al 2011).

3.3.2. Physico-chemical analysis

HC9 was evaluated for total ash content, ethanol soluble extractive, loss on drying at 105ºC, pH, specific gravity and bulk density (Table 3.2).

Table 3.2. Physiochemical characteristics of HC9

Parameters	Values
Total ash content	7.24%
Ethanol extractives	9.52%
Loss on drying	7.18%
pH	6.1 ± 0.2
Bulk density	0.50 g/ml
Specific gravity	0.801

Physico-chemical analysis of HC9 revealed that the total ash content present in HC9 was 7.24%. The total ash values of the individual plant materials of HC9 have been reported to be in the range of 2-8% (Table 3.3).

The percentage yield of alcohol-soluble extractive of HC9 was found to be 9.52% w/w. The alcohol-soluble extractive values of the individual plant materials in HC9 have been reported to be in the range of 4.5-18% (Table 3.3). Loss on drying at 105ºC of HC9 was found to be 7.18%. This value is indicative of amount of moisture content present in the drug (Saraf S et al 2010). The pH conventionally represents the acidity or alkalinity. HC9 (1% w/v solution) showed a pH of 6.1 indicating that the formulation was acidic in nature. Bulk density, a measure used to describe packing of particles or granules, of HC9 was found to be around 0.50 g/ml. Lower value of density indicates good flow and higher value indicates poor flow properties of formulation (Chandel HS et al 2011). The specific gravity of HC9 was found to be 0.801. All these values indicated that HC9 exhibited good flow properties.

Table 3.3: Permissible limits of physico-chemical parameters of individual components in HC9

Plant materials in HC9	Total ash content (%)[a]	Ethanol extractives (%)[b]	Loss on drying (%)	References
Picrorhiza kurroa	NMT 7	NLT 10	NMT 13	Ayurvedic Pharmacopoeia of India; Volumes I,III and IV
Cyperus rotundus	NMT 8	NLT 5		
Zingiber officinale	NMT 6	NLT 4.5	NMT 7.13	
Cedrus deodara	NMT 2	NLT 7		
Tinospora cordifolia	NMT 7	NLT 6	NMT 7.5	
Holarrhena antidysenterica	NMT 7	NLT 18		

Swertia chirata	NMT 6	NLT 10
Cissampelos pareira	NMT 7	NLT 11
Hemidesmus indicus	NMT 4.3	NLT 15

[a]NMT: not more than; [b]NLT: not less than, --: not available

3.3.3. Preliminary phytochemical evaluation

The preliminary phytochemical screening of HC9 demonstrated the presence of alkaloids, carbohydrates, flavanoids, saponins, tannins and phenolic compounds and absence of terpenoids. These qualitative tests are used to detect the presence of functional groups, which play an important role in the biological activity of the drug (Baragi UC et al., 2011).

3.3.4. HPTLC analysis of HC9 and ethanolic extracts of its individual components

TLC fingerprinting profile followed by HPTLC analysis of HC9 and its component plant materials was performed by using respective marker compounds (Table 3.4). The finger printing profiles were developed in respective solvent systems as given in Table 2 and their corresponding peaks were recorded at respective Rf values. Table 5 summarizes the marker compounds, mobile phases, wavelength (λ_{max}) and R_f values of spots visible in the HPTLC profiles of each herb.

The individual plant extracts showed Rf values of 0.22, 0.97, 0.6, 0.83, 0.29, 0.68, 0.66 and 0.91 corresponding to their marker compounds picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin and 2-hydroxy-4-methoxybenzaldehyde in the extracts (Table 3.5). Similarly, HC9 showed Rf values corresponding to the presence of respective marker compounds in the formulation.

Thus, all the component herbs of HC9 were authenticated and found to be present in the formulation based on HPTLC analysis.

The amount of marker compounds present in the extracts of individual plants of HC9 and the formulation was also evaluated. *P. kurroa***,** *C. rotundus, Z. officinale***,** *C. deodara***,** *T. cordifolia***,** *H. antidysenterica***,** *S. chirata***,** *C. pareira*and *H. indicus* was found to have 27.76, 6.59, 46.03, 30.01, 10.36, 6.58, 12.43, 5.95 and 13.78% of picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde, respectively (Table 3.5). on the other hand, HC9 was found to have 18.41, 4.59, 12.04, 8.89, 4.06, 1.66, 4.41, 4.2 and 12.72% w/w of picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde, respectively (Table 3.5). The overall results indicated that HC9 contained more amount of picroside-I, 6-gingerol, matairesinol and 2-hydroxy-4-methoxybenzaldehyde compared to the other marker compounds. Thus, the activity of HC9 could be due to the presence of high amounts of picroside-I, 2-hydroxy-4-methoxybenzaldehyde, 6-gingerol and matairesinol.

Table 3.4. Major chemical compounds present in HC9 with the markers used in the study

Plant materials	Major chemical compounds	Selected marker compound	References
P. kurroa	**Picroside I**, Picroside II, Picroside IV and 6-ferulloylcatalpol	Picroside-I	23-24, 26
C. rotundus	α-copaene , cyperene, β-selinene, valerenal, β-cyperone**,** **nootkatone,** caryophyllene oxide, α-selinene	Nootkatone	27, 52
Z. officinale	**6-gingerol**, 8-gingerol 10-gingerol and, 6-shogaol	6-Gingerol	30-31
C. deodara	Wikstromol, **matairesinol,** dibenzylbutyrolactol	Matairesinol	33-34
T. cordifolia	Cordifolioside A, tinocordifolin, tinosporadine, tinocordifolioside, makisterone,cordifol, **berberine**	Berberine	53-54
H. antidysenterica	**Conesine**, antidysentericine	Conesine	39,55
S. chirata	Mangiferin, **swertiamarin,** sweroside,amarogentin, Swertinin, swertianin, swerchirin	Swertiamarin	40, 56-57
C. pareira	Mensmine, pareirine, hayatinine, bebeerine, **beberine,** tetrandrine	Berberine	42, 58-59
H. indicus	**2-hydroxy-4methoxy-benzaldehyde,** Hemidesmin 1 and 2**,** α-amyrin,β-amyrin, lupeol	2-hydroxy-4-methoxybenzaldehyde	44-46

Table 3.5. HPTLC analysis of HC9 and individual plant materials

Marker compounds	Mobile phase	λ_{max} (nm)	Rf Value	Amount (%) of marker compound in HC9	Amount (%) of marker compound inindividual herbs
Picroside-I	Choloform: ethanol [8.8:1.2]	282	0.22	18.41	27.76
Nootkatone	N-hexane: EtOAc [3:7]	249	0.97	4.59	6.59
6-Gingerol	N-hexane: EtOAc [6:4]	282	0.6	12.04	46.03
Matairesinol	EtOAc:MeOH:FA:H_2O [7:1.5:0.5:1]	284	0.83	8.89	30.01
Berberine	*n*-but: EtOAc: GAA:H_2O [3:5:1:1]	350	0.29	4.06	10.36
Conesine	Toulene: EtOAc: diethylamine [6.5:2.5:1]	520	0.68	1.66	6.58
Swertiamarin	EtOAc:MeOH:H_2O [7.5:1.5:1.2]	244	0.66	4.41	12.43
Berberine	*n*-but: EtOAc: GAA: H_2O [3:5:1:1]	350	0.29	4.2	5.95
2-hydroxy-4-methoxybenzaldehyde	Toulene: EtOAc: GAA [7:2:1]	282	0.91	12.72	13.78

EtOAc: ethyl acetate; **MeOH:** methanol; **FA:** formic acid; **H_2O**: water; ***n*-but:** n-butanol; **GAA:** glacial acetic acid

Discussion

The therapeutic value of any herbal drug depends on its quality, clinical efficacy and lack of toxic side effects (Samarakoon SR et al., 2010). In the present chapter, the standardization of polyherbal formulation HC9 was done according to Ayurvedic Pharmacopoeia of India guidelines (Department of AYUSH, Government of India). HC9 was standardized by evaluating different parameters such as organoleptic characters, physicochemical parameters, preliminary phytochemical screening and HPTLC profiles. Organoleptic properties of powdered form of HC9 were evaluated by color, odor, taste, texture etc for identification of right variety and search of adulterants. These parameters form the basic criteria for selecting a raw drug. Fine texture of powdered HC9 indicated the smoothness and surface uniformity.

Physicochemical properties of HC9 were evaluated by determining total ash content, ethanol soluble extractive value, loss on drying at 105ºC, pH, specific gravity and bulk density of HC9 extract. Determination of total ash value is an important criteria to judge the authenticity and purity of the crude drug (Chandel HS et al 2011). It indicates total amount of inorganic material present in the drug after its complete incineration. A high ash value indicates contamination, substitution, adulteration during the preparation of drug (Saraf S et al 2010). The results indicated that HC9 has low inorganic material. Moreover, an extractive value indicates the amount of active ingredient present in the given amount of plant material when extracted with respective solvent (Chandel HS et al 2011). Less extractive value indicates addition of exhausted material, adulteration or incorrect processing during drying, storage or formulation preparation. The alcohol-soluble extractive of HC9 was found to be within the acceptable range. The test for loss on drying actually determines water as well as volatile matter content in drug when subjected to heat. The high moisture

content in herbal drugs endorses microbial as well as insect contamination. The low moisture content is always desirable for higher stability of drugs. Our results showed that the formulation could be stored for a long period and would not be easily contaminated with microbes (Saraf S et al 2010).

The preliminary phytochemical screening of HC9 extract was carried out to determine the various phytocompounds present in the extract (Samarakoon SR et al., 2010). Our phytochemical screening revealed that ethanolic extract of HC9 contained secondary metabolites such as alkaloids, flavanoids, saponins, tannins, carbohydrates and phenolic compounds. However, terpenoids were absent.

HPTLC chromatographic analysis of ethanolic extracts of individual herbs and HC9 formulation was carried out to identify the authenticity of the HC9 formulation based on their marker compounds. HPTLC profiles showed appropriate peaks for standard compounds at particular Rf. The similar peak profiles were observed in all respective individual extracts as well as in whole HC9 formulation. This indicated that, all the component herbs of HC9 are authentic and all are present in the HC9 formulation.

These results could be used as a reference standard for quality control and standardization of polyherbal formulations that could help in strengthening the use of medicinal herbs for their possible clinical application.

Chapter 4

Pre-clinical safety and toxicity evaluation of HC9 in Swiss albino mice

The research work in this chapter has been communicated

Abstract

In the current study, we have reported the acute and sub-acute oral toxicity of HC9 in Swiss albino mice. In acute toxicity study, the mice were orally given two different doses (1750 and 2000 mg/kg) of HC9 at one time only and were monitored for 14 days. In the sub-acute toxicity study, mice were given oral gavages of HC9 of 250, 500 and 1000 mg/kg doses per day for 28 days, followed by examination using biochemical, hematological, urine and histopathological parameters. Our results demonstrated that HC9 was well-tolerated by mice up to 2000 mg/kg as it did not produce any adverse effects in them in terms of mortality as well as behavioral or clinical signs in acute toxicity study. In sub-acute toxicity study, no treatment-related adverse effects were found in the mice upto 1000 mg/kg/day dose. HC9 did not produce any adverse effects in biochemical, hematological, urine and histopathological parameters indicating that HC9 was safe for its possible therapeutic applications.

4.1. Introduction

Traditionally, the herbal medicines are given in the form of polyherbal formulations (PHFs) as each ingredient is supposed to have different pharmacological function (Aggarwal et al., 2011; Kennedy et al., 2011; Kumar et al., 2011). Since, PHFs have a combination of compounds, it may be possible that one compound may either potentiate the effect or increase the bioavailability or decrease the toxicity of other pharmacologically active compound(s) (Aggarwal et al., 2011). PHFs are usually prescribed to be taken for a longer period of time and hence may cause adverse effects in the patients, thereby warranting evaluation of their efficacy and safety profile (Efferth et al., 2011; Hussin, 2001).

Considering the importance of the safety aspect of herbal formulations, in the present chapter we have performed the acute and sub-acute oral toxicity of HC9 in both the sexes of Swiss albino mice to evaluate its toxicity, safety and tolerability profile. The samples were analyzed for hematological, biochemical and histopathological parameters. The studies were done according to the Organization for Economic Cooperation and Development (OECD) guidelines for testing of chemicals and were in accordance with modern Good Laboratory Practice Regulations.

4.2. Materials and Methods

4.2.1. Plant materials and preparation of ethanolic extract

Ethanolic extract of HC9 was prepared as described in chapter 2 section 2.1.2.2

4.2.2. Animals and maintenance

Healthy Swiss albino mice, 6-8 weeks old, of either sex, having body weights in the range of 20±3 g were procured from the animal house of Bioscience (Pune, Maharashtra, India). They were randomly divided into different experimental groups.

The animals were housed in polypropylene cages at an ambient temperature of 21±3^{0}C and 30-70% relative humidity, with a 12:12 h light/dark rhythm. Animals were acclimatized to laboratory conditions for at least one week prior to the start of the experiment. They were provided with commercial food pellets (Nutrivet, Pune) and water ad libitum unless stated otherwise. The study was approved by the Institutional Ethics Committee (CPCSE Reg.No.258/CPCSE) of the Medical College of Bharati Vidyapeeth University, Pune.

4.2.3. Acute toxicity study

The acute toxicity of HC9 was evaluated in female Swiss albino mice having body weights in the range of 18-22g. The study was performed according to OECD guideline 423. The animals were divided into 3 groups, with three animals per group.Group I was kept as a vehicle control while Groups II and III served as test groups. All the mice were fasted prior to oral gavage with HC9 for 1-2 h. Individual body weights of animals were taken before dosing. HC9 was administered orally at 1750 and 2000 mg/kg dose of body weight, whereas the control group received distilled water only. Food or water was withheld for 2 h after drug treatment. The animals were closely monitored for initial 4 h after the administration of HC9 and then daily for 14 days to record any signs of toxicity such as tremors, convulsions, salivation, hyperactivity, ataxia, diarrhea, lethargy, sleep, coma, mydriasis, piloerection, gasping as well as mortality (Chandra et al., 2012; Rashid et al., 2012). At the end of the study, all the animals were sacrificed to analyze the effect of HC9 on different organs of the mice.

4.2.4. Sub-acute toxicity study

The sub-acute toxicity study of HC9 was performed in Swiss albino mice of either sex having body weights in the range of 18-22 g. All the animals were randomly distributed into 6 different groups (Group I-VI) comprising of ten animals (5 males and 5 females) per group (Table 4.1). The sub-acute toxicity study was performed according to OECD guideline 407. Group I served as a vehicle control and received only distilled water. Group III, IV and V received HC9 orally at the doses of 250, 500, 1000 mg/kg, respectively, everyday for 28 days. Group II and VI served as satellite control and high dose reversible groups, respectively. Group II received only distilled water and Group VI received 1000 mg/kg dose of HC9 orally for 28 days. The satellite/reversible groups were further observed for next 2 weeks post-treatment to observe the reversibility, persistence, or delayed occurrence of toxic effects of HC9 and were sacrificed on 43rd day (Table 4.1).

Table 4.1: Study design for sub-acute toxicity of HC9 treatment in Swiss albino mice

Groups	HC9 dose (mg/kg/body weight)	No. of mice	Treatment Days	Sacrificed on day
I (Untreated control)	0	10	28	29
II (Reversal/satellite control)	0	10	28	43
III (Low dose treatment)	250	10	28	29
IV(Inter dose treatment)	500	10	28	29
V(High dose treatment)	1000	10	28	29
VI (High dose reversal/satellite)	1000	10	28	43

Body weight of animals as well as their food and water consumption was recorded weekly throughout the study period. The animals were observed for signs of toxicity and mortality throughout the experimental period. The urine samples and blood samples of all the animals were taken prior to necropsy. After sacrificing the animals, different organs were collected for histopathological analysis.

4.2.5. Urine analysis

During the last week of the study period, urine samples from all the animals were collected and their analysis was done to evaluate various parameters such as appearance, specific gravity, pH, protein, ketone bodies, glucose, nitrite, urobilinogen, leucocytes and occult blood. Dx Urine test 10 (Piramal healthcare Limited, Mumbai) reagent strips were used for urine analysis.

4.2.6. Hematological analysis

The animals were fasted overnight prior to necropsy and blood collection (Rashid et al., 2012). Blood samples were collected from retro-orbital sinus puncture technique. Different parameters such as hemoglobin (Hb), platelet count (P), lymphocyte count (L), eosinophil count (E), monocyte count (M), basophil count (B), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were estimated.

4.2.7. Biochemical analysis

Biochemical analysis of serum samples was performed to analyze various parameters such as serum creatinine (CREA), triglycerides (TG), total protein (TP), albumin (ALB), total bilirubin (T-BIL), direct bilirubin (D-BIL), total cholesterol (TCHOL), blood urea, glucose, sodium (Na), potassium (K), serum glutamate oxaloacetete trasaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP).

4.2.8. Histopathological Analysis

After blood collection, animals were sacrificed and different organs such as brain, kidney, adrenal gland, liver, heart, spleen, thymus, lungs, testis/uterus, and epididymis/ovaries were collected from each mouse to observe histopathological changes in the organs. The absolute weight of all the organs was recorded and the relative organ weight was calculated as:

$$\text{Relative organ weight } = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of mouse on sacrificed day (g)}} \times 100$$

The organs were then fixed in 10% neutral buffered formalin for 18 h at 4°C and processed by conventional techniques. Paraffin sections were stained with hematoxylin and eosin, following the standard laboratory procedures. The stained sections were examined under microscope for any cellular damage or change in morphology.

4.2.9. Statistical Analysis

Data has been presented as mean ± S.D. Statistical analysis was performed with Sigma Stat 3.5 program (Systat Software, Inc.) by using two-way ANOVA with α=0.05.

4.3. Results

4.3.1. Acute Toxicity Study

In the acute toxicity study, female Swiss albino mice were orally administrated with two different doses (1750 and 2000 mg/kg) of HC9 and monitored for 14 days. The treated mice didn't exhibit mortality, body weight change, any signs of behavioral changes or toxicity compared to the control group. Morphological characteristics such as fur, skin, eyes, and nose appeared normal. There were no signs

of tremors, convulsion, salivation, diarrhea, lethargy or unusual behavior such as self mutilation or walking backward and so forth, change in gait and posture; reactivity to handling or sensory stimuli, or change in grip strength in the treatment groups.

4.3.2. Sub-acute Toxicity Study

4.3.2.1. General behavior and mortality

Daily oral administration of HC9 at doses of 250, 500 and 1000 mg/kg for 28 days did not produce any abnormality and toxicity symptoms in mice of either sex. The treated mice did not show any abnormal clinical signs such as tremors, convulsions, salivation, hyperactivity, ataxia, diarrhea, lethargy, sleep, coma, mydriasis, piloerection and gasping. No mortality was recorded in any mice due to HC9 treatment. In high dose reversible/satellite group treated with 1000 mg/kg HC9, there was no post-treatment related toxicity symptoms or mortality in any of the mice compared to the satellite control.

4.3.2.2. Changes in body weight and food consumption

Changes in body weight and food consumption of male and female mice were recorded weekly during the treatment period as given in Table 4.2 and Table 4.3, respectively. No significant difference ($p>0.05$) in body weights of male and female groups was recorded between control and HC9 treated groups (Table 4.2a and 4.2b, respectively). There was non-significant difference ($p>0.05$) within the groups compared to their initial body weights.

In food consumption studies, there was no significant difference ($p>0.05$) in food consumption pattern of HC9 treated groups compared to their respective controls

and also within the groups compared to their initial food consumption of either male or female mice (Table 4.3a and b, respectively).

Table 4.2a. Body weights of male Swiss albino mice treated with HC9

Weeks	Body weight change(gm)					
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
0	23.40±02.08	24.40±4.04	25.80±4.72	24.20±3.51	24.00±5.03	24.40±7.02
1	23.00±2.34	26.20±5.93	25.20±3.11	26.80±4.14	24.20±4.49	24.20±2.77
2	29.60±2.60	27.20±4.32	25.80±4.60	30.80±4.94	25.20±4.66	24.80±1.82
3	28.20±4.96	27.40±6.10	25.60±4.03	30.60±5.54	26.20±2.48	23.40±1.67
4	29.80±7.25	27.80±6.68	26.50±3.10	30.20±6.37	26.20±2.48	24.20±1.64
5		25.60±6.38				25.40±1.14
6		27.20±6.64				26.60±1.14

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared to the control mice ($p>0.05$).

Table 4.2b. Body weights of female Swiss albino mice treated with HC9

Weeks	Body weight change(gm)					
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
0	22.20±4.08	22.20±3.11	23.00±200	22.80±3.03	24.00±5.14	24.40±5.63
1	21.80±3.89	22.00±2.64	23.20±1.92	22.8±2.58	23.80±3.56	21.60±2.70
2	23.80±4.26	22.80±3.70	25.20±1.64	25.2±2.68	23.80±3.42	22.40±2.19
3	24.40±3.50	22.40±3.13	25.20±0.44	24.00±1.58	23.00±3.80	22.00±2.34
4	24.80±2.58	22.20±3.34	24.20±1.30	24.4±1.14	22.40±2.50	22.60±3.04
5		23.40±2.96				24.40±3.20
6		25.00±2.82				25.80±3.11

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05).

Table 4.3a. Food consumption of the male Swiss albino mice treated with HC9

Weeks	Food consumption (gm)					
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
1	5.75±0.59	6.24±1.41	6.30±0.78	5.36±0.83	8.07±1.50	6.05±0.69
2	7.40±0.65	8.50±1.76	6.45±0.96	5.70±0.92	7.41±0.64	6.89±0.46
3	8.00±0.53	6.52±1.45	6.10±0.96	6.65±1.21	6.55±0.62	8.36±0.60
4	6.21±1.51	6.62±1.59	7.00±0.71	5.39±1.14	5.95±0.57	6.72±0.46
5		4.13±1.03				5.77±0.26
6		4.69±1.75				6.94±1.70

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05).

Table 4.3b. Food consumption of the female Swiss albino mice treated with HC9

Weeks	Food consumption (gm)					
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
1	6.67±1.59	7.29±1.25	6.41±0.71	6.60±1.04	6.36±1.10	6.09±0.43
2	6.98±0.92	7.16±0.68	6.89±0.61	7.08±1.71	6.60±1.13	6.71±1.89
3	7.77±2.29	8.19±1.96	7.45±1.20	7.87±1.21	7.35±1.08	7.18±1.35
4	5.99±0.88	6.31±0.68	6.24±0.52	6.21±0.53	6.02±1.00	6.10±0.99
5		4.68±0.59				5.19±0.62
6		5.00±0.57				5.38±0.65

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05).

4.3.2.3. Urine Analysis

The urine analysis revealed no adverse effects due to HC9 treatment in any mice of either sex compared to the vehicle control group in the 28-day study (Table 4.4a-l). The urine analysis parameters such as appearance, blood, nitrate, leukocyte, glucose, pH, protein and specific gravity did not show any significant differences in HC9 treated mice of either sex compared to their respective control groups. All values were in normal range in control and treatment groups of both the sexes (Table 4.4a-h). The urine analysis of mice under high dose reversible/satellite groups also didn't reveal any significant differences compared to satellite control groups of either sex (Table 4.4i-l). Thus, the oral administration of HC9 did not affect urine parameters in mice

Table 4.4a: Urine analysis findings of the untreated control male mice (Group I)

Animal No	1	2	3	4	5	Normal range
Appearance	Yellow	Yellow	DY	PY	PY	PY to DY
Urobilinogen	1	0.2	1	0.2	0.2	0.2-1.0
Ketone	-	-	-	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	-	Trace	-	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.03	1.03	1.025	1.015	1.005-1.030
pH	5	8	6	6.5	6.5	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4b: Urine analysis findings of the untreated control female mice (Group I)

Animal No	1	2	3	4	5	Normal range
Appearance	DY	Yellow	Yellow	PY	Yellow	PY to DY
Urobilinogen	++	1	0.2	1	0.2	0.2-1.0 (Ehr U/dl)
Ketone	-	-	-	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	Trace	Trace	Trace	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.025	1	1.025	1.005	1.005	1.005-1.030
pH	6.5	8.5	6.5	7.5	7.5	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4c: Urine analysis findings of 250 mg/kg HC9 treated male mice (Group III)

Animal No	1	2	3	4	5	Normal range
Appearance	DY	PY	DY	PY	Yellow	PY to DY
Urobilinogen	1	0.2	1	1	1	0.2-1.0
Ketone	-	-	±5	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	Trace	-	-	Trace	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.03	1.03	1.03	1.03	1.005-1.030
pH	6.5	6	6.5	7	6	4.6-8.0

- **-:** Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4d: Urine analysis findings of 250 mg/kg HC9 treated female mice (Group III)

Animal No	1	2	3	4	5	Normal range
Appearance	Yellow	PY	DY	Yellow	DY	PY to DY
Urobilinogen	0.2	1	1	1	0.2	0.2-1.0
Ketone	-	-	-	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	-	Trace	-	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.02	1.025	1.025	1.03	1.005-1.030
pH	6	6.5	7	7.5	8	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4e: Urine analysis findings of 500 mg/kg HC9 treated male mice (Group IV)

Animal No	1	2	3	4	5	Normal range
Appearance	DY	DY	DY	DY	PY	PY to DY
Urobilinogen	++	1	1	1	1	0.2-1.0
Ketone	-	-	-	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	Trace	-	-	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.03	1.03	1.03	1.03	1.005-1.030
pH	6.5	6	6.5	7	6	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4f: Urine analysis findings of 500 mg/kg HC9 treated female mice (Group IV)

Animal No	1	2	3	4	5	Normal range
Appearance	Yellow	DY	PY	DY	PY	PY to DY
Urobilinogen	1	1	1	1	1	0.2-1.0
Ketone	-	-	-	-	±5	Negative
Blood	-	-	-	-	-	Negative
Protein	-	Trace	-	Trace	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.02	1.025	1.025	1.03	1.005-1.030
pH	6	6.5	7	7.5	8	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4g: Urine analysis findings of 1000 mg/kg HC9 treated male mice (Group V)

Animal No	1	2	3	4	5	Normal range
Appearance	DY	DY	DY	DY	DY	PY to DY
Urobilinogen	1	1	1	++	1	0.2-1.0
Ketone	-	-	-	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	Trace	Trace	Trace	-	-	Negative
Nitrate	-	-	+	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.03	1.025	1.025	1.025	1.005-1.030
pH	6.0	6.0	6.5	6.5	6.5	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4h: Urine analysis findings of 1000 mg/kg HC9 treated female mice (Group V)

Animal No	1	2	3	4	5	Normal range
Appearance	Yellow	Yellow	Yellow	PY	Yellow	PY to DY
Urobilinogen	1	1	0.2	0.2	1	0.2-1.0
Ketone	-	-	±5	-	-	Negative
Blood	-	-	Trace	-	-	Negative
Protein	-	-	-	Trace	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.010	1.025	1.025	1.025	1.025	1.005-1.030
pH	6.5	7.0	6.5	7.5	6.5	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4i: Urine analysis findings of the untreated reversal/satellite control male mice group (Group II)

Animal No	1	2	3	4	5	Normal range
Appearance	DY	PY	DY	DY	DY	PY to DY
Urobilinogen	1	1	1	1	1	0.2-1.0
Ketone	±5	-	-	±5	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	-	-	-	Trace	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.03	1.03	1.025	1.03	1.005-1.030
pH	6	6.5	6.5	7.5	6	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4j: Urine analysis findings of the untreated reversal/satellite control female mice group (Group II)

Animal No	1	2	3	4	5	Normal range
Appearance	DY	DY	DY	DY	DY	PY to DY
Urobilinogen	0.2	1	1	1	0.2	0.2-1.0
Ketone	±5	-	-	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	-	Trace	-	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.03	1.015	1.02	1.005	1.005-1.030
pH	6.5	6.5	6.5	8	8	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4k: Urine analysis findings of 1000 mg/kg HC9 treated reversal/satellite male mice (Group VI)

Animal No	1	2	3	4	5	Normal range
Appearance	PY	DY	DY	DY	PY	PY to DY
Urobilinogen	0.1	0.2	0.2	1	0.2	0.2-1.0
Ketone	-	-	-	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	Trace	-	-	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.025	1.03	1.03	1.005	1.005-1.030
pH	6.5	6.5	6.5	6.5	7	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

Table 4.4l: Urine analysis findings of 1000 mg/kg HC9 treated reversal/satellite female mice (Group VI)

Animal No	1	2	3	4	5	Normal range
Appearance	DY	PY	DY	DY	DY	PY to DY
Urobilinogen	1	1	1	1	1	0.2-1.0
Ketone	-	-	-	-	-	Negative
Blood	-	-	-	-	-	Negative
Protein	-	-	Trace	-	-	Negative
Nitrate	-	-	-	-	-	Negative
Leukocyte	-	-	-	-	-	Negative
Glucose	-	-	-	-	-	Negative
S.G.	1.03	1.03	1.03	1.03	1.03	1.00-1.03
pH	6	6	6	6.5	6	4.6-8.0

-: Negative; **+:** Positive; DY: Deep Yellow; PY: Pale Yellow

4.3.2.4. Hematological analysis

HC9 did not induce any abnormal changes in hematological parameters such as Hb, MCH, MCHC, PCV, MCV and differential cell counts (P, L, M, E, B) in male and female groups compared to their respective vehicle controls (Table 4.5a and b, respectively). In male groups, all the values, except for eosinophil count (E) of group II (satellite control group) were in the normal range (Table 4.5a). In female groups, all the values except, for eosinophil count (E) of IV group and MCV value in control group (Group I) were in the normal range (Table 4.5b). The slight differences in MCV or eosinophil (E) values in control or treatment mice of male and female groups could be due to some inherent variations in the mice as it is not reflected in other groups. Interestingly, the mice under high dose reversible/satellite groups did not show any significant differences in hematological parameters compared to the satellite control groups of either sex. These results suggest that administration of HC9 in mice did not affect the hematological parameters.

Table 4.5a: Hematological parameters of the male mice treated with HC9

Parameters[#]	I	II	III	IV	V	VI	Normal range
Hb (g/dl)	12.53±0.81	13±0.46	12.33±0.68	13.33± 0.47	13.4±0.2	12.41±0.46	10.2-16.6
P ($\times10^3$/µl)	320.66±6.03	370.66±15.53	390.00±5.00	343.33±5.13	371.66±8.62	400.00±8.00	140-450$\times10^3$
L ($\times10^3$/µl)	61.33±6.66	58.66±8.74	57.33±4.04	61.66±6.11	58.66±8.74	52.23±3.61	55.0-95.0
E (%)	2.66±0.58	4.33±1.53	2.08±1.00	2.00±1.00	2.33±0.58	2.09±1.00	0.0-3.9
M (%)	1.33±0.58	2.66±0.58	1.66±0.58	2.45±0.00	1.33±0.58	2.66±1.53	1.0-4.0
B (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.0-1.0
PCV (%)	39.7±1.44	40.53±1.80	38.1±2.91	41.2±2.1	40.93±0.85	38.33±1.08	39.0-49.0
MCV (fl)	53.86±0.55	53.06±2.06	51.46±1.39	53.09±1.23	50.60±0.44	51.43±1.66	45.4-60.3
MCH (pg)	14.96±0.40	15.16±0.40	14.46±0.42	14.76±0.55	14.43±0.42	15.06±0.21	14.1-19.3
MCHC (g/dl)	30.13±1.28	30.73±0.74	29.50±1.64	31.43±0.12	31.26±0.57	28.60±0.89	30.2-34.2

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice ($p>0.05$).

[#]**Hb**: Hemoglobin; **P:** Platelet counts; **L**: Lymphocyte count; **E**: Eosinophil count; **M**: Monocyte count; **B**: basinophil count; **PCV**: packed cell volume; **MCV**: Mean Corpuscular Volume; **MCH**:Mean Corpuscular Haemoglobin; **MCHC**: Mean Corpuscular Haemoglobin Concentration

Table 4.5b: Hematological parameters of the female mice treated with HC9

Parameters[#]	I	II	III	IV	V	VI	Normal range
Hb (g/dl)	12.80±1.10	12.13±0.87	12.33±0.93	12.27±0.57	12.83±0.93	12.83±0.64	10.2-16.6
P ($\times10^3$/µl)	403.00±3.61	405.67±5.03	400.67±3.79	290.00±4.58	390.00±2.65	353.33±3.06	140-450$\times10^3$
L ($\times10^3$/µl)	56.67±3.21	56.33±2.52	56.00±2.65	64.67±4.51	62.67±8.50	55.33±3.79	55.0-95.0
E (%)	1.67±1.15	3.00±2.00	2.00±1.00	4.67±0.58	3.00±1.00	3.33±0.58	0.0-3.9
M (%)	1.67±0.58	3.33±0.58	1.33±0.58	1.67±0.87	2.00±1.0	2.67±0.65	1.0-4.0
B (%)	0.00±0.00	0.00±0.00	0.00±0.0	0.00±0.00	0.00±0.00	0.00±0.00	0.0-1.0
PCV (%)	41.40±2.36	36.93±2.84	38.47±1.72	38.17±2.71	39.97±2.87	39.23±3.23	39.0-49.0
MCV (fl)	63.57±17.61	51.03±2.05	52.03±0.12	52.17±0.40	51.90±1.50	52.97±0.57	45.4-60.3
MCH (pg)	14.90±0.53	14.80±0.95	15.47±0.65	14.77±0.06	15.07±0.68	15.57±0.25	14.1-19.3
MCHC (g/dl)	30.40±1.42	28.87±2.20	30.30±1.04	30.20±0.26	29.80±1.80	29.77±0.92	30.2-34.2

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice ($p>0.05$).

[#]**Hb**: Hemoglobin; **P:** Platelet counts; **L**: Lymphocyte count; **E**: Eosinophil count; **M**: Monocyte count; **B**: basinophil count; **PCV**: packed cell volume; **MCV**: Mean Corpuscular Volume; **MCH**:Mean Corpuscular Haemoglobin; **MCHC**: Mean Corpuscular Haemoglobin Concentration

4.3.2.5. Biochemical analysis

Biochemical parameters of control and HC9 treated male and female groups are shown in Table 4.5a and 4.5b, respectively. HC9 did not cause any statistically significant changes ($p>0.05$) in liver and kidney function tests such as serum glutamate oxaloacetete trasaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), serum creatinine (CREA), triglycerides (TG), total protein (TP), albumin (ALB), total bilirubin (T-BIL), D-BIL, total cholesterol (TCHOL), blood urea, glucose as well as levels of electrolytes such as sodium (Na) and potassium (K) in treated groups compared to their respective vehicle controls. All the values were within normal range in both male and female mice (Table 4.6a and 4.6b, respectively).

In male control groups, the values of urea, albumin, SGOT and SGPT were found to be outside the normal range. However, differences in SGPT levels between control group I and group IV and V; and between satellite control group II and group VI (high dose reversible) were found to be non-significant ($p>0.05$) (Table 4.6a). In female control groups also, the values of urea, albumin and SGPT were outside the normal range. Interestingly, upon HC9 treatment, the values of SGPT decreased in treatment groups compared to their respective controls (Table 4.6b). These results suggest that control mice had some inherent problem with liver function but overall HC9 did not significantly affect the biochemical parameters in treated mice.

Table 4.6a: Biochemical findings of the male mice treated with HC9

Parameters[#]	I	II	III	IV	V	VI	Normal range
TCHOL (mg/dl)	83.33±17.39	73.00±4.58	73.00±25.12	69.33±10.69	76.00±9.17	70.33±4.04	40.0-130.0
GLU (mg/dl)	102.67±37.07	144.00±23.58	85.00±17.69	114.00±8.72	80.67±9.45	122.67±43.75	62.0-175.0
UREA (mmol/l)	42.00±8.19	39.67±5.13	40.00±3.00	38.00±3.0	37.67±3.21	36.67±4.73	8.0-33.0
CREAT(µmol/l)	0.70±0.11	0.53±0.08	0.62±0.12	0.58±0.06	0.51±0.03	0.47±0.05	0.2-0.9
TG (mg/dl)	29.00±1.73	37.67±4.04	30.67±17.21	24.67±11.02	30.67±6.11	31.33±6.66	-
NA (mmol/l)	147.67±6.43	147.67±2.52	153.33±6.43	149.67±1.53	152.67±8.74	148.67±4.51	140.0-160.0
K (mmol/l)	5.57±0.31	6.17±0.21	5.80±0.30	5.73±0.57	6.03±0.49	5.67±0.25	5.0-7.5
TP (g/l)	7.17±0.12	7.17±0.38	7.17±0.38	6.83±0.06	6.60±0.61	6.73±0.12	3.5-7.2
ALB (g/l)	3.57±0.25	3.93±0.15	3.83±0.12	3.60±0.36	3.80±0.44	3.17±0.15	2.5-3.0
T-BIL (µmol/l)	0.40±0.10	0.47±0.05	0.37±0.06	0.37±0.06	0.40±0.10	0.46±0.10	0.0-0.9
D-BIL (µmol/l)	0.22±0.01	0.24±0.04	0.23±0.06	0.21±0.02	0.22±0.03	0.23±0.06	< 0.3
SGOT (U/L)	308.33±140.56	305.67±112.59	188.00±8.89	304.67±123.71	270.33±77.73	249.00±88.54	54.0-298.0
SGPT (U/L)	119.67±9.87	176.67±11.59	114.33±33.72	156.33±22.50	188.33±16.86	210.00±40.29	17.0-77.0
ALP (U/L)	47.33±13.65	67.67±4.73	70.00±9.85	67.67±16.04	75.00±15.10	62.67±8.50	35.0-96.0

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice ($p>0.05$).

[#]**TCHOL**: Total Cholesterol; **Glu**: Glucose; **UREA**: Blood Urea; **CREA**: Serum Creatinine; **TG**: Triglycerides; **NA**: Sodium; **K**: Potassium; **TP**: Total Protein; **ALB**: Albumin; **T-BIL**: Total Bilirubin; **D-BIL**: Direct Bilirubin; **SGOT:** Serum Glutamate Oxaloacetete Trasaminase; **SGPT**: Serum Glutamate Pyruvate Transaminase; **ALP**: Alkaline Phosphatase

Table 4.6b: Biochemical findings of the female mice treated with HC9

Parameters[#]	I	II	III	IV	V	VI	Normal range
TCHOL (mg/dl)	80.33±11.02	78.67±10.50	64.00±12.77	81.33±16.07	76.33±5.69	83.33±8.74	40.0-130.0
GLU (mg/dl)	104.33±28.59	114.33±43.66	113.67±43.00	120.67±13.87	96.67±19.50	99.67±13.32	62.0-175.0
UREA (mmol/l)	40.00±3.61	45.00±5.20	34.67±6.66	39.67±2.52	36.33±3.21	37.67±4.16	8.0-33.0
CREAT (µmol/l)	0.49±0.03	0.64±0.11	0.64±0.17	0.41±0.03	0.49±0.03	0.43±0.05	0.2-0.9
TG (mg/dl)	32.33±4.16	35.00±3.61	28.67±7.09	35.67±15.53	33.67±5.03	36.33±5.03	-
NA (mmol/l)	148.67±4.16	148.00±1.00	150.33±4.16	149.33±3.51	149.00±6.24	154.67±1.53	140.0-160.0
K (mmol/l)	6.23±0.40	6.00±0.26	5.50±0.26	5.90±0.40	5.90±0.56	6.17±0.25	5.0-7.5
TP (g/l)	6.20±0.36	7.00±0.10	6.77±0.59	6.83±0.15	6.53±0.40	6.77±0.21	3.5-7.2
ALB (g/l)	3.33±0.45	3.83±0.12	3.77±0.25	3.57±0.29	3.37±0.40	3.50±0.46	2.5-3.0
T-BIL (µmol/l)	0.33±0.06	0.44±0.10	0.39±0.19	0.40±0.08	0.36±0.07	0.40±0.04	0.0-0.9
D-BIL (µmol/l)	0.18±0.09	0.20±0.06	0.21±0.12	0.26±0.05	0.18±0.03	0.22±0.02	< 0.3
SGOT (U/L)	298.07±107.05	195.33±42.52	190.67±11.06	302.00±110.96	244.33±23.09	149.00±28.28	54.0-298.0
SGPT (U/L)	237.00±86.28	269.33±64.44	151.00±26.91	173.33±4.51	224.67±31.94	166.33±8.33[**]	17.0-77.0
ALP (U/L)	64.67±7.77	55.67±24.66	66.00±6.56	71.00±12.12	74.00±0.12	85.67±7.57	35.0-96.0

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice ($p>0.05$). [**]Significantly different from the control at $p<0.01$.

[#]**TCHOL**: Total Cholesterol; **Glu**: Glucose; **UREA**: Blood Urea; **CREA**: Serum Creatinine; **TG**: Triglycerides; **NA**: Sodium; **K**: Potassium; **TP**: Total Protein; **ALB**: Albumin; **T-BIL**: Total Bilirubin; **D-BIL**: Direct Bilirubin; **SGOT:** Serum Glutamate Oxaloacetete Trasaminase; **SGPT**: Serum Glutamate Pyruvate Transaminase; **ALP**: Alkaline Phosphatase

4.3.2.6. Organ Weights

The relative organ weights of the mice treated with HC9 are shown in Table 4.7. There was no statistically significant difference ($p>0.05$) in relative and absolute organ weights of male and female mice (Table 4.7a-b and Table 4.8a-b, respectively) of the non-reversible and high dose reversible treated groups compared to their respective controls.

Table 4.7a: Relative organ weight of male Swiss albino mice treated with HC9

Relative organ weights of male mice (mg)						
Organs	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
Brain	1.91±0.19	2.02±0.87	1.56±0.24	1.56±0.37	1.63±0.28	1.59±0.16
Liver	7.92±0.92	7.32±2.95	6.04±1.20	7.35±2.86	6.47±0.88	5.87±0.32
Kidney	1.51±0.34	1.56±0.65	1.39±0.41	1.50±0.51	1.46±0.27	1.38±0.14
Adrenal	0.03±0.01	0.03±0.01	0.02±0.00	0.03±0.01	0.02±0.00	0.02±0.00
Heart	0.54±0.13	0.64±0.27	0.51±0.06	0.57±0.12	0.57±0.07	0.60±0.10
Thymus	0.23±0.03	0.20±0.04	0.18±0.01	0.19±0.05	0.19±0.04	0.19±0.04
Lung	1.11±0.22	1.13±0.34	0.97±0.27	1.08±0.35	0.68±0.19	0.95±0.18
Spleen	0.57±0.22	0.58±0.12	0.46±0.13	0.46±0.15	0.58±0.19	0.40±0.07
Testis	0.77±0.23	0.93±0.44	0.72±0.09	0.59±0.09	0.71±0.08	0.88±0.08
Epididymids	0.27±0.06	0.32±0.12	0.25±0.03	0.28±0.10	0.27±0.10	0.35±0.02

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice ($p>0.05$).

Table 4.7b. Relative organ weight of female Swiss albino mice treated with HC9

Relative organ weights of female mice (mg)						
Organs	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
Brain	1.83±0.36	1.64±0.32	1.73±0.06	1.62±0.04	1.83±0.06	1.69±0.13
Liver	7.16±0.39	5.61±1.01	6.69±0.86	6.71±0.20	6.13±0.61	5.46±0.42
Kidney	1.20±0.21	1.18±0.26	1.05±0.08	1.15±0.14	1.10±0.12	1.11±0.21
Adrenal	0.02±0.00	0.02±0.00	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01
Heart	0.55±0.11	0.57±0.09	0.56±0.12	0.53±0.03	0.54±0.09	0.55±0.02
Thymus	0.30±0.16	0.29±0.07	0.23±0.04	0.27±0.05	0.28±0.04	0.27±0.06
Lung	1.31±0.10	1.21±0.59	1.23±0.16	1.29±0.26	1.27±0.19	1.18±0.12
Spleen	0.49±0.12	0.48±0.05	0.45±0.10	0.46±0.05	0.42±0.12	0.49±0.06
Ovaries	0.08±0.03	0.06±0.01	0.07±0.03	0.09±0.02	0.08±0.00	0.06±0.00
Uterus	0.33±0.06	0.31±0.07	0.30±0.03	0.25±0.02	0.32±0.05	0.28±0.07

Values are expressed as mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice ($p>0.05$).

Table 4.8a. Absolute organ weight of male Swiss albino mice treated with HC9

Organ weights of male mice (mg)						
Organs	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
Brain	0.42±0.02	0.49±0.08	0.40±0.02	0.43±0.02	0.43±0.04	0.42±0.03
Liver	1.76±0.46	1.77±0.29	1.56±0.28	1.99±0.39	1.71±0.15	1.54±0.11
Kidney	0.37±0.03	0.38±0.06	0.36±0.09	0.36±0.04	0.38±0.03	0.36±0.02
Adrenal	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0
Heart	0.15±0.04	0.15±0.03	0.15±0.01	0.13±0.01	0.18±0.03	0.16±0.02
Thymus	0.09±0.06	0.04±0.02	0.04±0.01	0.05±0.01	0.04±0.0	0.05±0.01
Lung	0.24±0.04	0.28±0.05	0.16±0.10	0.29±0.03	0.18±0.01	0.25±0.06
Spleen	0.12±0.03	0.15±0.02	0.12±0.02	0.13±0.02	0.15±0.04	0.11±0.01
Testis	0.19±0.03	0.22±0.04	0.19±0.02	0.17±0.03	0.18±0.03	0.23±0.02
Epididymids	0.06±0.0	0.08±0.02	0.07±0.01	0.08±0.01	0.07±0.02	0.09±0.01

Values expressed are mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice ($p>0.05$).

Table 4.8b. Absolute organ weight of female Swiss albino mice treated with HC9

Organ weights of female mice (mg)						
Organs	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
Brain	0.44±0.05	0.42±0.04	0.43±0.02	0.39±0.00	0.40±0.03	0.43±0.02
Liver	1.75±0.21	1.44±0.17	1.67±0.21	1.63±0.03	1.34±0.06	1.67±0.27
Kidney	0.24±0.01	0.30±0.02	0.26±0.02	0.28±0.04	0.28±0.03	0.30±0.05
Adrenal	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0
Heart	0.18±0.08	0.14±0.01	0.14±0.03	0.13±0.01	0.12±0.02	0.13±0.01
Thymus	0.07±0.03	0.07±0.01	0.06±0.01	0.06±0.01	0.07±0.01	0.06±0.01
Lung	0.33±0.04	0.30±0.10	0.28±0.04	0.29±0.09	0.28±0.02	0.27±0.04
Spleen	0.12±0.04	0.12±0.01	0.11±0.02	0.10±0.01	0.09±0.03	0.13±0.01
Ovaries	0.02±0.01	0.01±0.00	0.02±0.01	0.02±0.01	0.02±0.00	0.01±0.00
Uterus	0.08±0.01	0.08±0.01	0.06±0.01	0.06±0.01	0.07±0.01	0.07±0.02

Values expressed are mean ± standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice ($p>0.05$).

4.3.2.7. Histopathological Analysis

Histological examinations of different organs from control and HC9 treated mice were done to confirm whether there were any alterations in cell structure (Jothy et al., 2011). At necropsy, no treatment-related macroscopic changes were observed in any of the treated mice. Histopathological analysis of brain, kidney, adrenal gland, liver, heart, spleen, thymus, lungs, testis/uterus and epididymis/ovaries of HC9 treated groups did not reveal any microscopic changes (Table 4.9a-b) Some mild cloudy changes observed in kidney sections of male and female groups in both control and treatment groups were considered to be normal as these were also observed in vehicle controls of either sex (Table 4.9a-b). Mild swelling was observed in one of the mouse in high-dose male group (Group V) and not in other mice within the same group or other treated groups.

Table 4.9a. Histopathological findings of HC9 treated male mice

Organs	Group I			Group II			Group III			Group IV			Group V			Group VI		
Brain	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Liver	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Kidney	N	C	N	N	C	C	N	N	N	C	N	N	N	N	S	N	N	C
Adrenal Gland	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Heart	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Thymus	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Lung	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Spleen	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Testies	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Epididymids	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

N: Normal; C: Cloudy change; S: Swelling

Table 4.9b. Histopathological findings of HC9 treated female mice

Organs	Group I			Group II			Group III			Group IV			Group V			Group VI		
Brain	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Liver	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Kidney	N	C	N	N	C	C	N	N	N	C	N	N	N	N	S	N	N	C
Adrenal Gland	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Heart	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Thymus	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Lung	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Spleen	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Testies	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Epididymids	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

N: Normal**; C:** Cloudy change

Figures4.1a and 4.1b show representative histological sections of heart, kidney, liver, and lung from male and female mice from each group, respectively. The histological sections of all the treated mice showed normal cardiac muscle fibers in heart and normal glomeruli, tubules and interstitium in kidney. The sections showed normal heptatocytes and portal tracts in liver as well as normal architecture of lungs in all doses of HC9 compared to the vehicle control. Histological pictures of all the vital organs of other mice from respective groups were similar (data not shown) to that observed in Figure 4.1a-b.

Figure 4.1a. Histopathological findings of HC9 treated male mice

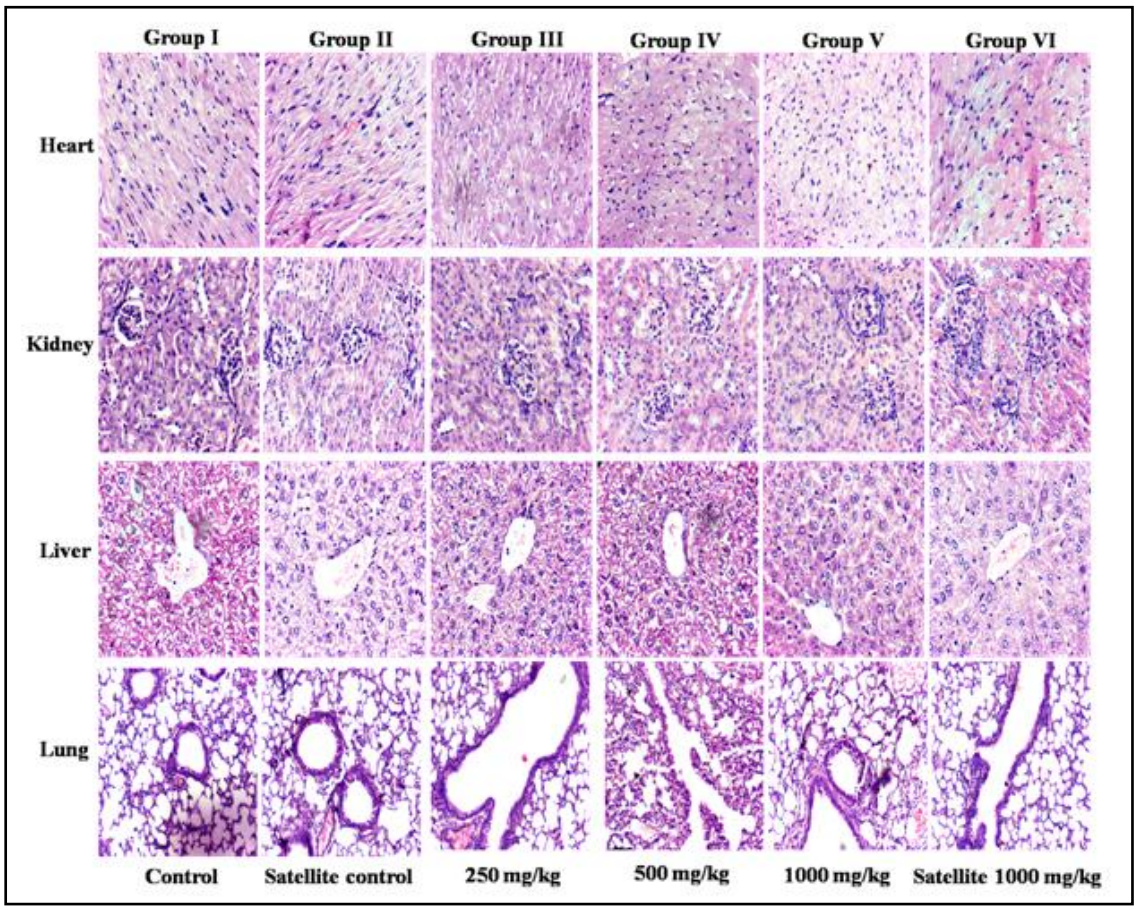
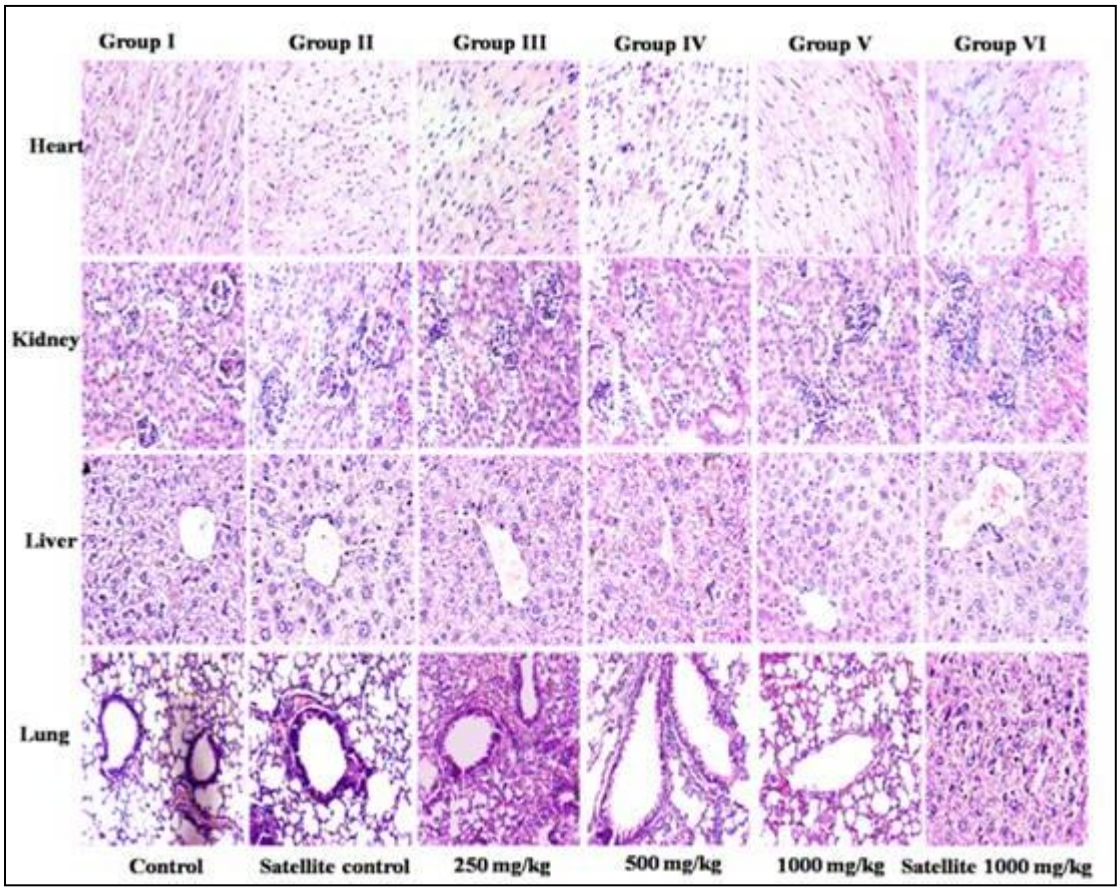


Figure 4.1b. Histopathological findings of HC9 treated female mice



4.4. Discussion

The safety and toxicity assessment of herbal medicines is rarely done before their human consumption as they are considered to be inherently safe (Bent S, 2008). The clinical use of herbal drugs without adequate scientific evidence has raised concerns regarding their toxicity status (Neergheen-Bhujun, 2013).Thus, toxicity evaluation of herbal medicines in various experimental animal models to predict their safety is extremely important for selecting a 'safe' dose for future human use (Jothy et al., 2011; Singh et al., 2013).In the previous part of thesis work, we have reported that HC9 has significant antioxidant potential and cytotoxic activity in breast cancer cell lines (Suryavanshi et al., 2011).We have also standardized the formulation with

respect to its marker compounds (Accepted in pharmacognosy journal, 2014). Since, HC9 is effective against breast cancer cell lines, we wanted to evaluate its safety using various biochemical, histological and other parameters in animal studies.

The acute toxicity of HC9 was mainly performed to identify a safe dose regimen and the therapeutic index of the drug for its possible future use. HC9 did not reveal any treatment related adverse effects on vital functions of the mice that included cardiovascular, central nervous and respiratory systems. Sub-acute toxicity study is a widely accepted test to evaluate any possible health hazards due to treatment with the drugs (Neergheen-Bhujun, 2013; Gautam et al., 2012). Interestingly, the 28-day sub-acute toxicity study did not show any adverse effects of HC9 on body weights, food consumption, urine analysis, hematology, serum biochemistry, gross pathology, organ weights or histopathology.

The hematological system is an important indicator of the physiological and pathological status of animals or humans (Adeneye et al., 2006). It is highly sensitive to toxic compounds and small changes in the hematological system could have higher predictive value for drug associated toxicity (Adeneye et al., 2006). All the hematological parameters in the treatment groups were within the normal range, except for eosinophil count (E) of 500 mg/kg treatment group of female mice. However, it could be due to some inherent variations in the mice as it is not reflected in other groups. Besides, other related parameters such as Hb, MCH, MCHC, PCV, MCV, P, L, M and B of the mice in the same group were normal. Moreover, the mice under high dose reversible/satellite groups didn't show any significant difference in hematological parameters compared to the satellite control groups of either sex. HC9 could be considered to be safe as it had no adverse effect on the hematological parameters in the mice.

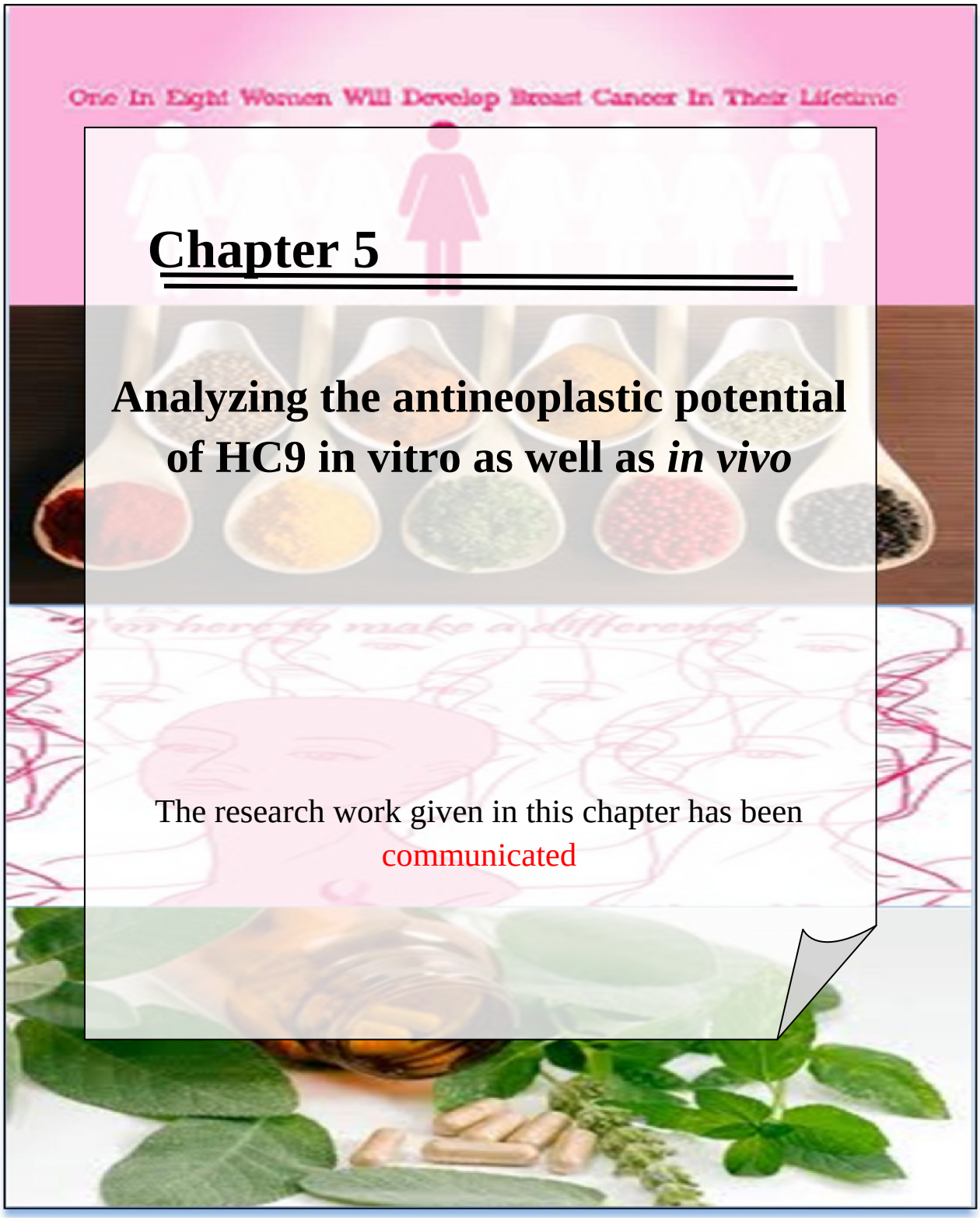
After hematological analysis, the biochemical parameters were evaluated using serum and urine sample of treated and untreated mice. Clinical biochemistry is mainly performed to evaluate the effect of drugs on hepatic and renal functions, serum electrolytes as well as glucose and total cholesterol levels (Ping et al., 2013). Hepatic biochemical parameters provide valuable information on the status of liver in terms of its functionality, cellular integrity, synthesis and its link with biliary tract (Agbaje et al., 2009). Enzymes such as SGPT, SGOT and ALP are well-known indicators of liver function that predict drug related toxicity (Singh et al., 2011). Since, liver is the major site of synthesis of cholesterol, protein and albumin as well as for cholesterol disposal or degradation; any changes in these parameters could be suggestive of liver dysfunction that may be due to drug toxicity (Singh et al., 2011; Pillai et al., 2011). HC9 did not cause any significant change in biochemical parameters for hepatic and renal functions such as SGOT, SGPT, ALP, triglycerides, protein, albumin, bilirubin and cholesterol. Though the values of urea, albumin, SGOT and SGPT of control and treated groups were found to be outside the normal range, however, upon HC9 treatment, the values of urea, albumin and SGOT were found to decrease in treatment groups compared to their respective controls. These results suggest that control mice may have some inherent problem with liver function. An increase in hepatic SGPT levels was observed at 500 mg/kg and 1000 mg/kg dose in male mice. Since SGOT levels did not exhibit any significant change compared to control groups even at higher doses, the increase in SGPT should not reflect any problem with liver function by HC9 treatment.

HC9 did not affect the serum levels of blood urea and creatinine in any mice of either sex at any dose compared to their respective controls. Blood urea and creatinine are important markers of renal toxicity (Neergheen-Bhujun, 2013, Fuchs et

al., 2012; Abotsi et al., 2011; Gowda et al., 2010). There are several reports of kidney toxicity related to the use of phytotherapeutic drugs, as kidneys eliminate many drugs and their metabolites (Agbaje et al., 2009). Our data shows that HC9 does not alter the kidney functions of mice.

Organ weight is an important index of physiological and pathological status of animals. HC9 didn't affect the absolute and relative organ weights of treated mice of either sex. Histological examinations also supported the conclusions from clinical biochemistry studies that oral administration of HC9 did not induce any renal or liver damage even at higher doses (1000 mg/kg). HC9 did not induce any pathological changes in heart, brain, adrenal gland, thymus, lung, spleen and reproductive organs such as testis/uterus and epididymis/ovaries of the mice.

Based on the biochemical, hematological and histological studies in Swiss albino mice, HC9, could be considered to be non-toxic and safe for its possible clinical applications.



Chapter 5

Analyzing the antineoplastic potential of HC9 in vitro as well as *in vivo*

The research work given in this chapter has been communicated

Abstract

The present study aimed to investigate the mechanisms underlying anticancer potential of standardized HC9 formulation in breast cancer cell lines in vitro as well as *in vivo* in mouse melanoma model. Our in vitro findings suggested that HC9 significantly altered growth kinetics of MCF-7 and MDA MB-231. It blocked the cell cycle progression at S phase in MCF-7 by up regulating the expression of p53, p21 and p16 proteins. Contrarily, it induced G1 phase arrest in MDA MB-231 by up regulating p53, p21 and pRb proteins with simultaneous decrease in phospo-Rb (ppRb) protein. In addition, HC9 significantly reduced migration and invasion in both the cell types that was accompanied by decreased expression of MMP-2/9, HIF-1α and VEGF proteins. HC9 decreased the expression of inflammatory markers (NF-κB, COX-2), and altered the expression of chromatin modulators (SMAR1 and CDP/Cux) in the breast cancer cells. In animal studies, HC9 significantly retarded the tumor volume of B16F10 induced tumors in C57BL/6 mice compared to the untreated tumor control group. All these data suggested that HC9 exhibited anticancer activity and could prove a potential drug candidate in breast cancer.

5.1. Introduction

The components of HC9 are known to exhibit different activities including anticancer, anti-inflammatory, antioxidant and immunomodulatory (Chapter-1). In earlier part of the thesis, we have reported that HC9 exhibited significant antioxidant potential and cytotoxic activity in breast cancer cell lines (Chapter-2). We standardized the formulation with respect to its marker compounds (Chapter-3) and carried out its toxicity evaluation in Swiss albino mice, which indicated that HC9 was non-toxic and safe for its possible therapeutic applications (Chapter-4). In this chapter, an attempt has been made to demonstrate the anticancer activity of HC9 in vitro in breast cancer cell lines as well as in mouse melanoma model.

5.2. Materials and Methods

5.2.1. Chemicals and reagents

Tissue culture plasticwares were purchased from BD Biosciences, (CA, USA); Axygen Scientific Inc, CA, USA and Nunc, Roskilde, Denmark. Dulbecco's Modified Eagles Medium (DMEM), Penicillin and streptomycin were obtained from Gibco BRL, CA, USA. Fetal bovine serum (FBS) was purchased from Moregate (Biotech, Australia), N. Z. SMAR1, p53, p21, p16, CDP/Cux, MMP-2, MMP-9, pRb, ppRb, VEGF, NF-κB, COX-2, tubulin and Donkey anti-Mouse IgG and donkey anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology, CA, USA; propodium iodide (PI) and FM® 4-64 dye were purchased from Invitrogen (CA, USA). Doxorubicin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse Th1/Th2 ELISA Ready-SET-Go kit was purchased from eBioscience (CA, USA). All other common reagents were procured from Qualigen fine chemicals (Mumbai, India).

5.2.2. Cell Culture

Two well-characterized human breast cancer cell lines MCF-7 (ER/PR+) and MDA-MB-231 (ER/PR-) as well as highly metastatic mouse melanoma cell line B16F10 were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 2mM L-glutamine supplemented with 10% fetal bovine serum (FBS) and 100U/ml penicillin-streptomycin antibiotics in tissue culture flasks under a humidifying atmosphere containing 5% CO2 and 95% air at 37°C. The cells grown in monolayers were washed with phosphate-buffered saline (PBS) followed by a brief incubation with trypsin/EDTA. The washed cells were isolated by centrifugation and resuspended in culture medium for plating or counting. Only cells growing at the exponential phase were used to perform all the experiments.

5.2.3. Cell growth analysis

Proliferation of MCF-7 and MDA MB-231 cells was determined by dye exclusion method. Briefly, the cells were seeded at a density of 1×10^5 cells/ml in 24-well plate in triplicates and were incubated for 24 hrs at 37^0C. After 24 hrs, the MCF-7 cells were treated with doses of 0, 10, 20, 40 µg/ml and MDA MB-231 cells were treated with 0, 20, 40, 80 µg/ml concentrations of HC9 respectively. The cells were counted after 24, 48 and 72 hrs by tryphan blue dye. The cell growth kinetics was obtained by plotting concentrations of HC9 against viability of cells at various time intervals (Choudhari et al., 2013).

5.2.4. Colony formation assay

Clonogenic survival determination assay was performed as explained previously (Choudhari et al., 2013). Briefly, log growth phase of MCF-7 and MDA-MB-231cells were trypsinized and plated at a density of 1×10^3 cells/ml in 6-well plates and incubated for 24 h at 37^0C. Next day, the medium was removed and fresh medium was added with the different concentrations of HC9 (0, 10, 20, 40 µg/ml for MCF-7 and 0, 20, 40, 80 µg/ml for MDA MB-231) followed by incubation for 7-10 days at 37^0C. The plates were observed under inverted compound microscope until sufficient colonies were formed. Once the colonies were formed, the media was removed and the cells were fixed with 4% paraformaldehyde followed by staining with 0.5% crystal violet dye. The colonies were photographed with Sony DSC-S75 cyber-shot camera (Choudhari et al., 2013).

5.2.5. Soft agar assay

MCF-7 and MDA-MB-231cells (5×10^3 cells/ml) treated with different concentrations of HC9 extract (0, 5, 10, and 20 µg/ml and 0, 20, 40, 80 µg/ml respectively) were suspended in culture medium containing 0.35% agarose (DNA grade, GIBCO BRL, CA, USA), mixed and plated over a previously gelled layer of 0.5% agarose in culture medium in 6-well plates. After incubation for 10 days, colonies were photographed directly using an Axiovert 200 M microscope (Carl Zeiss, Germany) and counted (Choudhari et al., 2013).

5.2.6. Wound healing assay

Scratch wound assay was used for mobility analysis. Cells were seeded into 24-well plates at a seeding density of 4×10^5/ml cells and were allowed to adhere overnight at

37°C in 5% CO_2 incubator. The confluent monolayer was carefully wounded using a sterile 10µl micropipette tip after 6 h serum starvation cells and the cellular debris was gently washed with 1XPBS. The wounded monolayer MCF-7 cells were treated with 0-40 µg/ml doses and MDA MB-231 cells were treated with 0-80µg/ml doses of HC9 extract. Migrating cells were examined and images of the cells at 0 hr as well as after 16 hrs were photographed using an Axiovert 200 M microscope (Carl Zeiss, Germany).

5.2.7. Matrigel transmembrane invasion assay

Cell migration across the matrigel barrier was determined by Boyden matrigel invasion Chambers (BD Bioscience, Bedford, MA). The matrigel coated inserts were re-hydrated with serum free DMEM for 30 min at 37°C in 5% CO_2 followed by the washing of inserts with serum free media. Exponentially growing breast cancer cells were harvested by trypsin/EDTA and suspended in serum free medium. The cells were seeded at the seeding density of 5×10^4/ml with or without treatment of HC9 extract into the invasion chambers and 10% DMEM medium was added as a chemo attractant to the lower chambers. The Boyden chambers were incubated at 37°C in 5% CO_2 and allowed to invade across the Matrigel-coated membrane for 24 hrs. Next day, non-invading cells in the upper chambers were removed by gently wiping with the wet cotton swabs. The invading cells that were transversed to the matrigel and attached to the bottom of the membrane were fixed with 10% formalin and stained with 0.5% crystal violet. The membranes were removed from the inserts and examined under microscope and the numbers of invading cells were counted.

5.2.8. Reverse Transcription PCR analysis

The total cellular RNA was extracted from control as well as cells treated with different concentrations of HC9 (10-80 μg/ml) by a one-step acid guanidine isothiocyanate-phenol method using TRI reagent (Invitrogen) from both the cell lines. RNA was precipitated with isopropanol and the concentration was quantified by using Nanodrop instrument (Eppendorff BioPhotometer plus). Total RNA was extracted and reverse transcribed as described previously (Deshpande et al., 2013).The expression of hif1α was examined by amplifying cDNA template using gene-specific primers by PCR method. The primers used were β-actin-F: 5'-taccactggcatcgtgatg-gact-3'; β-actin-R: 5'-tttctgcatcctgtcggaaat-3'; hif1α. The PCR was run as the following 25–30 cycles at 95°C for 5min, 95°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 1 min. and a final extension. 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 phosphor imager (Alpha Imager, Alpha Innotech) and normalized with respect to the β-actin product.

5.2.9. Gelatin zymography

The serum free condition medium collected from control as well as HC9 treated breast cancer cells (10-80μg/ml) were analyzed for pro-matrix metalloproteinase-9 (pro-MMP-9 or z-MMP-9) and pro-matrix metalloproteinase-2 (pro-MMP-2 or z-MMP-2) activity by gelatin zymography as described previously (Koppikar et al., 2010). Briefly, MCF-7 as well as MDA MB-231 cells were seeded at 5×10^5/ml seeding density in 6 well plates. The cells were treated with HC9 (0-80μg/ml) in serum free media for 24 hrs. The conditioned media from control as well as treated

cells were collected and concentrated in Centricon YM-30 tubes (Millipore, MA). The samples were subjected to 7.5% SDS-polyacrylamide gel containing gelatin (0.5 mg/ml) under non-reducing conditions. The gel was washed with 0.25% Triton X-100 followed by overnight incubation at 37°C in incubation buffer containing 150 mM NaCl, 100 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5, 1% Triton X-100 and 0.02% NaN_3. The gel was stained with 0.1% Coomassie Brilliant blue R-250 in 40% isopropanol and destained in 7% acetic acid, examined and photographed. Gelatinase activation was visualized as unstained clear bands against the blue-stained gelatin background. The quantification of bands in control and treated samples was performed by densitometric analysis on Alpha Imager using Alpha Ease FC software, Alpha Innotech.

5.2.10. Western blotting

For western blotting, the control as well as treated cells (10-80 µg/ ml) were harvested, washed with 1X PBS and lysed by lysis buffer as previously described (Choudhari et al., 2013). 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 TBST and incubated at room temperature for 3 hrs or overnight at 4ºC with mouse monoclonal antibodies against p53, SMAR1, p16, MMP-2 and tubulin (Santacruz, CA, USA) as well as with rabbit polyclonal antibodies against CDP/Cux, p21, Rb, phospo-Rb, VEGF, NFκB and goat polyclonal antibody against COX-2 (Santacruz, CA, USA) at 1:2000 dilutions respectively. The membrane was washed in TBST and incubated with secondary IgG HRP conjugate at

1:5000 dilutions. Proteins were visualized using a chemiluminescence kit (Amersham ECL Advance western blotting detection kit, GE Healthcare, UK) and densitometric analysis of X-ray films was performed by Image J gel analysis software.

5.2.11. Cell cycle analysis

Cell cycle distribution was determined using flow cytometry analysis. Briefly, MCF-7 and MDA MB-231 cells were seeded at 5×10^5/ml seeding density in 6 well plates and incubated overnight at 37°C. After treatments with HC9 extract (0-40 µg/ml for MCF-7 and 0-80 µg/ml for MDA MB-231) for 24 hrs, the cells were trypsinized, washed twice with 2 ml of 1X-PBS and centrifuged at 180g. The cells were then fixed with 70% ice cold ethanol and kept at 4°C for 30 mins followed by the incubation with RNase A (50 µg/ml) at 37°C for 30 minutes and staining with propidium iodine (50 µg/ml) for 1 hour at 0°C (Choudhari et al., 2013). Fluorescence intensities were measured at 525 nm by FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). The cell cycle profiles were modeled by using CellQuest software (Becton Dickinson) for the proportions of cells in G0-G1, S phase, and G2-M phases of the cell cycle.

5.2.12. Animals and maintenance

Healthy female C57BL/6 mice (average weight 18-22 g; 6-8 weeks n=6) were procured from National Institute of Health (NIH), Hyderabad, India and were housed in polypropylene cages at an ambient temperature of 21±3°C and 30-70% relative humidity, with a 12:12h light/dark rhythm and fed with commercial food pellets (Nutrivet Pune) and water ad libitum. Animals were acclimatized to laboratory conditions for at least one week prior to start of the experiment. This study was

approved by the Institutional Ethics Committee of the Medical college of Bharati Vidyapeeth, Pune. All the experiments were conducted in accordance with the guidelines of the Animal Ethics Research Board.

5.2.13. Tumor retardation study

B16F10 cells (5×10^5) were mixed with 1XPBS and injected subcutaneously into the right flank of female C57BL/6mice (average weight 18-22 g; 6-8 weeks n=6) (National Institute of Health (NIH), Hyderabad, India). The tumors were raised in one week and then mice were randomly divided into groups of six animals each. Group I was kept as only control with no tumors, group II was kept as tumor control who received the vehicle alone in parallel whereas group III was kept as positive control administrated with Doxorubicin (2 mg/kg bw) intravenously on every 1^{st}, 5^{th} and 9^{th} day after tumors were raised. Simultaneously, Group IV, V, VI were orally gavaged with the HC9 at concentration of 100 mg/kg, 200mg/kg, 400mg/kg bw respectively everyday for 3 weeks. The mice were weighed and tumors were measured using digital Vernier calipers once a week. Tumor volume was calculated using the formula: $1/2(\text{length} \times \text{width}^2)$. The urine samples and blood samples for cytokine study of all animals were taken prior to necropsy. All animals were sacrificed and different organs were collected for histopathological analysis (Zhou C et al., 2014).

5.2.14. Statistical analysis

All experiments were performed in triplicates and repeated at least three times to confirm the results and the data points shown in the figures were presented as mean ± SD. Statistical analysis was conducted with the Sigma Stat 3.5 program (Systat Software, Inc.) using one-way ANOVA with $\alpha=0.05$. Comparisons among groups

were made by analysis of variance. Probability values $p<0.05$ were considered statistically significant.

5.3. Results

5.3.1. HC9 modulated growth kinetics of MCF-7 and MDA MB-231

In earlier chapters, we have reported some important leads with the standardized HC9 formulation. It exhibited significant antioxidant potential and cytotoxic activity in breast cancer cell lines without affecting non-tumorigenic MCF-10A. In view of this finding, we used non-cytotoxic concentrations of HC9 extract (0-80 μg/ml) for our further studies and focused on elucidating the anticancer mechanism of HC9. We first determined the effect of HC9 on cell proliferation of MCF-7 and MDA-MB-231. Both the cell types were incubated with different concentrations of HC9 (0-40 μg/ml for MCF-7 and 0-80 μg/ml for MDA MB-231) for 24, 48 and 72 h before being harvested and assayed for cell viability by trypan blue dye exclusion assay.

HC9 exhibited a dose- and time-dependent anti-proliferative effect on MCF-7 and MDA MB-231 as compared to untreated control cells (Figure 5.1 A and B). In MCF-7, there was significant reduction in cell proliferation at 40 μg/ml in 24 hrs (~4.33 fold; $p=0.100$), 48 h (~3.11 fold; $p=0.004$) and 72 h (~2.65 fold; $p\leq0.001$) compared to that observed in the untreated control (Figure 5.1A). Similarly, HC9 significantly reduced cell growth of MDA MB-231 cells at 80 μg/ml concentration in 24 h (~2.67 fold; $p=0.100$), 48 h (~2.58 fold; $p=0.005$) and 72 h (~2.35 fold; $p=0.001$) compared to the untreated control cells (Figure 5.1B).

The anti-proliferative activity of HC9 on MCF-7 and MDA MB-231 cells was determined and verified by colony forming assay. The cells were plated at a very low

seeding density ($1x10^3$cells/ml) and were treated with different concentrations of HC9 for two weeks. HC9 significantly reduced the number of growing colonies compared to the untreated control cells (Figure 5.1C-D), the decrease being more prominent at the dose of 40 and 80 µg/ml of HC9 in MCF-7 (Figure 5.1D)and MDA MB-231(Figure 5.1D), respectively.

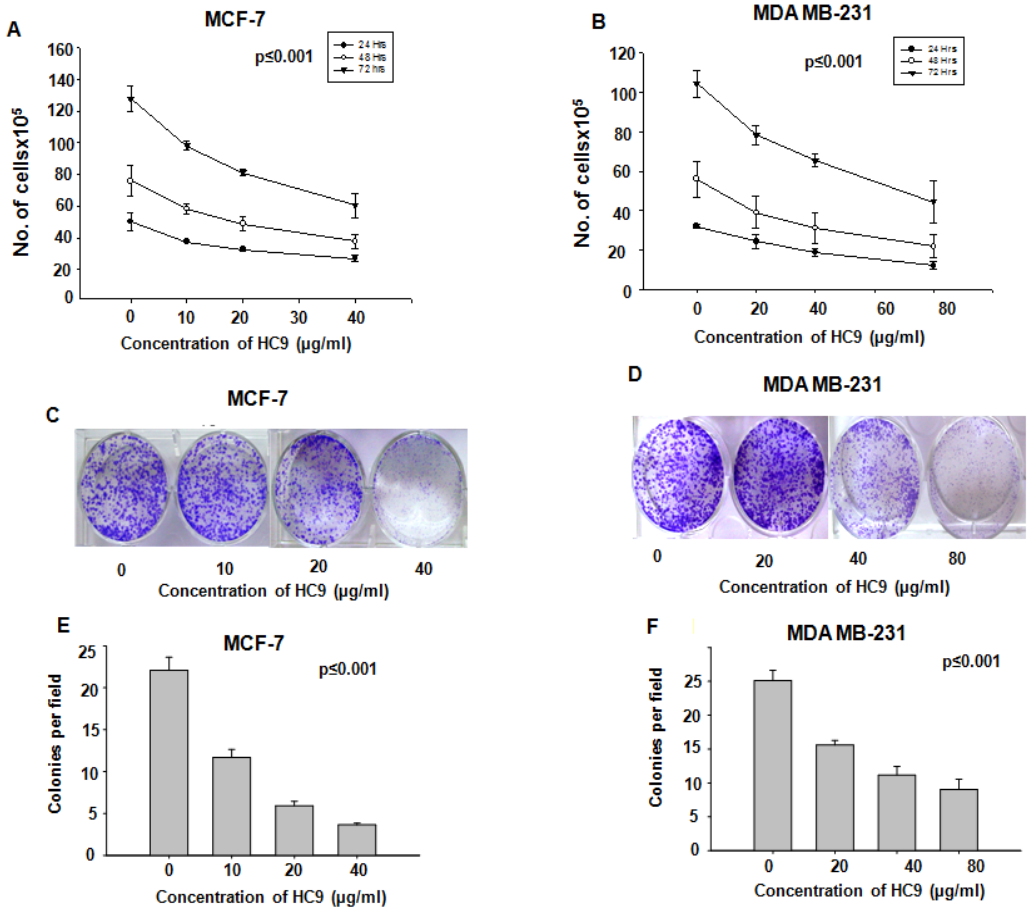


Figure 5.1: HC9 altered the growth of breast cancer cells. *MCF-7(**A**) and MDA MB-231 (**B**) were treated with HC9 (0–80 µg/ml) for 24, 48 and72 h and assayed for cell viability by trypan blue dye exclusion assay. Data represents mean ± S.D of three independent experiments. MCF-7 (**C**) and MDA MB-231 **(D)** cells were plated at very low seeding density ($1x10^3$ cells/ml) and were treated with different concentrations of HC9 for two week.The colonies were stained with crystal violet and photographed. The experiments were repeated three times. MCF-7(**E**) and MDA MB-231 **(F)** cells were mixed with HC9 (0-80 µg/ml) and 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 ± S.D of three independent experiments.*

This was further confirmed by soft agar assay that showed an inverse correlation between the number of soft agar colonies and the concentration of HC9 (Figure 5.1E and F). A significant dose dependent reduction in the number of soft agar colonies was found in both MCF-7 and MDA MB-231 compared to the control cells. At 40 µg/ml concentration of HC9 (Figure 5.1E), there was almost 6.09-fold reduction in MCF-7 ($p \leq 0.001$), whereas at 80 µg/ml concentration (Figure 5.1F), there was 2.80-fold reduction ($p \leq 0.001$) in MDA MB-231 compared to the untreated control cells. These findings suggested that HC9 significantly regulated the growth kinetics of the breast cancer cells.

5.3.2. HC9 induced S phase arrest in MCF-7 and altered the expression of cell cycle regulatory proteins

We further wanted to determine whether HC9 had any effect on the cell cycle. Both the cell types were treated with HC9 (0-80 µg/ml) for 24 h and analyzed for cell cycle phase distribution by flow cytometry. HC9 arrested the progression of MCF-7 in S phase compared to untreated control cells (Figure 5.2A-B). In particular, at 40 µg/ml, HC9 treatment induced accumulation of MCF-7 cells in S phase (from 18.62% in untreated cells to 24.73% in treated; $p=0.010$) and simultaneously resulted in the decrease in G2/M phase (from 23.77% in untreated control to 13.26% in treated; $p= 0.114$) (Figure 5.2A).

To understand the effect of HC9 on the molecular events involved in S phase arrest in MCF-7, we investigated its effect on expression of p53, p21 and p16 proteins by western blot analysis. Our data demonstrated a substantial dose-dependent increase in the expression of p53, its downstream target p21 and p16 compared to the untreated control cells (Figure 5.2C).

At 40 µg/ml of HC9, there was ~1.4 (p=0.067), ~4.5 (p=0.010) and ~4.56-fold (p=0.010) increase in the expressions of p53, p21 and p16, respectively, as determined by densitometric scanning (Figure 5.2C).

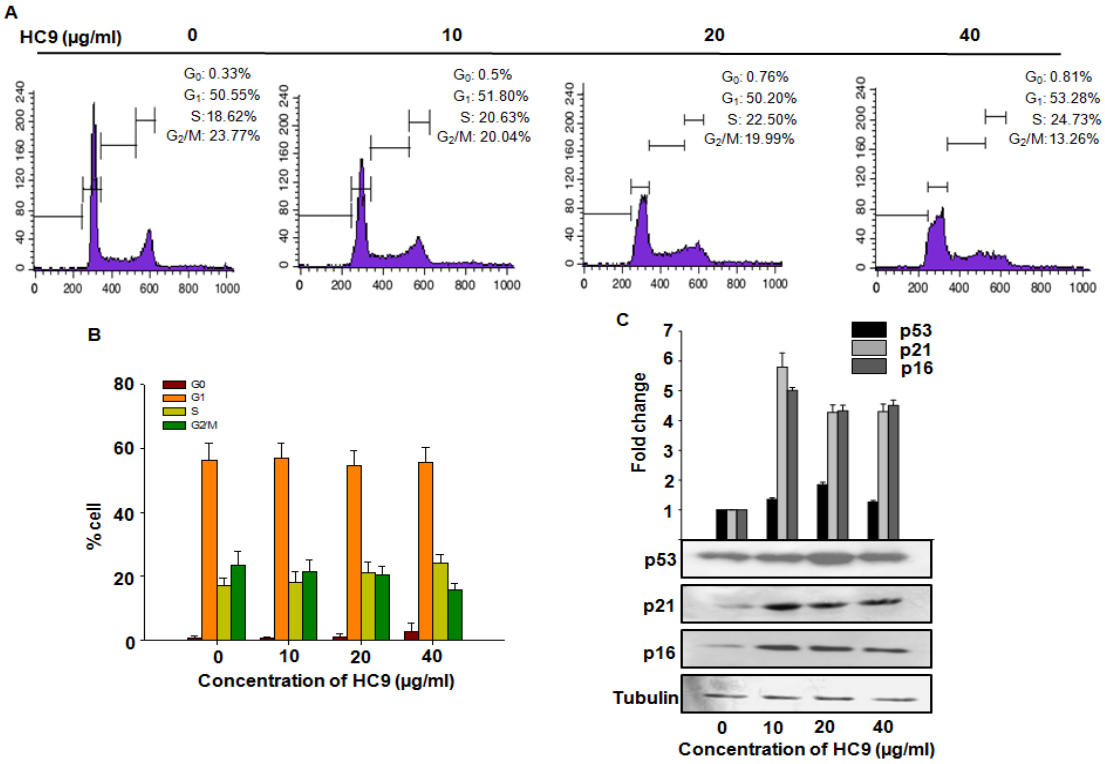


Figure 5.2: HC9 induced S phase arrest in MCF-7 cells and and altered the expression of cell cycle protein. *MCF-7 cells were treated with HC9 (0-40 µg/ml) for 24 h.* ***(A)*** *HC9 treatment induced accumulation of cells in S phase with simultaneous decrease in G2/M phase population.* ***(B)*** *Bar graph of cell cycle analysis of three independent experiments.* ***(C)*** *Densitometric analysis and western blotting analysis showing levels of p53, p21 and p16 proteins upon HC9 treatment in MCF-7. Tubulin was used as the loading control. The bands were quantified by densitometry scanning using ImageJ 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).*

5.3.3. HC9 induced G1 phase arrest in MDA MB-231 and modulated the expression of cell cycle regulatory proteins

We further analyzed effect of HC9 on cell cycle progression of MDA MB-231 cells. The flow cytometric analysis indicated that HC9 arrested the progression of MDA MB-231 in G1 phase compared to the untreated control cells (Figure 5.3A and B). Interestingly, at 80µg/ml concentration of HC9, there was significant increase in

number of cells in G_1 phase (from 60.21% in untreated control to 77.65% in treated cells; p=0.038) with a simultaneous reduction in the percentages of cells in the S phase (from 16.88% in untreated control to 11.53% in treated cells; p=0.333) and G2/M phase (from 22.89% in untreated control to 10.49% in treated cells; p=0.038).

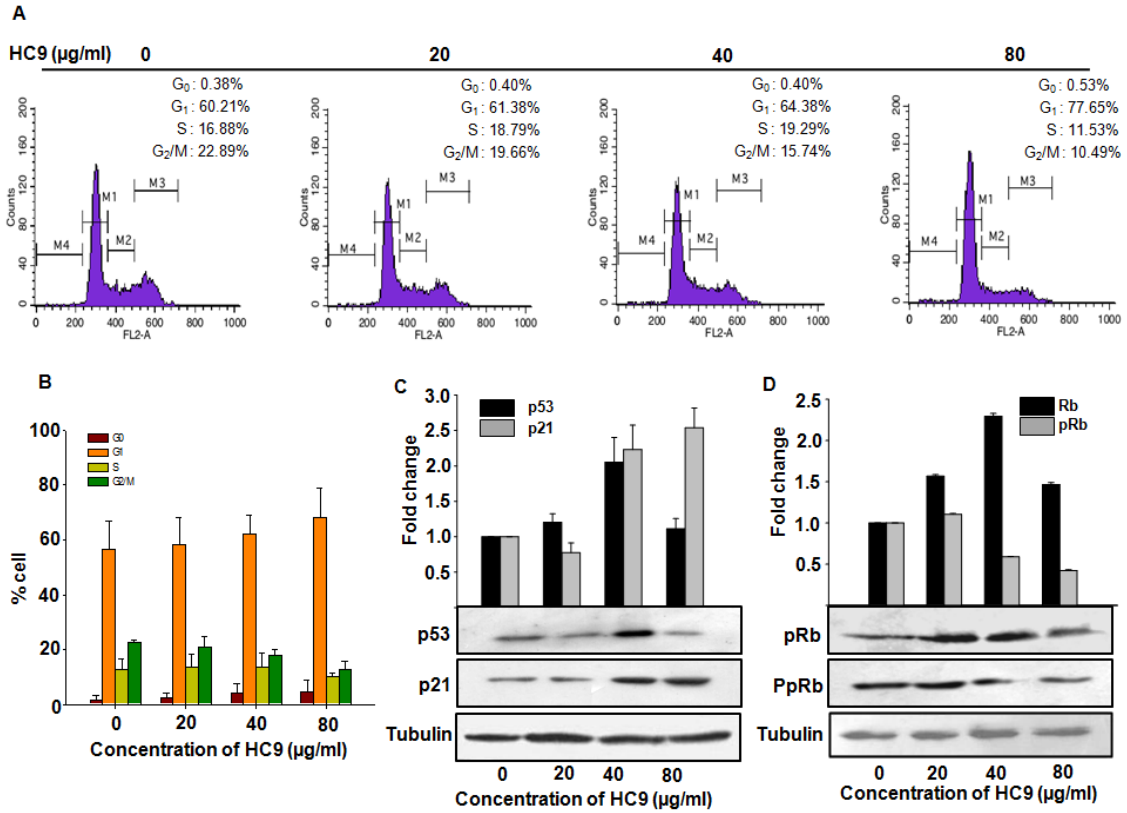


Figure 5.3: HC9 induced G1 phase arrest in MDA MB-231 and regulated the expression of cell cycle regulatory proteins. *MDA MB-231 cells were treated with HC9 (0-80 µg/ml) for 24 h.* ***(A)*** *HC9 induced G1 phase cell cycle arrest in MDA MB-231, with simultaneous reduction in the percentage of cells in the S and G2/M phase.* ***(B)*** *Bar graph of cell cycle analysis of three independent experiments.* ***(C)*** *p53 and p21 and* ***(D)*** *pRb and ppRb proteins in MDA MB-231 upon HC9 treatment. Tubulin was used as the loading control. The bands were quantified by densitometry scanning using ImageJ 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).*

To understand the effect of HC9 on the molecular events involved in G1 phase arrest, the expression of cell cycle regulatory proteins, p53, Rb, phospo-Rb, and p21 was determined in MDA MB-231 cells. Our western blot analysis showed that HC9 significantly increased the expression of p53 (~1.34-fold; p=0.038) and p21 (~2.34-fold; p=0.038) proteins (Figure 5.3C). HC9 also increased the expression of pRb (~1.45-fold; p=0.008) and simultaneously reduced the expression of its phosphorylated form phospo Rb (ppRb) (~1.91-fold; p≤0.001) (Figure 5.3D). These results indicated that HC9 induced G_1 phase cell cycle arrest in MDA MB-231 and modulated the expression of G_1 regulatory proteins.

5.3.4. HC9 inhibited migration and invasion of breast cancer cells and altered the expression of associated proteins

Inhibition of cancer cell migration and invasion is one of the efficient approaches in anti-metastatic therapy (Banyard J et al., 2014). In view of this, we studied the effect of HC9 on migration and invasion of MCF-7 and MDA MB 231. The effect on cell migration was examined by the wound healing assay. Confluent MCF-7 and MDA MB-231 cells were scraped with a sterilized tip and cells were then allowed to migrate into the gap created by the scraping either in the absence or presence of HC9. After 16 h incubation, the gap remained unfilled in cells treated with HC9 compared to the control untreated cells (Figure 5.4 A and B). At 40 and 80 μg/ml concentrations, HC9 significantly decreased the migration rate of MCF-7 (~3.02-fold; p≤0.001) (Figure 5.4C) and MDA MB-231 (~2.86-fold; p≤0.001), respectively, (Figure 5.4D). We further evaluated the effect of HC9 on cell invasion by using BD BioCoat Matrigel invasion chambers. The number of cells invaded through the Matrigel-coated filter from the upper to the lower chamber was significantly inhibited by HC9, at 40 and 80 μg/ml concentration in both MCF-7

(~2.92; p≤0.001) (Figure 5.4E) and MDA MB-231 (~3.97-folds; p≤0.001), respectively, compared to the untreated control cells (Figure 5.4F).

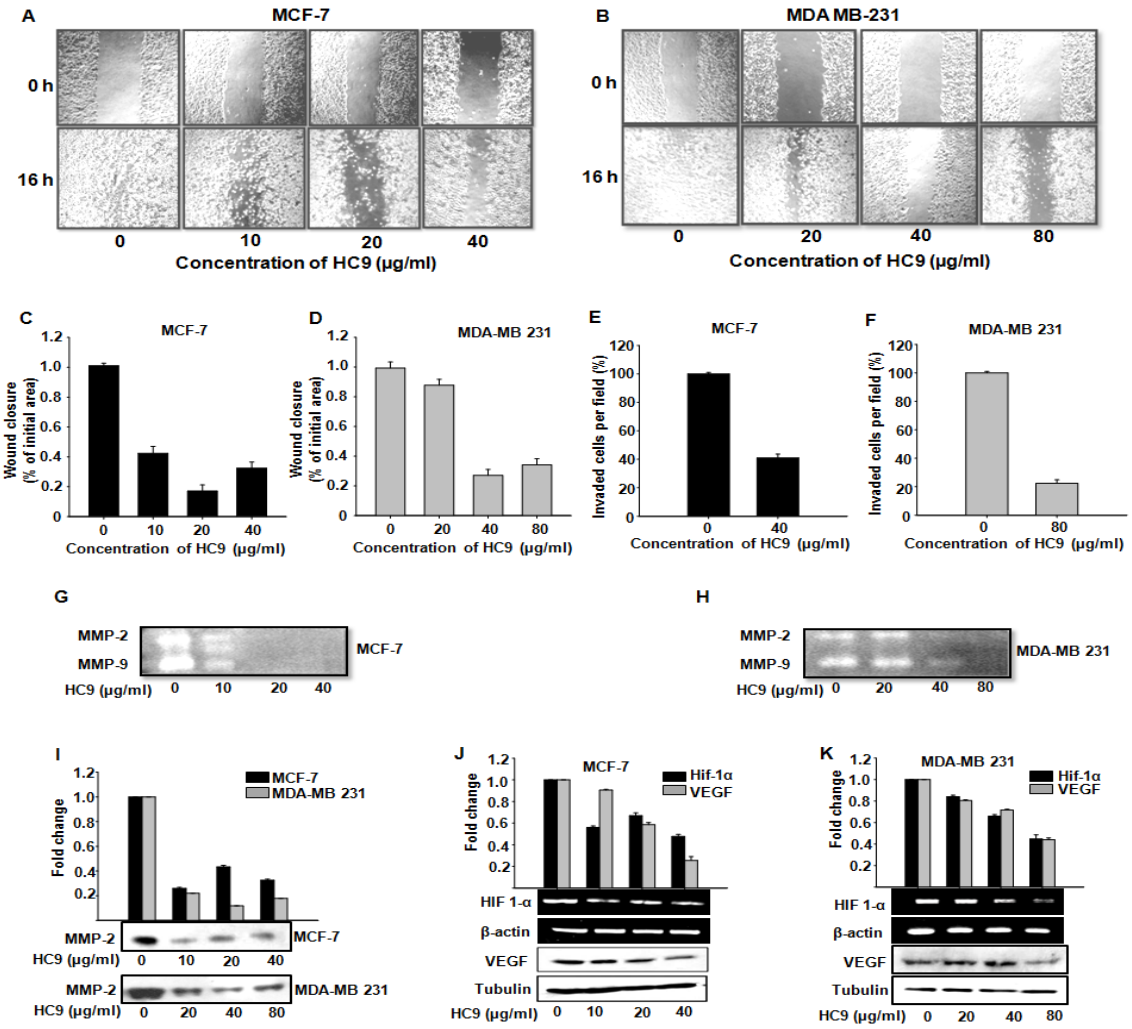


Figure 5.4: HC9 regulated migration and invasion of breast cancer cells. *Analysis of cell migration in MCF-7* ***(A)*** *and MDA MB-231****(B)*** *treated with HC9 (0-80 µg/ml) was measured by wound-healing assay. The upper panel of the image shows the wound made at 0 h. The lower panel shows the migration of cells corresponding to the distance travelled at 16 h. Graphical representation of wound closure in MCF-7* ***(C)*** *and in MDA MB-231* ***(D)*** *cells at 16 h after HC9 treatment has been shown. Values were represented as mean ± SD for three independent experiments. Cell invasion assay showing the percentage of MCF-7****(E)*** *and MDA MB-231* ***(F)*** *cells invaded per field in the presence or absence of HC9. The invaded cells were counted in ten random fields and the values have been expressed as mean ± SD for three independent experiments. Gelatin zymography showing the expression of MMP-2 and MMP-9 protein in HC9 (0-80 mg/ml) treated MCF-7* ***(G)*** *and MDA MB-231****(H)***. *Densitometric analysis and western blotting of MMP-2 in MCF-7 and MDA MB-231****(I)***. *Densitometric*

analysis of HIF-1α at transcriptional level and VEGF at translational level, in MCF-7 ***(J)*** *and in MDA MB-231* ***(K)*** *cells. The bands were quantified by densitometry using ImageJ 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).*

To understand the effect of HC9 on the molecular events involved in migration and invasion, we investigated its effect on migration and invasion associated proteins; MMP-2, MMP-9, Hif-1α and VEGF. Gelatin zymography assays indicated that HC9 reduced the secretion of MMP-2 and MMP-9 proteins in both MCF-7 (Figure 5.4G) and MDA MB-231 (Figure 5.4H) breast cancer cells compared to the untreated control cells. The expression of MMP-2 was further confirmed by western blotting which demonstrated reduced expression upon HC9 treatment in both the cell types (Figure 5.4I). In MCF-7, at 40 µg/ml of HC9, there was ~3.13-fold ($p \leq 0.001$) decrease in MMP-2, whereas in MDA MB-231, there was ~5.59-fold ($p \leq 0.001$) decrease in MMP-2 at 80µg/ml. In MCF-7, at 40 µg/ml, there was ~2.16 ($p=0.010$) and ~2.10-fold ($p=0.010$) decrease in the expressions of Hif-1α and VEGF, respectively, (Figure 5.4J). HC9 also inhibited the expression of HIF-1α at transcriptional level and VEGF at translational level, in both MCF-7 (Figure 5.4J) and MDA-MB-231 cells (Figure 5.4K). Similarly, in MDA-MB-231, at 80µg/ml concentration of HC9, there was ~3.57 ($p=0.010$) and ~2.21-fold ($p=0.010$) decrease in the expressions of Hif-1α and VEGF, respectively, as determined by densitometric scanning (Figure 5.4K). These results indicated that HC9 inhibited the migration and invasion of breast cancer cells and altered the expression of associated proteins.

5.3.5. HC9 altered the expression of inflammatory markers and chromatin modulators

Inflammatory tumor markers such NF-κB and COX-2 are over expressed in aggressive breast cancers and play a critical role in cancer development and

progression (Malonia SK et al., 2011). We studied the effect of HC9 on expression of NF-κB and COX-2 proteins in both MCF-7 and MDA MB 231. Immunoblotting analysis showed that HC9 significantly down regulated the expression of NF-κB and COX-2 in a dose dependent manner in both cell types (Figure 5.5 A and B). In MCF-7 at 40 µg/ml of HC9 treatment, there was ~3.77-fold (p=0.067) and ~7.38-fold (p=0.010) reduction in NF-κB and COX-2 expression respectively, compared to untreated control (Figure 5.5A). Similarly, in MDA-MB-231, at 80µg/ml concentration of HC9 treatment, there was ~2.74-fold (p=0.038) and ~3.88-fold (p=0.010) reduction in NF-κB and COX-2, respectively, compared to the untreated control cells (Figure 5.5B).

MAR (matrix attachment region) binding proteins SMAR1 and CDP/Cux are chromatin modulating proteins that are known to regulate breast cancer (Malonia SK et al 2011). To test whether HC9 altered the expression of these chromatin modulators, we evaluated their expression in HC9 treated cells. Our results showed that HC9 increased the expression of SMAR1 and simultaneously reduced the expression of CDP/Cux proteins in breast cancer cells. In MCF-7, at 40 µg/ml of HC9 treatment, there was ~2.99-fold (p=0.010) increase in expression of SMAR1 (Figure 5.5 C) with ~4.15-fold decrease in CDP/Cux protein expression, respectively, (Figure 5.5 D). Similarly, in MDA-MB-231, at 80 µg/ml concentration of HC9 treatment, there was ~6.8-fold (p=0.010) increase in expression of SMAR1 (Figure 5.5 E) with ~2.91-fold decrease in CDP/Cux expression, respectively, (Figure 5.5 F). These findings strongly suggest that HC9 effectively altered the expression of inflammatory markers and chromatin modulators in breast cancer cells.

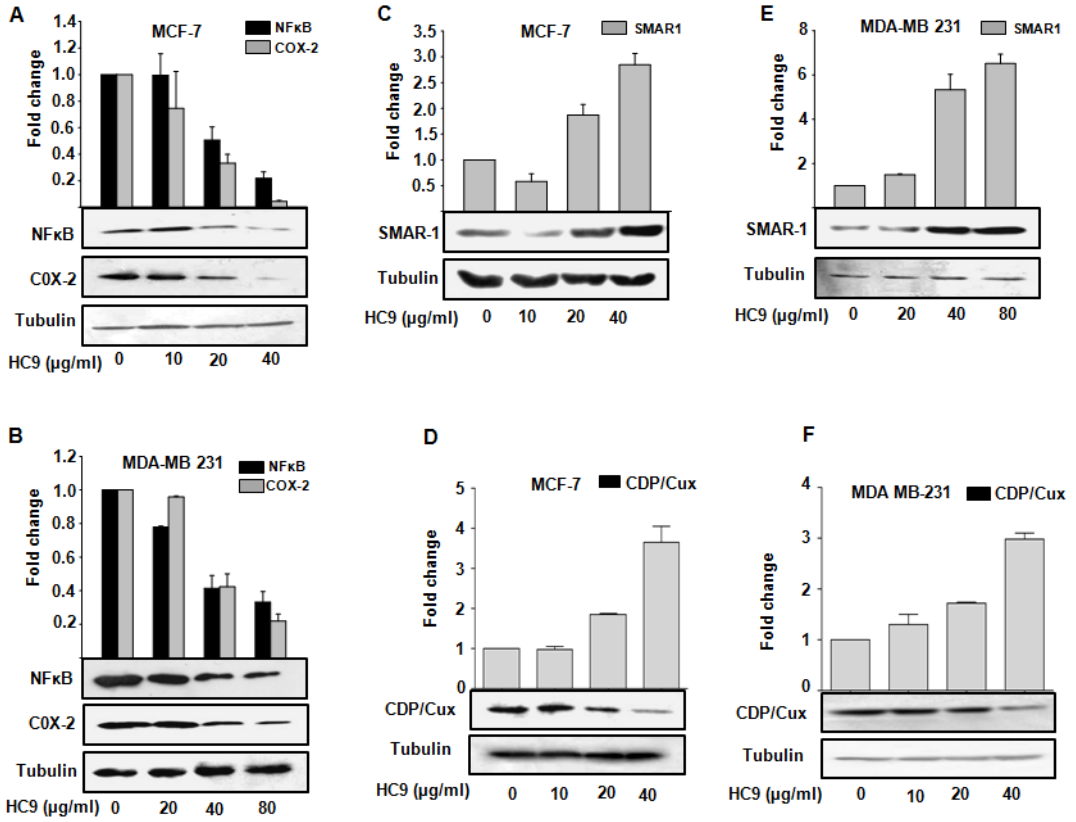


Figure 5.5: HC9 regulated the expression of inflammatory markers and chromatin modulators in breast cancer cells. *Densitometric analysis and western blot showing fold change in protein levels and expression of NFκB and COX-2 in* ***(A)*** *MCF-7 and* ***(B)*** *MDA MB-231 respectively. Densitometric analysis and western blot showing fold change in protein levels and expression of* ***(C)*** *SMAR1 as well as* ***(D)*** *CDP/Cux in MCF-7. Densitometric analysis and western blot showing fold change in protein levels and expression of* ***(E)*** *SMAR1 and* ***(F)*** *CDP/Cux in MDA MB-231. Tubulin was used as a loading control. The bands were quantified by densitometry scanning using ImageJ 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).*

5.3.6. HC9 reduced growth of subcutaneous melanoma tumors in C57BL/6 mice

Our in vitro studies prompted us to investigate the anti-cancer activity of HC9 *in vivo*. For this, C57BL/6 mouse melanoma model was used. Subcutaneous tumors of B16F10 melanoma were induced in mice by the injection of 5×10^5 viable tumor cells. One week after development of subcutaneous tumors, mice were given oral gavages of HC9 at doses of 100, 200, 400 mg/kg bw for 15 consecutive days (Figure

5.6A). HC9 treated groups showed significant retardation of B16F10 mouse melanoma tumor growth in female C57BL/6 mice (Figure 5.6B). At 200 and 400 mg/kg, the tumor volume decreased almost 2.82 and 3.11-fold ($p \leq 0.001$), respectively, compared to the untreated tumor control (Figure 5.6 B). There were no signs of toxicity as reflected by 100% survival rate, unaltered body weights (Figure 5.8 C) and food consumption (Figure 5.8 D).

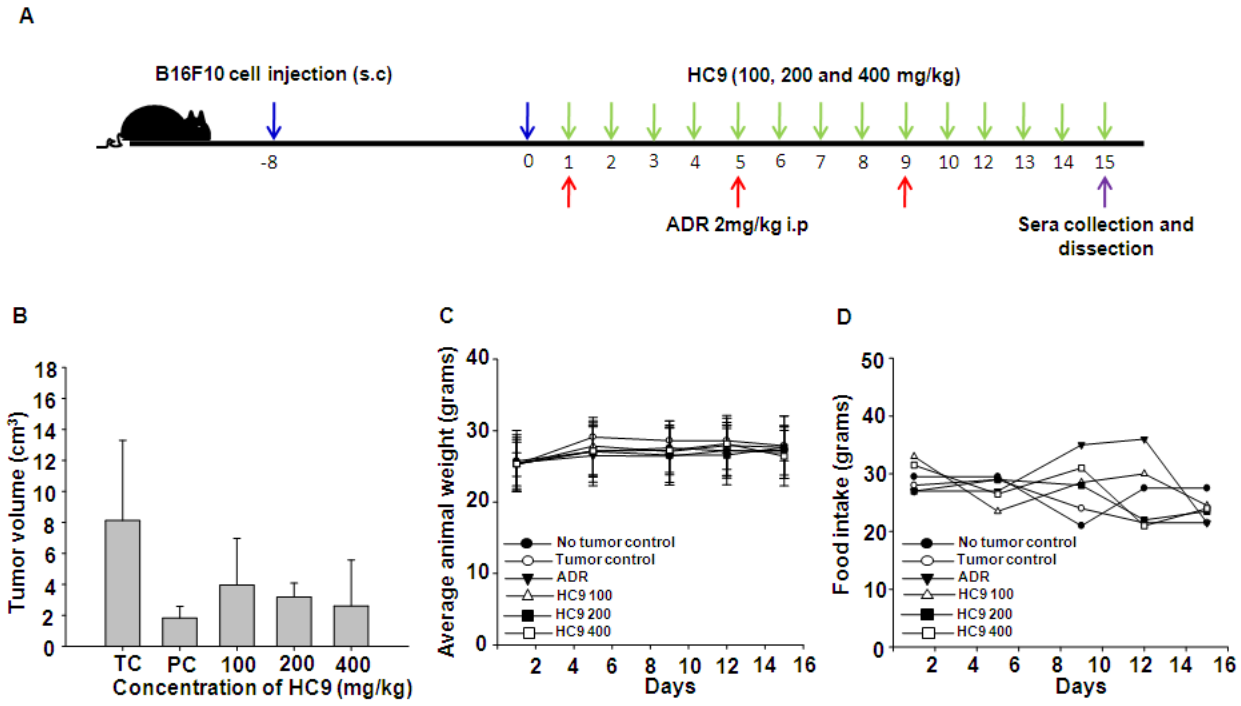


Figure 5.6: HC9 reduced growth of subcutaneous melanoma tumors in C57BL/6 mice. (A) *B16F10 cells (5×10^5) were injected subcutaneously into the right flank of female C57BL/6mice. Group I was kept as only control with no tumors, group II was kept as tumor control who received the vehicle, group III was kept as positive control administrated with Doxorubicin (2 mg/kg bw) intravenously on every 1st, 5th and 9th day and simultaneously, Group IV, V, VI were orally gavaged with the HC9 at concentration of 100 mg/kg, 200mg/kg, 400mg/kg bw respectively everyday for 15 days after tumors were raised.* ***Effect of HC9 on (B)Tumor growth:*** *The tumor volume was measured every 3 days; ***p<0.001, *p<0.05 between HC9 and tumor control at same day,* ***(C) Body weight:*** *The mean ± SD body weight of mice in each group was calculated, and* ***(D) Food consumption:*** *The average consumption of food by each group was calculated. Data represent mean ± SD; ($^{***}p \leq 0.001$).*

5.4. Discussion

Herbal remedies in the form of single herb or polyherbal formulations are increasingly playing prime role in the healthcare system because of their wide biological activities, easy accessibility, cost effectiveness and safe usage (Gatkal S et al., 2012; Osemene KP et al., 2011; Bent S., 2008). Due to complex interaction between different phytochemicals present in polyherbal formulations, they may target multiple dysregulated pathways in cancer cells and provide an extremely promising, way to block the development of cancer (Gatkal S et al., 2012). However, these remedies have not yet been integrated into modern clinical practice due to lack of experimental and clinical evidences on their safety, efficacy, and pharmacological mechanisms (Zhang AL et al., 2011; Firenzuoli F et al., 2007). Thus, it is necessary to evaluate the polyherbal formulations for their safety and therapeutic efficacy at in vitro, and at *in vivo* before they could be taken up for clinical trials.

Estrogen (ER) and progesterone (PR) receptors are important prognostic markers of aggressive breast cancer (Baglietto L et al., 2010). Therefore, two representative breast cancer cell lines, namely MCF-7$^{ER(+)/PR(+)}$ and MDA MB-231$^{ER(-)/PR(-)}$ were selected to investigate the anti-proliferative effect of HC9 on them. HC9 significantly reduced proliferation in breast cancer cells by arresting them in S (MCF-7) and G1 (MDA MB-231) phase without any induction of apoptosis. The difference in sensitivity and pattern of cell cycle arrest between MCF-7 and MDA MB-231 cells could be attributed to several factors such as growth rate, ER/PR status and basal p53 activity among the cell lines. We investigated the mechanism behind the cell cycle arrest by analyzing the effect of HC9 treatment on cell cycle regulatory proteins. Most of the chemopreventive drugs regulate the growth of cancer cells by targeting cell cycle regulatory proteins (Königsberg R et al., 2008). Our results showed that HC9

caused S phase arrest in MCF-7 and increased expression of p53, p21 and p16 proteins. Transcriptional activation of p53 results into activation of its downstream target gene p21,that blocks the cell cycle progression in the S phase by inhibiting CDK-cyclin activity (Qin H et al., 2007). Thus, HC9 mediated up regulation of p53 and p21 might have resulted into activation of downstream effectors of p53-dependent S phase arrest. Similarly, in MDA MB-231, there was an increase in the expression of p53, p21 and pRb and down regulation of ppRb. The decrease in phopshorylation of Rb and increase in expression of p21 and p53 could have led to G1 phase arrest in MDA MB-231cells.

Our study has indicated that HC9 reduced the migration and invasion properties of breast cancer cells by significantly reducing the expression of MMP-2, MMP-9 and VEGF proteins as well as Hif-1α mRNA levels. Metastasis is an aggressive phase in cancer and one of the main causes of mortality in cancer patients (Khamis ZI et al., 2012). Metastasis consist of a series of steps which involve altered expression of different proteolytic enzymes (matrix metalloproteinases) associated with degradation of the extracellular matrix (Moss LA et al., 2012), acquisition of a motile phenotype and chemotactic activity of the cells (Bravo-Cordero et al., 2012). Hif-1α, a hypoxia response gene, activates the expression of angiogenic cytokine VEGF whose over-expression is correlated with tumor progression by formation of new blood vessels and increased oxygen supply to tumor cells (Raja R et al., 2014). Thus, drugs that could inhibit/decrese the tumor metastasis and its regulatory markers would be potential chemo-preventive or chemotherapeutic candidates for the management of cancer (Sagar SM., et al., 2004; Khamis ZI et al., 2012).

HC9 significantly reduced the expression of inflammatory markers, NF-κB and COX-2. Inflammation has been recognized as an important factor to regulate the

development and progression of cancer (Ben-Neriah Y et al., 2011). NF-κB is a key transcription factor that promotes the cancer cell cycle progression, migration, invasion and angiogenesis by activating its downstream transcriptional target genes (Yodkeeree S et al., 2010). Cyclooxegenase 2 (COX-2) is a downstream target of NF-κB, which is over expressed in aggressive breast cancers and may play a critical role in cancer progression through up regulation of MMPs (Larkins TL et al., 2006). Accumulated data suggests that chronic inflammation is usually associated with altered chromatin structure and aberrant expression of chromatin modulators (Dawson MA et al., 2012). MAR (Matrix Attachment Region) binding proteins (MARBPs) such as SMAR1 and CDP/Cux are known to play an important role in chromatin modulation that is required for regulation of transcription of genes involved in growth regulatory mechanisms (Malonia SK et al., 2011; Singh K et al., 2007). The expression of SMAR1, a tumor suppressor MARBP, is reduced in higher grades of breast cancer. It has been reported that its expression is inversely correlated with CDP/Cux (Michl P et al., 2005). HC9 significantly increased the expression of SMAR1 and simultaneously reduced the expression of NF-κB, COX-2 and CDP/Cux in breast cancer cells. Recent studies have shown that SMAR1 down modulated the expression of NF-κB and CDP/Cux (Singh K et al., 2009). HC9 mediated up regulation of SMAR1 could have inhibited NF-κB and CDP/Cux expression that might have resulted into regulation of migration of breast cancer cells.

HC9 significantly decreased the tumor growth in C57BL/6 melanoma tumor model. It is a widely used model in cancer research to investigate the anticancer activity of drugs (Yu KF et al., 2012). HC9 did not induce any adverse effects on the body weight and organ weight of mice and mice did not show any significant mortality till the end of the experiment. HC9 is rich in polyphenols (Suryavanshi et

al., 2011) and also contains flavonoids, phenols, saponins, alkaloids, tannins and carbohydrates (Suryavanshi et al., 2014). Due to their chemopreventive properties, polyphenols can modulate the process of carcinogenesis either towards protective or therapeutic side depending upon either the amount of the drug being used or upon the cellular phenotype (D'Archivio M et al., 2008). Thus, owing to the presence of polyphenols and other chemopreventive components, HC9 could exhibit improved bioavailability and reduced toxicity in breast cancer cells. Such property of the formulation could provide a strong basis for further exploration of HC9 as a therapeutic drug against breast cancer. These results warrant further investigation on the use of HC9 as an adjunct to conventional chemotherapy.

In conclusion, this chapter has demonstrated that HC9 formulation induced cell cycle arrest, decreased migration and invasion and altered the expression of inflammatory markers and chromatin modulators. HC9 also retarded tumor growth in mice, thereby indicating its potential as a possible chemopreventive drug which could be explored the management of breast cancer.

THESIS SUMMARY AND FUTURE PROSPECTS

Summary

The focus of the present thesis was to analyze the chemopreventive potential of a polyherbal formulation (HC9) in breast cancer and elucidate its anticancer mechanism. The study included both in vitro as well as *in vivo* experiments. As a first step, comparison of the antioxidant potential and cytotoxicity of aqueous ($HC9_{aq}$) and ethanolic ($HC9_{et}$) extracts of HC9 was carried out to know which one was more efficient. The results demonstrated that, $HC9_{et}$ (IC_{50}=162.41μg/ml) exhibited significant DPPH radical scavenging activity compared to $HC9_{aq}$ (IC_{50}=172.89 μg/ml). Both the extracts showed a concentration-dependent increase in ferric reducing power activity. However, $HC9_{et}$ exhibited slightly higher activity than $HC9_{aq}$. The total polyphenol content of $HC9_{aq}$ and $HC9_{et}$ were 144.06 and 193.34mg/g, respectively, equivalent to gallic acid concentration. The $HC9_{et}$ (IC_{50}=66.7 μg/ml) exhibited significant anti-lipid peroxidation (ALP) activity in a dose-dependent manner than $HC9_{aq}$ (IC_{50}=84.7 μg/ml). Interestingly, $HC9_{et}$ showed significant cytotoxic activity at lower doses in breast cancer cell lines, MCF7 and MDA MB 231. These results suggest that the ethanolic extract of HC9 ($HC9_{et}$) was more potent than the aqueous extract ($HC9_{aq}$) and could be explored for its further use.

As a next step, $HC9_{et}$ (referred to as HC9) and its individual herbal components were standardized on the basis of botanical validation and physico-chemical profiling using specific markers. The individual herbs and the whole formulation showed the presence of marker compounds such as picroside-I, nootkatone, 6-gingerol, matairesinol, swertiamarin, berberine, connesine and 2-hydroxy-4-methoxybenzaldehyde. Further, a thorough comparative analysis of cytotoxic activity of standardized HC9 with its individual components was performed

in breast cancer cell lines and non-cancerous transformed cells. Results from MTT assay demonstrated that at 160 µg/ml concentration, $HC9_{et}$ exhibited cytotoxicity in breast cancer cells, MCF7 and MDA MB231, with no cytotoxicity in non-cancerous transformed cells, MCF-10A, HaCaT and HEK-293. In contrast, at this concentration, the individual herbs of $HC9_{et}$ exhibited cytotoxicity not only in cancerous cells, but also in non-cancerous cells. These results suggested that the standardized HC9 formulation was safe for non-cancerous cells and specifically affected the viability of breast cancer cells.

Acute toxicity study in Swiss albino mice demonstrated that HC9 was safely tolerated by mice up to 2000 mg/kg and did not produce any adverse effects in terms of mortality or behavioral and clinical signs. In sub-acute toxicity study, no treatment-related adverse effects were found in the mice upto 1000 mg/kg/day dose of HC9. It did not produce any adverse effects in biochemical, hematological, urine and histopathological parameters, indicating that HC9 was safe for its possible therapeutic applications.

The mechanism of antineoplastic potential of the standardized HC9 was evaluated in vitro as well as *in vivo*. The results demonstrated that HC9 significantly altered cell proliferation by modulating cell growth kinetics and inducing cell cycle arrest in MCF-7 and MDA MB-231. Moreover, it altered the expression of chromatin modulators and inflammatory markers of breast cancer. It also inhibited migration and angiogenic markers in breast cancer cells. HC9 significantly retarded tumor growth in C57BL6 mouse melanoma model, thereby proving its anticancer activity.

First time findings of the study on polyherbal composition HC9

Demonstration of significant antioxidant activity of $HC9_{et}$ compared to $HC9_{aq}$

1. $HC9_{et}$ demonstrated significant DPPH free radical scavenging activity
2. $HC9_{et}$ exhibited significant ferric reducing power activity
3. $HC9_{et}$ exhibited significant anti-lipid peroxidation (ALP) activity
4. $HC9_{et}$ showed presence of high total phenol content

Comparison of effect of HC9 versus its individual components on cell viability of cancerous and non cancerous cells

1. HC9 reduced the viability of only breast cancer cell lines without affecting the non-cancerous cells.
2. Individual components of HC9 were cytotoxic to both cancerous and non-cancerous cells.

Standardization of HC9 and its components

1. Organoleptic characters, physico- chemical and preliminary phytochemical parameters were analyzed to establish the quality of HC9

2. Qualitative and quantitative HPTLC analysis of component herbs and HC9 was done by using specific markers

Demonstration of no adverse effect of HC9 in Swiss albino mice

1. HC9 did not induce any apparent toxicity in Swiss albino mice at acute and sub-acute oral doses
2. HC9 did not produce any adverse effects in biochemical, hematological, urine and histopathological parameters

HC9 exhibited anticancer activity

1. HC9 induced S phase arrest in MCF-7 and altered the expression of cell cycle regulatory proteins (p53, p21 and p16)
2. HC9 induced G1 phase arrest in MDA MB-231 and modulated the expression of cell cycle regulatory proteins (p53, p21,pRb and ppRb)
3. HC9 inhibited migration and invasion of breast cancer cells and altered the expression of associated proteins (MMP-2, MMP-9, HIF1-α and VEGF)
4. HC9 altered the expression of inflammatory markers (NFκB and COX-2) and chromatin modulators (SMAR1 and CDP/Cux)
5. Oral administration of HC9 reduced growth of subcutaneous melanoma tumors in C57BL/6 mice

Future prospects

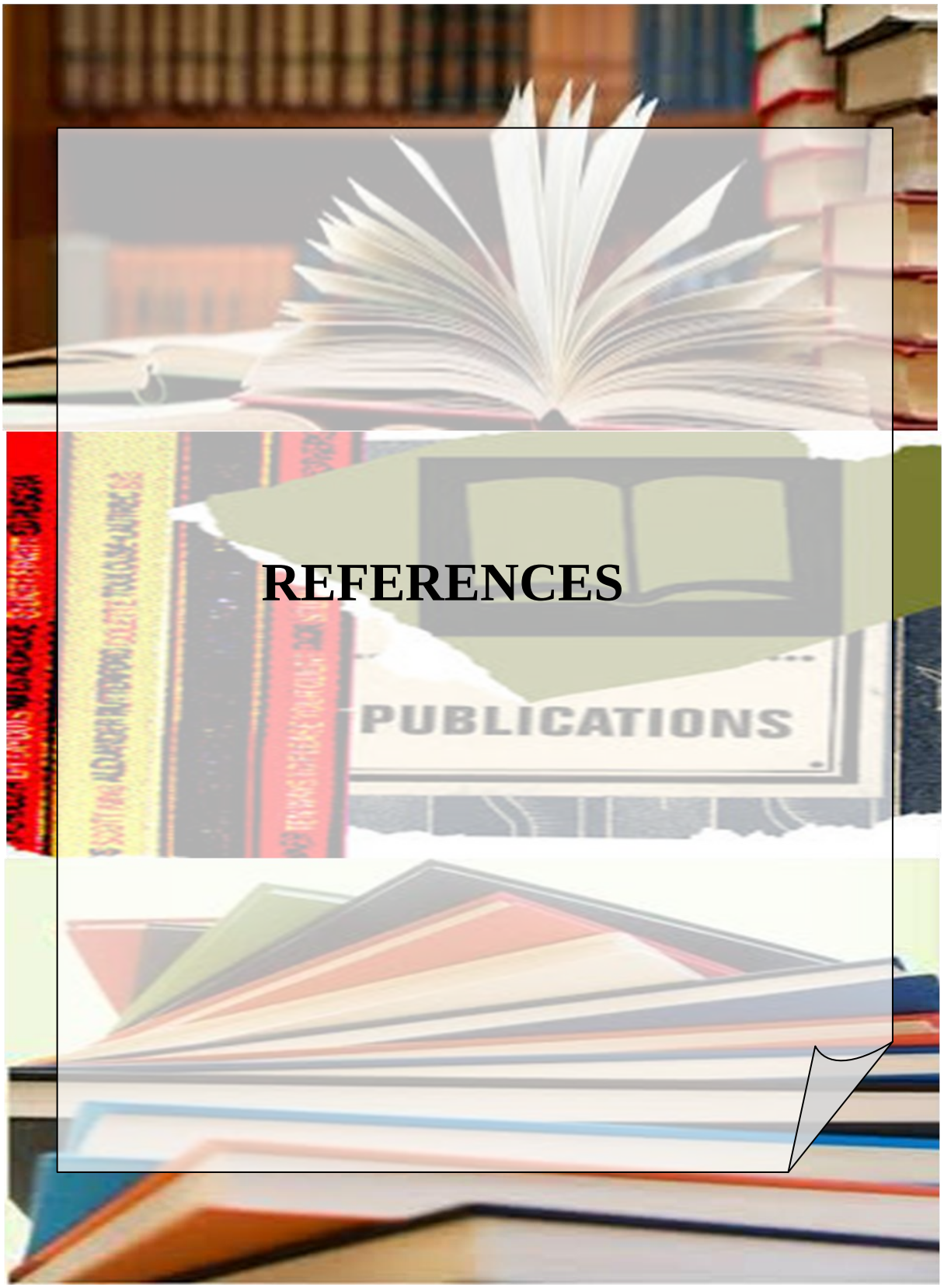
The following experimental strategies could be considered to be undertaken in future that would further strengthen the claims of usage of HC9 in treatment/ prevention of breast cancer.

1. The present study utilized mouse melanoma model, a general one used to study anticancer activity of any drug. In future the efficacy of HC9 could be tested in EO771 induced tumors in C57BL/6 breast cancer model. EO771 is a mammary adenocarcinoma cell line, derived from a spontaneous mammary tumor in a female C57BL/6 mouse (Casey et al., 1951).

2. Combinatorial studies of HC9 with well known chemotherapeutic drugs such as doxorubicin or others could be done to test whether the dose of the chemotherapeutic drugs could be reduced that in effect would reduce the toxicity to normal cells.

Moreover, whether HC9 could enhance the effectiveness of the chemotherapeutic drugs could also be evaluated.

This study may provide a clue for development of effective polyherbal interventions in the management and prevention of breast cancer, and would reduce the side effects of conventional therapy. This would further validate our experimental studies and would translate our findings from bench to bedside.



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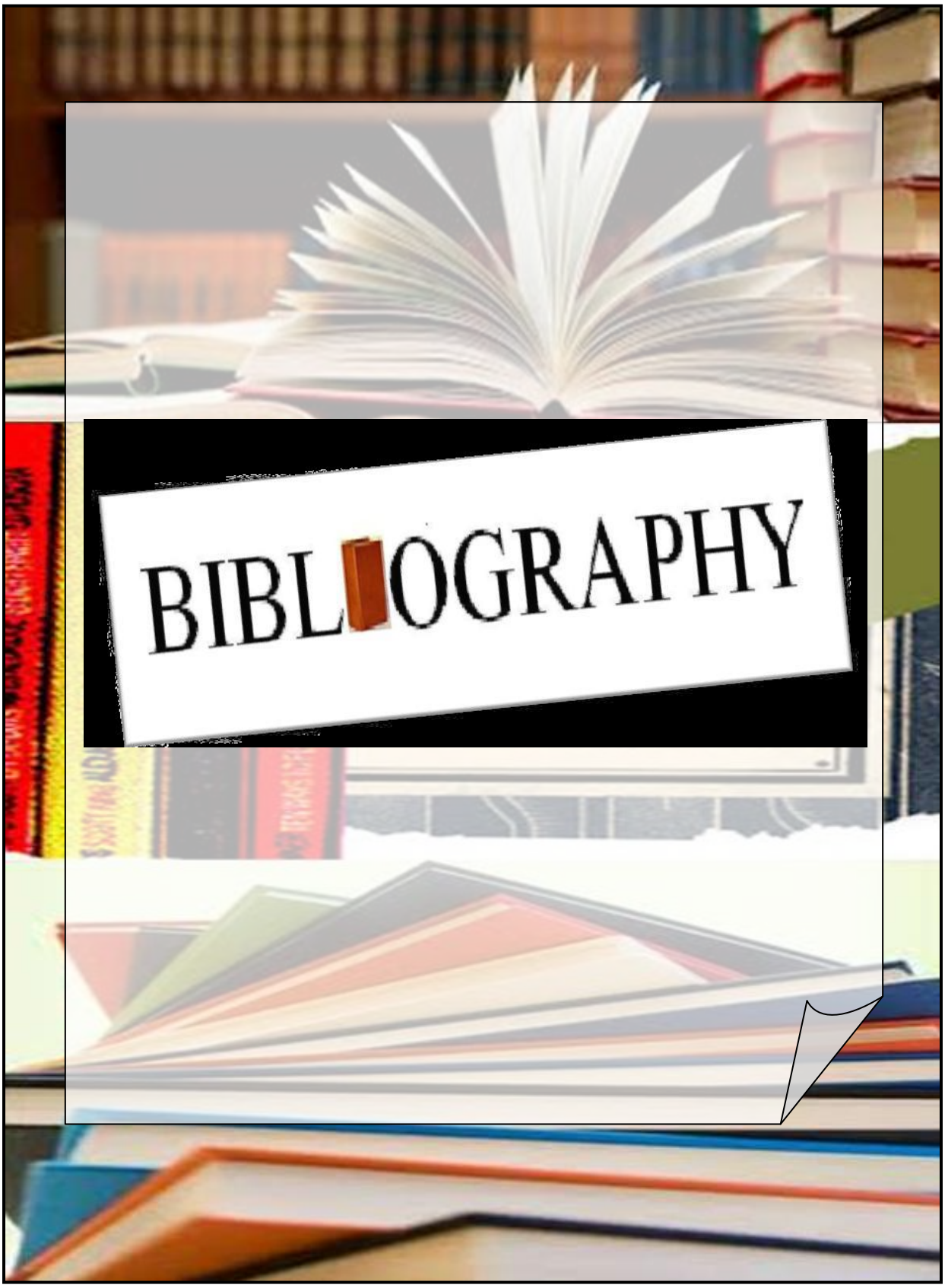
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