ANTICANCER PROPERTIES OF SELECTED PHYTOCHEMICALS AGAINST COLORECTAL CANCER

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CERTIFICATE

This is to certify that the work incorporated in this Ph. D. thesis entitled "**Anticancer properties of selected phytochemicals against colorectal cancer**" submitted by **Ms. Sithara Thomas** to the Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Biological Sciences embodies original research work carried out by her under my supervision. I further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.It is also certified that this work done by the student, under my supervision, is plagiarism free.

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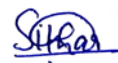
DECLARATION

I hereby declare that the matter embodied in the thesis entitled: "**Anticancer properties of selected phytochemicals against colorectal cancer**" is the result of the work carried out by me at the Agro-Processing and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram, under the supervision of Dr. P. Nisha and the same has not been submitted elsewhere for any other degree.



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Sithara Thomas

Dedicated to

my family members.......

CONTENTS

Chapters **Page No.**

1. Chapter 1: Introduction and Review of Literature 1-57

1. Introduction 2
1.1.1. Non-communicable diseases (NCDs) 2
1.1.2. Non-communicable diseases (NCDs) in India 3
1.1.3. Risk factors for NCDs 3
1.2.1. Cancer 5
1.2.2. Colorectal cancer (CRC) 9
1.2.3. Colorectal cancer in India 10
1.2.4. Stages of colorectal cancer 10
1.2.5. Symptoms of colorectal cancer 11
1.2.6. Diagnosis of colorectal cancer 12
1.2.7. Risk factors for CRC 12
1.2.7.1. Non- modifiable risks factors 13
1.2.7.1.1. Age 13
1.2.7.1.2. Personal history of inflammatory bowel disease 14
1.2.7.1.3. Family history of colorectal cancer or Adenomatous polyps 14
1.2.7.2. Modifiable risks factors 15
1.2.7.2.1. Diet 15
1.2.7.2.2. Physical inactivity and obesity 15
1.2.7.2.3. Cigarette smoking and heavy alcohol Consumption 16
1.3. Various conditions in the body which worsens the colorectal cancer condition 16
1.3.1. Alteration in glucose metabolism 16
1.3.1.1. Warburg effect 16
1.3.1.2. Role of PI3K/Akt signaling pathway 18
1.3.2. Inflammation and CRC 19
1.3.2.1. NF-κB signaling 20

1.3.2.2. MAPK (Mitogen-activated protein kinases) signaling 21
1.3.2.3. Role of inducible NOS (iNOS) and cyclooxigenase-2 (COX-2) 22
1.3.3. Colorectal cancer metastasis 23
1.3.3.1. Role of Matrix metalloproteinase (MMPs) in CRC metastasis 24
1.3.3.2. Role of Wnt/β-catenin signaling pathway in CRC metastasis 25
1.4. Treatment strategies available for colorectal cancer 25
1.5. Prevention and management of CRC through modulation of diet 26
1.5.1. Phytochemicals as anticancer agents 27
1.5.2. CRC and dietary fibre 28
1.6. Current study 31
1.6.1. Morin 32
1.6.2. Fisetin 33
1.6.3. Genistein 33
1.6.4. Luteolin 34
1.6.5. zerumbone 35
1.7. Objectives of the study 36
1.8. Work flow 36
2. REFERENCE 37

2. Chapter 2: Screening of selected phytochemicals for their anticancer activity against colorectal cancer 58-113

2.1. INTRODUCTION 59
2.2. MATERIALS AND METHODS 60
2.2.1. Materials 60
2.2.2. Cell Culture and Treatment 61
2.2.3. Experimental design 62
2.2.4.1. Cell viability by MTT assay 63

2.2.4.2. Acridine Orange – Ethidium Bromide
Double Staining (AO/EB staining) 63
2.2.4.3. Nuclear staining using Hoechst 33342 stain 64
2.2.4.4. Cell apoptotic analysis by flow cytometry 64
2.2.4.5. Assay for mitochondrial membrane potential
by JC-1 staining 65
2.2.4.6. Measurement of mitochondrial membrane
potential ($\Delta\Psi m$) 65
2.2.4.7. Measurement of intracellular Reactive
Oxygen Species (ROS) generation 66
2.2.4.8. Antioxidant assays 66
2.2.4.9. Immunoblot analysis 67
2.2.4.10. Statistical analysis 68
2.3. RESULTS 68
2.3.1. Cytotoxic effect of selected phytochemicals 68
2.3.2. Effects of selected phytochemicals on
plasma membrane integrity 70
2.3.3. Hoechst 33342 staining 72
2.3.4. Detection of cell apoptosis using flow cytometry 74
2.3.5. Mitochondrial membrane potential 78
2.3.6. Intracellular ROS production by DCFH-DA staining 87
2.3.7. Antioxidant level in SW480 cells
(catalase activity and glutathione levels) 94
2.3.8. Western blot analysis 95
2.3.9. Effect of morin and zerumbone on viability
of SW620 cells by MTT assay 98
2.3.10. Detection of SW620 cell apoptosis by Annexin
V-FITC staining and flow cytometry 99
2.3.11. Western blot analysis 101
2.3.12. Evaluation of cytotoxicity of morin on human
hepatoma cell line (Hep G2) 102
2.4. DISCUSSION 103
2.5. CONCLUSION 106

2.6. REFERENCES 107

3. Chapter 3: Effect of Morin on Warburg Effect (SW480 Colorectal Cancer Cells) 114-142

3.1. INTRODUCTION 115
3.2. MATERIALS AND METHODS 116
3.2.1. Materials 116
3.2.2. Cell culture and treatment 116
3.2.3. Experimental design 117
3.2.4.1. Fluorescence analysis of 2-NBDG uptake by flow cytometry 117
3.2.4.2. Extraction and measurement of Adenosine Tri Phosphate (ATP) levels 118
3.2.4.3. Immunoblot analysis 118
3.2.4.4. Molecular docking (*In silico* studies) 120
3.2.4.5. Statistical analysis 120
3.3. RESULTS 121
3.3.1. Effect of morin on 2-NBDG uptake by flow cytometry 121
3.3.3. Glucose transporter 1 (GLUT 1) level by western blotting 122
3.3.3. Determination of ATP production 123
3.3.4. Effect of morin on PI3K/Akt signalling decreased glucose uptake through GLUT 1 mediated mechanism 125
3.3.5. Molecular docking studies 128
3.4. DISCUSSION 133
3.5. CONCLUSION 136
3.6. REFERENCES 137

4. Chapter 4: Impact of Morin on Inflammatory Status of SW480 Colorectal Cancer Cells 143-169

4.1. INTRODUCTION 144
4.2. MATERIALS AND METHODS 146

4.2.1. Materials 146
4.2.2. Cell culture and treatment 146
4.2.3. Experimental design 147
4.2.4.2. Cytotoxicity assay 147
4.2.4.1. Electrophoretic Mobility Shift Assay (EMSA) 148
4.2.4.3. Quantification of inflammatory cytokines 148
4.2.4.4. NF-κ (p65) translocation assay 148
4.2.4.5. Western blotting 149
4.2.4.6. Total RNA isolation and quantitative real time PCR analysis 150
4.2.4.7. Statistical analysis 150
4.3. RESULTS 151
4.3.1. Combined effect of morin and LPS on cell viability 151
4.3.2. Effect of morin on nuclear levels of Nrf2 152
4.3.3. Inhibitory effects of morin on generation of inflammatory cytokines in SW480 cells 153
4.3.4. Effect of morin on LPS induced nuclear translocation of NF-κB (p65) 155
4.3.5. Effect of morin on MAPK signalling pathway 156
4.3.6. Impact of morin on iNOS and COX-2 gene expression 158
4.4. DISCUSSION 159
4.5. CONCLUSION 163
4.6. REFERENCE 164

5. Chapter 5: Effect of Morin on Metastasis of SW480 Colorectal Cancer Cells 170-193

5.1. INTRIDUCTION 171
5.2. MATERIALS AND METHODS 172
5.2.1. Materials 172
5.2.2. Cell culture and treatment 172
5.2.3. Experimental design 173

5.2.4.1. Scanning electron microscope (SEM) observation 173
5.2.4.2. Microfilament analysis 173
5.2.4.3. Anti-migration assay 174
5.2.4.4. Colony formation assay 174
5.2.4.5. Gelatin zymography 175
5.2.4.6. Western blot analysis 175
5.2.4.7. Statistical analysis 176
5.3. RESULTS 176
5.3.1. Morphological changes in the cells when treated with morin examined using SEM 176
5.3.2. Microfilament analysis 177
5.3.3. Migration of SW480 cells were inhibited by morin 178
5.3.4. Colony formation assay 179
5.3.5. MMP-2 and MMP-9 expression by gelatin zymography 180
5.3.6. MMP-2 and MMP-9 expression by western blotting 181
5.3.7. Wnt/β-catenin signaling pathway regulate the metastatic arrest of SW480 cells by morin 182
5.4. DISCUSSION 183
5.5. CONCLUSION 187
5.6. REFERENCES 188

6. Chapter 6: Encapsulation of Morin with Dietary Fiber (Inulin) & Anticancer Efficacy of the Encapsulated Product 194-231

6.1. INTRODUCTION 195
6.2. MATERIALS AND METHODS 198
6.2.1. Materials 198
6.2.2. Experimental design 198
6.2.3. Encapsulation by spray drying 199
6.2.3.1. Assessment of Product yield (PY) 199
6.2.3.2. Assessment of moisture content 199
6.2.3.3. Morphological analysis of spray dried product 199

6.2.3.4. Standardization and quantification of morin in spray dried product by HPLC 200
6.2.4. *In vivo study:* Anticancer efficacy of encapsulated product 200
6.2.4.1. Animals and maintenance condition 200
6.2.4.2. Carcinogen and treatment drug administration 200
6.2.4.3. Histopathological analysis 202
6.2.4.4. Examination for aberrant crypt foci (ACF) formation 202
6.2.4.5. Colon length and weight 203
6.2.4.6. Protein extraction and western blotting 203
6.2.4.7. Statistical analysis 204
6.3. RESULTS 204
6.3.1. Encapsulation of morin by inulin using spray drying 204
6.3.1.1. Product yield, Encapsulation efficiency, moisture content 205
6.3.1.2. Morphological analysis of spray dried product 205
6.3.1.3. Quantification of morin in spray dried product by HPLC 206
6.3.2. *In vivo study:* Anticancer efficacy of encapsulated product 207
6.3.2.1. Histopathology of liver 207
6.3.2.2. Effect of morin on the body weight of control and experimental groups of animals 208
6.3.2.3. Effect of morin on the ACF formation in control and experimental groups of animals 209
6.3.2.4. Effect of morin on the colon length of control and experimental groups of animals 210
6.3.2.5. Effect of morin on the colon weight/length ratio of control and experimental groups of animals 211
6.3.2.6. Effect of morin on apoptosis protein expression in control and experimental groups of animals 211
6.4. DISCUSSION 213
6.5. CONCLUSION 220

6.6. REFERENCES 221

7. Chapter 7: Summary and Conclusion 232-236

List of Publications 237-238

Conference proceedings 239

List of Tables

1. Table 2.1. The IC_{50} values of selected phytochemicals after 48 h treatment on SW480 cells
2. Table 3.1. Pharmacokinetic parameters of morin based on QikProp analysis
3. Table 3.2. G-score and D-score of morin and PI3k, morin and GLUT 1, LY294002 and PI3K
4. Table 6.1. Experimental groups
5. Table 6.2. Effect of morin on the body weight of control and experimental groups of animals

List of Figures

1. Figure 1.1.a. Cancer occurrence among males
2. Figure 1.1.b. Cancer occurrence among females
3. Figure 1.2. Global cancer data
4. Figure 1.3. Colon cancer and polyp
5. Figure 1.4. Stages of colorectal cancer
6. Figure 1.5. Genetic and non-genetic risk factors for colorectal cancer
7. Figure 1.6. Mechanisms of colitis-associated cancer (CAC) development
8. Figure 1.7. Organs involved most often in metastatic colon cancer
9. Figure 1.8. Chemical structure of morin
10. Figure 1.9. Chemical structure of fisetin
11. Figure 1.10. Chemical structure of genistein
12. Figure 1.11. Chemical structure of luteolin
13. Figure 1.12. Chemical structure of zerumbone
14. Figure 1.13. Outline of the work
15. Figure 2.1. Outline of Chapter 2
16. Figure 2.2. Cytotoxic effect of selected phytochemicals on SW480 cells after 48 h treatment
17. Figure 2.3a. Acridine orange/ethidium bromide staining (morin and camptothecin)
18. Figure 2.3b. Acridine orange/ethidium bromide staining (zerumbone, fisetin, genistein and luteolin)
19. Figure 2.4a. Chromatin condensation and nuclear fragmentation observed using Hoechst 33342 staining (morin and camptothecin)
20. Figure 2.4b. Chromatin condensation and nuclear fragmentation observed using Hoechst 33342 staining (zerumbone, fisetin, genistein and luteolin)
21. Figure 2.5a. Flow cytometry analysis of the percentage of apoptotic cells in each group by Annexin- V/FITC and PI staining (morin and camptothecin)
22. Figure 2.5b. Flow cytometry analysis of the percentage of apoptotic cells in each group by Annexin- V/FITC and PI staining (zerumbone, fisetin, genistein and luteolin)

23. Figure 2.6.1. Change in mitochondrial membrane potential by JC-1 staining (morin and camptothecin)
24. Figure 2.6b. Change in mitochondrial membrane potential by JC-1 staining (zerumbone and fisetin)
25. Figure 2.6c. Change in mitochondrial membrane potential by JC-1 staining (genistein and luteolin)
26. Figure 2.7a. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining (morin)
27. Figure 2.7b. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining (zerumbone)
28. Figure 2.7c. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining (fisetin)
29. Figure 2.7d. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining (genistein)
30. Figure 2.7e. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining (luteolin)
31. Figure 2.8a. Detection of ROS formation by DCFH-DA staining (morin)
32. Figure 2.8b. Detection of ROS formation by DCFH-DA staining
33. Figure 2.8b. Detection of ROS formation by DCFH-DA staining (zerumbone, fisetin, genistein and luteolin)
34. Figure 2.9a. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis (morin)
35. Figure 2.9b. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis (zerumbone)
36. Figure 2.9c. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis (fisetin)
37. Figure 2.9d. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis (genistein)
38. Figure 2.9e. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis (luteolin)
39. Figure 2.10. Treatment with selected phytochemicals resulted in a declined level of cellular reduced glutathione and catalase activity (morin, zerumbone, fisetin, genistein and luteolin)

40. Figure 2.11a. Effect of morin on the expression of apoptosis related proteins.
41. Figure 2.11b. Effect of zerumbone on the expression of apoptosis related proteins.
42. Figure 2.12. Cytotoxic effect of morin and zerumbone on SW620 cells after 48 h treatment
43. Figure 2.13. Flow cytometry analysis of the percentage of apoptotic cells in each group by Annexin- V/FITC and propidium iodide (PI) staining (morin and zerumbone)
44. Figure 2.14. Effect of morin and zerumbone on apoptosis of SW620 cells
45. Figure 2.15. Effect of morin on viability of Hep G2 cells.
46. Figure 3.1. Outline of Chapter 3
47. Figure 3.2. Flow cytometry analysis of 2-NBDG uptake (morin)
48. Figure 3.3. Effect of morin on GLUT 1 expression
49. Figure 3.4. Quantification of ATP level by HPLC method (morin)
50. Figure 3.5a. Effect of morin on PI3K level
51. Figure 3.5b. Effect of morin on PDK1 level
52. Figure 3.5c. Effect of morin on p-Akt level.
53. Figure 3.5d. Effect of morin on PIP5K level.
54. Figure 3.5e. Effect of morin and p-Akt activator (SC79) on p-Akt level
55. Figure 3.6a. 2D interaction diagram of morin with the protein 1E8Z (PI3K)
56. Figure 3.6b. High resolution model of interaction of morin with PI3K
57. Figure 3.6c. 2D interaction diagram of LY294002 with the protein 1E8Z (PI3K)
58. Figure 3.6d. High resolution model of interaction of LY294002 with PI3K
59. Figure 3.6e. 2D interaction diagram of morin with the protein 4PYP (Glut 1)
60. Figure 3.6f. High resolution model of interaction of morin with Glut 1
61. Figure 4.1. Outline of chapter 4
62. Figure 4.2. Effect of LPS and morin on Viability of SW480 cells by MTT assay
63. Figure 4.3. Effect of morin on Nrf2 in SW480 cells using EMSA
64. Figure 4.4. Effect of LPS and morin on generation of pro-inflammatory cytokines in SW480 cells
65. Figure 4.5. Effect of LPS and morin on NF-kB translocation in SW480 cells
66. Figure 4.6a. Effect of LPS and morin on p-p38 levels in SW480 cells

67. Figure 4.6b. Effect of LPS and morin on p-JNK levels in SW480 cells
68. Figure 4.6c. Effect of LPS and morin on p-ERK levels in SW480 cells
69. Figure 4.7a. Effect of LPS and morin on iNOS expression in SW480 cells
70. Figure 4.7b. Effect of LPS and morin on COX-2 expression in SW480 cells
71. Figure 5.1. Outline of chapter 5
72. Figure 5.2. Scanning electron microscopic images of SW480 cells after morin treatment
73. Figure 5.3. Microfilament analysis after morin treatment
74. Figure 5.4. Effect of morin on the migration of SW480 cells *in vitro*
75. Figure 5.5. Effect of morin on the colony formation of SW480 cells *in vitro*.
76. Figure 5.4. Effect of morin on MMP-2 and MMP-9 in SW480 cells by gelatin zymography
77. Figure 5.5. Effect of morin on MMP-2 and MMP-9 level
78. Figure 5.6. Effect of morin on MMP-7, β-catenin, c-myc and cyclin D1 level
79. Figure 6.1. Outline of chapter 6
80. Figure 6.2. Morphological analysis of spray dried product
81. Figure 6.3. Quantification of morin in spray dried product (HPLC)
82. Figure 6.4. Histopathology analysis of liver of mice of various groups at the end of the study
83. Figure 6.5. Number of ACF formed among mice of various groups
84. 6.6 Colon length among mice of various groups
85. Figure 6.7. Colon weight/length ratio among mice of various groups
86. Figure 6.8. Protein expression analysis of key proteins involved in apoptotic pathways

Abbreviations

µg	Micro gram
µM	Micro molar
AACC	American Association for Clinical Chemistry
5-FU	5- Fluorouracil
AARs	Annual incidence rates
ABPs	Actin Binding Proteins
ACF	Aberrant crypt foci
ADP	Adenisone diphosphate
AMP	Adenisone monophosphate
AO	Acridine orange
AOM	Azoxymethane
APC	Adenomatous polyposis coli
ATCC	American type culture collection
ATP	Adenisone triphosphate
Bad	Bcl 2 associated death promoter
Bax	Bcl 2 associated X protein
B. bifidum	*Bifidobacterium bifidum*
BCA	Bicinchoninic acid
Bcl 2	B-cell lymphoma 2
CD	Crohn’s disease
CFU	Colony formation unit
CO_2	Carbon dioxide
COX-2	Cyclooxigenase-2
c-PARP	Cleaved PARP
CRC	Colorectal cancer
CT	Computed tomography
DCF	Dichlorofluorescein
DCFDA	2’,7’dichlorofluorescin diacetate
DCFH	Dichloro dihydro fluorescein
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbent assay
ERK	Extra cellular signal-regulated kinase
EtBr	Ethidium bromide
FAP	Familial adenomatous polyposis
FACS	Fluorescence-activated cell sorting
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GI	Gastro intestine
GLUT 1	Glucose transporter 1
H_2O_2	Hydrogen peroxide
H_2S0_4	Sulphuric acid
HCl	Hydro chloric acid
Hep G2	A human liver cancer cell line
HIF-1	Hypoxia inducible factor-1
HK	Hexokinase
HNPCC	Hereditary nonpolyposis colon cancer
HPLC	High pressure liquid chromatography
IκB	Inhibitor of κB
IBD	Inflammatory bowel disease
IC_{50}	50% Inhibition concentration
ICMR	Indian council of medical research
IGF1	Insulin-like growth factor 1
IL-1ß	Interleukin-1ß
IL-6	Interleukin-6
NOS	Nitric oxide synthase
JNK	Jun N-terminus kinase
KH_2PO_4	Potassium dihydrogen phosphate

KOH	Potassium hydroxide
L. casei	*Lactobacillus casei*
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinases
MMPs	Matrix metalloproteinase
mg	Milli gram
mL	Milli litre
mm	Milli molar
MMR	Mismatch repair genes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate
NADH	Nicotineamide adenine dinucleotide
NaOH	Sodium hydroxide
2-NBDG	2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose
NCD	Non communicable diseases
nm	Nano molar
NO	Nitric oxide
OD	Optical density
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PDH	Pyruvate dehydrogenase
PI3K	Phosphoinositide 3-kinase
PK	Pyruvate kinase
Rh123	Rhodamine 123
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
STAT3	Signal transducer and activator of transcription 3
TBS	Tris-buffered saline

TBST	Tris-buffered saline containing 0.1% Tween 20
TCA	Tri chloro acetic acid
TGF	Transforming growth factor
TNF-α	Tumour necrosis factor-α
TIMP	Tissue inhibitor of metalloproteinase
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
WHO	World health organization
$\Delta\psi m$	Mitochondrial membrane potential

CHAPTER 1

Introduction and Review of Literature

1. 1. INTRODUCTION

We all live in an era with improved global average living standards and increased access to adequate health care. These measures have an influence on the average life expectancy in most regions of the world. Even though improvements are achieved in all the areas of life, appearance of various types of diseases are the huge burdens against the betterment of today's human life. Among various types of diseases, some that were once rare have become common, others have disappeared and new varieties have emerged (Blaser, 2006).

Mainly three categories of burden of disease conditions are distinguished

1. Communicable, maternal, perinatal and nutritional diseases
2. Non-communicable diseases (NCDs)
3. Injuries (which include violence and conflict)

It is expected that, by the year 2020, non-communicable diseases account for seven out of every ten deaths in the developing regions, compared with less than half of today. Injuries, both unintentional and intentional, are also growing in importance and it is expected that by 2020, ill health arising from non-communicable diseases may be higher than that of infectious diseases (Murray et al., 1996).

1.1.1. Non-communicable diseases (NCDs)

Chronic non-communicable diseases are presently the main reason of both disability and death worldwide and are the result of a combination of genetic, physiological, environmental and behaviour factors. It is a heterogeneous group of diseases, including cardiovascular conditions, cancers, chronic respiratory conditions, diabetes etc., affect people of all ages and all social classes (Organization, 2002). It is estimated that non-communicable diseases mortality is about 41 million in each year, equivalent to 71% of all deaths globally. In every year, about 15 million people die from NCD having 30

and 69 years of age; over 85% of these "premature" deaths occur in low and middle income countries. Cardiovascular diseases are responsible for most NCD deaths, affecting about 17.9 million people annually followed by cancers (9.0 million), respiratory diseases (3.9 million) and diabetes (1.6 million) (Riley and Cowan, 2014).

1.1.2. Non-communicable diseases (NCDs) in India

In India, non-communicable diseases (NCDs) are the main reason for mortality. Non Communicable Diseases Country Profiles 2018, released by WHO (Organization, 2018) point out that, in India NCDs are the biggest global killers accounting for 63% of all deaths. It is distributed in such a way that 27% by cardiovascular diseases, 9% by cancers, 11% by chronic respiratory disease, 3% by diabetes and 13% by other NCDs. It is also noticed that these statistical values are higher than that of 2014 report, in which 60% of the total mortality reported annually in India was from NCDs (Organization, 2014). This massive rise in the disease pattern of NCDs is really a serious public health issue. It is also noticed that more than 20% of the population in India has at least one chronic disease and more than 10% of the people have more than one.

1.1.3. Risk factors for NCDs

Risk factors for NCDs includes modifiable behavioural risk factors like unhealthy diet, harmful use of alcohol, tobacco use, physical inactivity etc. where, 4.1 million annual deaths have been ascribed to excess salt/sodium intake. More than half of the 3.3 million yearly deaths attributable to alcohol use are from NCDs, including cancer. Insufficient physical activity contributes to about 1.6 million deaths annually. Metabolic risk factors like raised blood pressure, overweight/obesity, hyperglycemia (high blood glucose levels) and hyperlipidemia (high levels of fat in the blood)

contribute to four key metabolic changes that increase the risk of NCDs (Forouzanfar et al., 2016b). This burden is expected to worsen in the coming years.

Among several risk factors for NCDs, unhealthy diet plays the key role. Global diet is undergoing a frightening transition with staple foods are becoming more refined and processed, fat and meat intakes are increasing, more processed dairy products and other foods are consumed and more numbers of meals are consumed outside the home, making households more reliant on the food industry. Dietary behaviours of peoples have been influenced by urbanisation, economic development, market globalisation and industrialisation where the move from a traditional to a western-type diet is observed as the characteristic feature (Drewnowski and Popkin, 1997). Consequently, this transition has been associated with an increase in the global prevalence of non-communicable diseases (Vorster and Gibney, 2009).

A huge number of literatures point towards to the fact that unhealthy diets and specific nutrient deficiencies influence the development of NCDs and appropriate dietary changes may reduce the risk of NCDs (Vorster and Gibney, 2009). Globally, calories gained from sugars, meat, fats and oils have been increasing during recent times and those from fibre-rich foods such as pulses, whole grains and roots have been diminishing. Consumption of processed and convenience foods continue to rise rapidly, especially in low and middle income countries. Many studies have proven that the consumption of predominantly plant-based diets reduces the risk of developing obesity, diabetes, cardiovascular diseases and some forms of cancer. Plant-based diets are rich in vegetables, fruits, whole grains, pulses, nuts and seeds and have only modest amounts of meat and dairy. Such diets can help to attain and sustain a healthy weight, reduce blood pressure and are also rich in sources of dietary fibre. Dietary fibre rich

food can prevent many of the NCDs, especially cancer and more precisely colorectal cancer (Cecchini et al., 2010, Lim et al., 2012).

1.2.1. Cancer

Among various non-communicable diseases, cancer is the one of the major and most threatening non-communicable disease with high mortality rate and has become the major cause of death among the population in developed countries as well as in developing countries (Jemal et al., 2011). It is considered as a group of diseases having abnormal cell growth with the potential to invade or spread to other parts of the body (Edge and Compton, 2010)**.** The most common types of cancer that appears among males are lung cancer, prostate cancer, colorectal cancer and stomach cancer (McGuire, 2016) and among females, the most common types are cervical cancer, lung cancer, colorectal cancer and breast cancer **(Figure 1.1),** (Forouzanfar et al., 2016a).

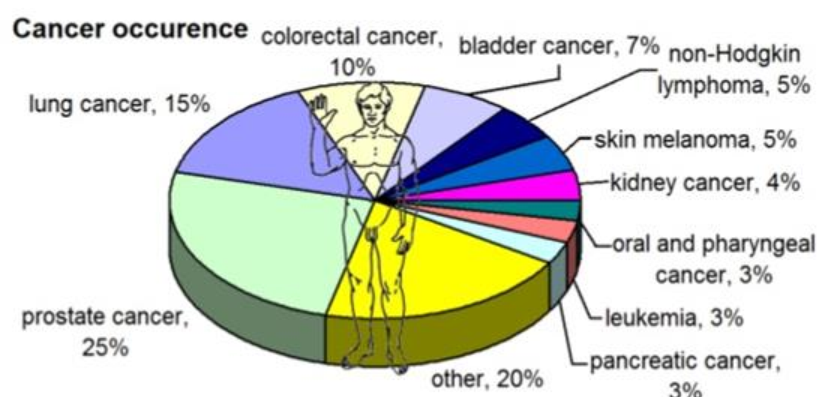

Figure 1.1.a. Cancer occurrence among males (*https://goo.gl/images/W8gY8K*)

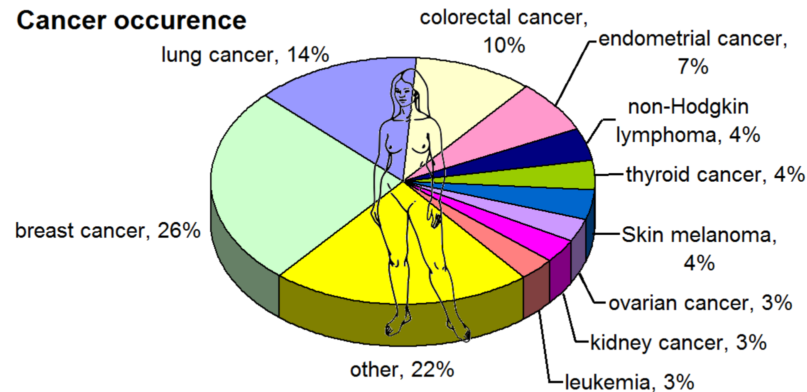

Figure 1.1.b. Cancer occurrence among females (*https://goo.gl/images/ HdcA48*)

Cancer develops as a result of the transformation of normal cells into tumour cells via a multistage process that generally progresses from a pre-cancerous lesion to a malignant tumour. These modifications are the result of the interactions of a person's genetic factors with 3 categories of external agents, including:

1. Physical carcinogens like ultraviolet and ionizing radiation
2. Chemical carcinogens like polycyclic aromatic hydrocarbons (benzo pyrene), aromatic amines (2-acetyl aminofluorene), amino azo dyes, N-nitroso compounds, carbamates, halogenated compounds (trichloroethylene) etc.
3. Biological carcinogens, like infections from certain bacteria, viruses or parasites.

According to global cancer report 2018 (Bray et al., 2018), cancer is the second leading cause of death globally and is responsible for about 9.6 million deaths in 2018 and which reflects that, about 1 in 6 deaths is due to cancer. About 70% of deaths from cancer happen in low and middle-income countries. Behavioural and dietary risks like high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use and alcohol use are the major five risk factors contributing towards cancer development. Tobacco use is the most important risk factor for cancer and is responsible for approximately 22% of cancer deaths (Forouzanfar et al., 2016b).

Cancer causing infections, such as hepatitis and human papilloma virus (HPV) infection, are responsible for up to 25% of cancer cases in low and middle-income countries (Plummer et al., 2016). In 2018, by combining all forms of cancer, excluding non-melanoma skin cancer, it is found that there were 17 million new cases of cancer worldwide; 8.8 million (52%) in males and 8.2 million (48%) in females, giving a male:female ratio of 10:9.3 (Forouzanfar et al., 2016b). The World age-standardised (AS) incidence rate item shows that there are 204.7 new cancer cases for every 100,000 men in the world, and 175.6 for every 100,000 females (Forouzanfar et al., 2016b). It is anticipated that, there will be 27.5 million new cancer cases worldwide each year by 2040, if recent drifts in incidence of major cancers and population growth are seen globally in the future. This is an increase of 61.7% from 2018 and is expected to be more in males (67.6% increase) than in females (55.3% increase), (**Figure 1.2**), (Bray et al., 2018).

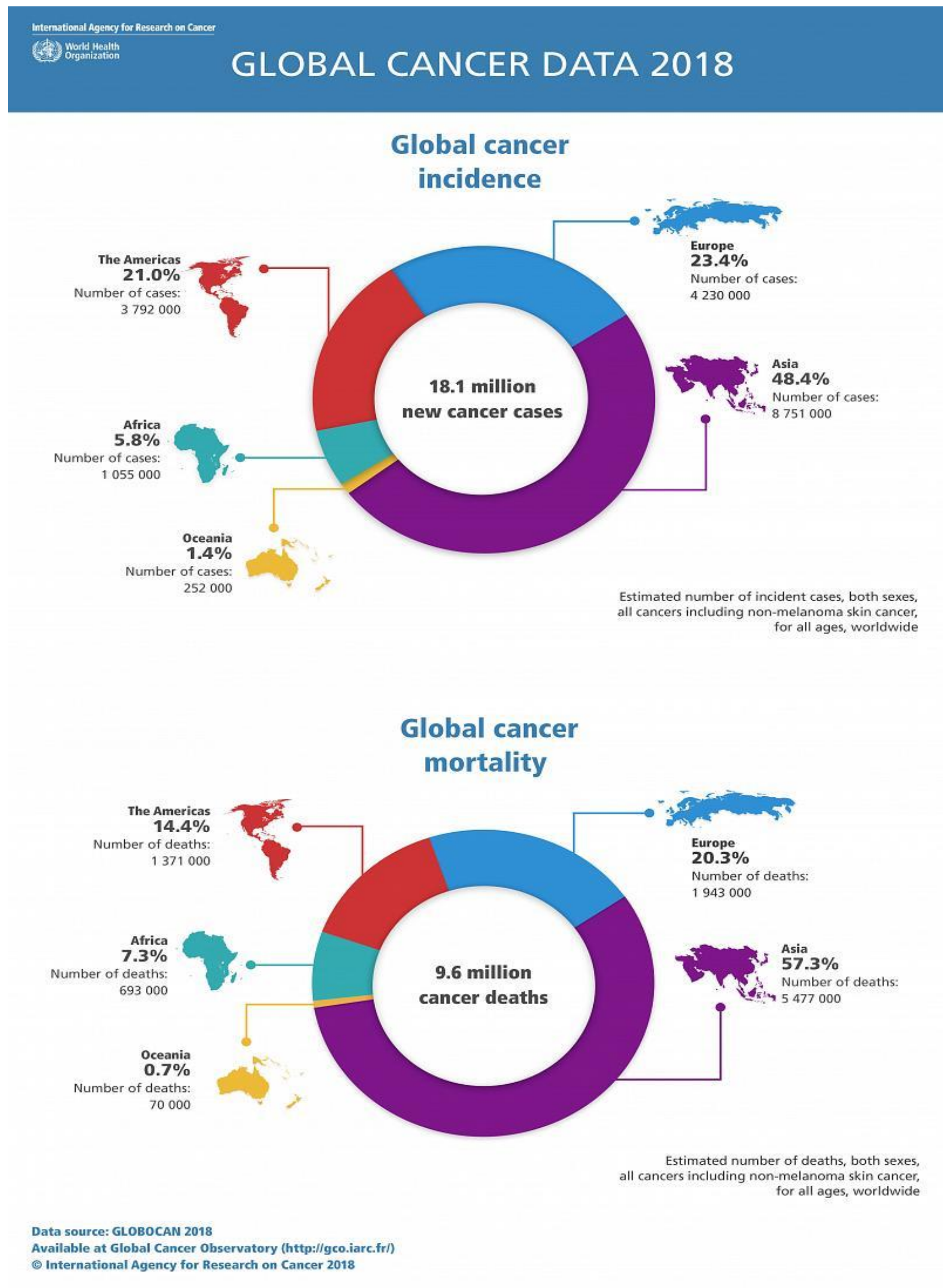

Figure 1.2. Global cancer data (*https://goo.gl/images/kVtCkn*)

1.2.2. Colorectal cancer (CRC)

Colorectal cancer, also known as colon cancer, rectal cancer or bowel cancer is a cancer that starts in the colon or the rectum. These cancers are called as colon cancer or rectal cancer, depending on where they start. Usually colon cancer and rectal cancer are grouped together because they have many features in common **(Figure 1.3)**. Among numerous types of cancers, colorectal cancer is one of the most frequently occurring malignancies worldwide (Murphy et al., 2018). It is the third most frequently diagnosed cancer after lung and breast cancers and the fourth most common cause of death (Spanos et al., 2008, Fund and Research, 2007). GLOBOCAN data reports that there were over 1.8 million new colorectal cancer cases and 881,000 deaths in 2018 (Bray et al., 2018), that accounts for more than 10% of all cancer incidence and almost 8% of total cancer deaths (Center et al., 2011). It is estimated that, about 56% of patients with CRC die from their cancer. The prevalence of CRC has amplified steadily in recent years and it is predicted that by the year of 2035, worldwide number of CRC cases will rise to 1.36 million for men and 1.08 million for women (Stewart and Wild, 2014).

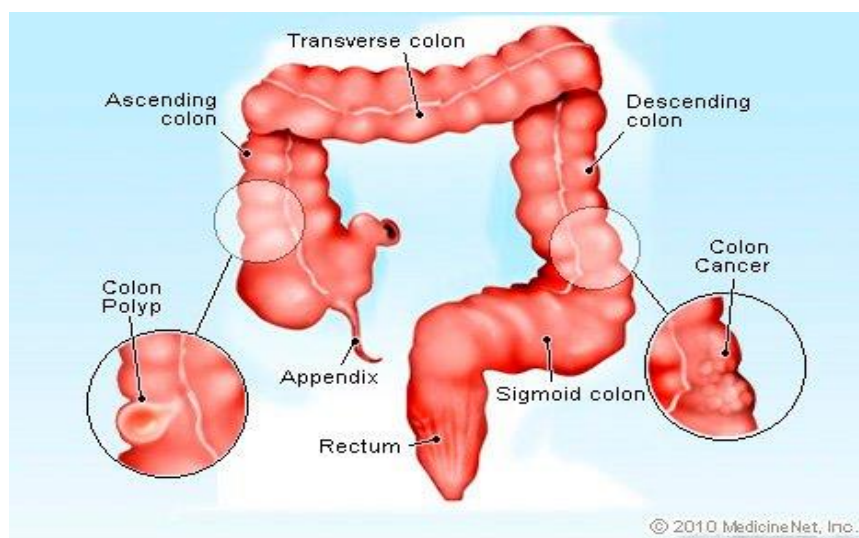

Figure 1.3. Colon cancer and polyp (*https://goo.gl/images/aj7xFE*)

1.2.3. Colorectal cancer in India

In India, the annual incidence rates of colon cancer and rectal cancer among men are 4.4 and 4.1 per 100000, respectively. The annual incidence rate of colon cancer among women is 3.9 per 100000. For men, colon cancer ranks 8th and rectal cancer ranks 9th among various types of cancers. Among women, rectal cancer does not figure in the top 10 cancers, whereas colon cancer ranks 9th. According to the ICMR (Indian Council of Medical Research) report in 2013, the highest annual incidence rate of CRC among men in India was recorded in Thiruvananthapuram (4.1) followed by Bangalore (3.9) and Mumbai (3.7). The highest annual incidence rate of CRC among women was recorded in Nagaland (5.2) followed by Aizwal (4.5) (Katoch, 2013).

1.2.4. Stages of colorectal cancer

The stage of a cancer delineates how far it has spread and by determining the stage of the cancer most apposite treatment can be given **(Figure 1.4).**

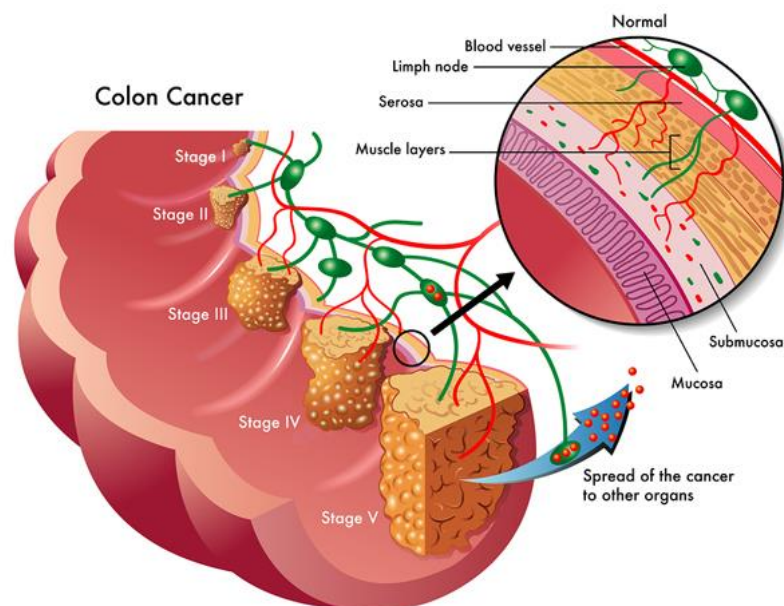

Figure 1.4. Stages of colorectal cancer (*https://goo.gl/images/dq65Pw*)

The different stages of colon cancer are:

Stage 0: This is the earliest stage and in this stage cancer is with in the inner layer of the colon or rectum or within the mucosa. It is also called as **Duke A stage** or carcinoma in situ.

Stage 1: At this stage cancer has grown through the inner layer of the colon or rectum but has not yet spread beyond the wall of the rectum or colon. It is also known as **Duke B stage**.

Stage 2: A stage in which cancer has grown through or into the wall of the colon or rectum, but it has not yet extended the nearby lymph nodes. It is also known as **Duke C stage**.

Stage 3: The adjacent lymph nodes have been invaded by the cancer, but it has not yet affected other parts of the body. It is also known as **Duke D stage**.

Stage 4: The cancer has spread to other parts of the body, including other organs, such as lung, the membrane lining the abdominal cavity, ovaries or liver. It is also known as **Duke E stage**.

Recurrent stage: The cancer has returned after treatment and can affect the rectum, colon or another part of the body.

1.2.5. Symptoms of colorectal cancer

- Changes in bowel habits
- Diarrhoea or constipation
- A sensation that the bowel does not empty correctly after a bowel movement
- Blood in faeces that makes stools look black
- Bright red blood coming from the rectum
- Pain and bloating in the abdomen

- A sense of fullness in the abdomen, even after not eating for a while.
- fatigue or tiredness
- Unexplained weight loss
- Detection of lump in the abdomen or the back passage felt by your doctor
- Presence of iron deficiency in men or in women after menopause

Since many of these symptoms may associated with other conditions also, it is important to see a doctor if symptoms persist for 4 weeks or more.

1.2.6. Diagnosis of colorectal cancer

Screening tests will help to detect polyps before they become cancerous and also to detect colon cancer during its early stages, when the chances of a cure are much higher.

The most common screening and diagnostic procedures for colorectal cancer are;

- Faecal occult blood test (blood stool test)
- Stool DNA test
- Flexible sigmoidoscopy
- Colonoscopy
- Barium enema X-ray
- CT colonography
- Ultrasound or MRI scans

1.2.7. Risk factors for CRC

Many risk factors are associated with the occurrence of colorectal cancer. The risk factors for the development of CRC are generally divided into modifiable risks factors and non-modifiable risks factors (**Figure 1.5**). Even though most CRCs are sporadic, genetic factors increase the risk considerably.

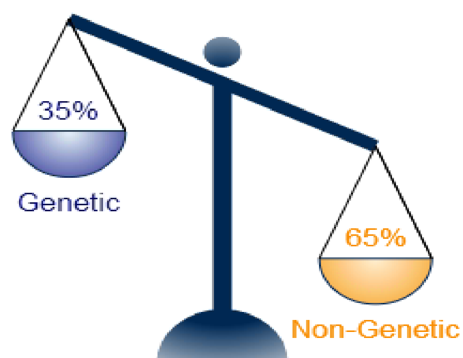

Figure 1.5. Genetic and non-genetic risk factors for colorectal cancer (*https://goo.gl/images/nCJmdu*)

Under non- modifiable risks factors; age, personal history of adenomatous polyps, personal history of inflammatory bowel disease, family history of colorectal cancer or adenomatous polyps (FAP) and its variants (Gardner, Turcot and attenuated FAP) and MYH-associated polyposis, inherited genetic risk are the major ones. These are coming under colonic polyposis category and hereditary nonpolyposis colon cancer (HNPCC) is coming under non-colonic polyposis category. Non-modifiable risk factors are playing lesser role compared to modifiable risk factors

1.2.7.1. Non- modifiable risks factors

1.2.7.1.1. Age

The chance for appearing colorectal cancer increases after the age of 40 years and a sharp rise is noticed after 50 years of age. More than 90% of colorectal cancer cases occur in people aged 50 years or older (AI and Hispanicb, 2008). The incidence rate is more than 50 times higher in persons aged 60 to 79 years than in those younger than 40 years (El-Shami et al., 2015).

1.2.7.1.2. Personal history of inflammatory bowel disease

It is found that inflammatory bowel diseases, ulcerative colitis and crohn disease increase an individual's overall risk of developing colorectal cancer and it is observed that in patients with inflammatory bowel disease, the relative risk for colorectal cancer is in between four to twenty folds (Haggar and Boushey, 2009).

1.2.7.1.3. Family history of colorectal cancer or Adenomatous polyps

Person having a history of adenomatous polyps or colorectal cancer in one or more first-degree relatives are at increased risk of developing colorectal cancer and if the first degree relative is younger than 60 years of age, again it is much higher (Boardman et al., 2007). It may be due to the shared environmental factors or inheritance of genes among the family members.

Mutations in the tumour suppressor gene, adenomatous polyposis coli (APC) results in appearance of Familial Adenomatous Polyposis (FAP) (Wilmink, 1997). Attenuated FAP is related with the same genetic mutation as FAP but is characterized by less adenomas and a later average age of CRC presentation. A variant of FAP, Turcot syndrome appears as a consequence of mutations in mismatch repair genes (MMR) or a germ line APC mutation. Fault in one of the MMR genes leads to an autosomal dominant condition called Lynch syndrome. Occurrence Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is associated with mutations in genes involved in the DNA repair pathway (MLH1 and MSH2 genes). Mutations in the base excision repair gene, mutY homologue is associated with MYH associated polyposis (Papadopoulos et al., 1994, van Vliet et al., 2011, Guarinos et al., 2010).

1.2.7.2. Modifiable risks factors

Physical inactivity, obesity, improper diet, cigarette smoking and heavy alcohol consumption are the major modifiable risk factors for colorectal cancer. It is found that modifiable risk factors are playing major role compared to non-modifiable risk factors.

1.2.7.2.1. Diet

Nutritional practices strongly affects the risk of colorectal cancer and variations in food habits might reduce up to 70% of this cancer burden (Willett, 2005). Diets low in fruits, vegetables and dietary fibre, high in animal fat, high red meat, meats are cooked at high temperatures are closely associated with development of colorectal cancer. Dietary fibre in the food can dilute faecal content, increase faecal bulk and reduce transit time. Fibre also helps in balancing intestinal pH and stimulates intestinal fermentation and production of short chain fatty acids, which intern reduce risk of colorectal cancers (Dhingra et al., 2012). Fat in food favours the development of a bacterial flora capable of degrading bile salts to potentially carcinogenic N-nitroso compounds (Larsson and Wolk, 2006). Heme iron in red meat as well as production carcinogenic compounds (heterocyclic amines and polycyclic aromatic hydrocarbons) on cooking meats at high temperatures augment the genesis of colorectal cancer (Santarelli et al., 2008).

1.2.7.2.2. Physical inactivity and obesity

Reduced physical activity and excess body weight are positively correlated with colorectal cancer incidence and the frequency and intensity of physical activity inversely associated with risk. Being overweight or obese, notably increases circulating estrogens and decreases the insulin sensitivity, reduces gut motility and physical activity, reduces metabolic rate and oxygen consumption and all these factors make it more prone to develop colorectal cancer (Lee et al., 2007, English et al., 2004).

1.2.7.2.3. Cigarette smoking and heavy alcohol consumption

It is observed that not only the lung cancer but also the colorectal cancer (especially both formation and growth rate of adenomatous polyps) is closely associated with the carcinogens found in tobacco (Ezzati and Riboli, 2013). Along with smoking, the regular alcohol consumption may be associated with increased risk for developing colorectal cancer. Aldehyde, the end product of alcohol metabolism can be carcinogenic and specific mutations induced by tobacco are less efficiently repaired in the presence of alcohol. Also, alcohol may function as a solvent, augmenting infiltration of other carcinogenic molecules in the tobacco into mucosal cells (Ferlay et al., 2013).

1.3. Various conditions in the body which worsens the colorectal cancer condition

1.3.1. Alteration in glucose metabolism

Targeting metabolic pathways for cancer therapy appears interesting at first glance, as enzymes are striking molecular targets. However, to be an attractive candidate for cancer therapy, there must be a significant alteration in the requirement for a given enzyme's activity between cancer cells and normal proliferating cells.

1.3.1.1. Warburg effect

According to the great Biochemist Otto Warburg, contrary to normal cell metabolism which principally depends on mitochondrial oxidative phosphorylation to generate Adenosine triphosphate (ATP), most cancer cells show amplified glycolysis rate. This is known as Warburg effect, is a hallmark in cancer and he won the Nobel Prize in 1931for this discovery (Warburg, 1956). Due to this increased glycolytic metabolism, cancer cells produces abundant ATP as the main source of energy to drive cell growth (Phan et al., 2014).

Cancer cells need high glucose uptake for energy production and for the production of metabolic intermediates for supporting cell survival, growth and metastasis. As a result

of this, a high glucose condition has been shown to promote progression in many cancer cells like colon, bladder, breast and prostate (Chen et al., 2015, Takatani-Nakase et al., 2014). Newest epidemiological studies revealed a connection between obesity and type II diabetes mellitus and increased appearance of new tumour cases (Vona-Davis et al., 2007).

Overexpression of glucose transporters, such as glucose transporter 1 (GLUT1) and a number of glycolytic enzymes, including hexokinase (HK), lactate dehydrogenase (LDH) and pyruvate kinase (PK), subsidises to the acquisition of metabolic profile of cancer cells (Cerella et al., 2013b, Cerella et al., 2013a). Upregulation of glucose transporters are observed in many tumours fulfilling the high glucose requirements. Strikingly GLUT-1 is considered as a master regulator of the Warburg effect during the course of neoplastic transformation (Macheda et al., 2005) and hence it can be used as a molecular marker in CRC to indicate the tumour hypoxia degree (Chung et al., 2009), expression level of hypoxia inducible factor-1 (HIF-1) and chemotherapy resistance of cancer cells (Evans et al., 2008, Song et al., 2014, Zhang et al., 2013).

Alterations in glucose metabolism leads to increased production of lactate (Walenta and Mueller-Klieser, 2004). Raised lactate within cancer cells shows a switch in glucose metabolism from aerobe to an anaerobe consumption of glucose. A stable increase of anaerobic glucose utilisation in primary tumours typifies more aggressive tumour cells (Walenta et al., 2001). Increased conversion of glucose into lactate, reduced mitochondrial respiration rate and enhanced secretion of lactate results in the acidification of the tumour as well as its environment (Pelicano et al., 2006). Even though in normal cells an acidic microenvironment is toxic and it will trigger induction of caspase mediated apoptosis, this will give an advantage for tumour cells with a resistance to acidosis, indicated by increased activity of H^+ transporter (Thews et al., 2008, Gatenby

et al., 2007). The condition of long term acidosis is leading to the damage of the extra cellular matrix surrounding the tumour and it is further supported by the acid-mediated activation of matrix metalloproteinases and thus helping migration of tumour cells to other parts of body from point of origin (Langbein et al., 2006).

1.3.1.2. Role of PI3K/Akt signaling pathway

It is also observed that many proto-oncogenes like Ras, c-Myc and tumour suppressor gene like p53 has effect on cancer metabolism and mutations in these genes up-regulate glucose uptake in cancer cells and stimulates a metabolic phenotype, which aids tumour cell growth and proliferation. Many signaling pathways like phosphoinositide 3-kinase (PI3K), hypoxia inducible factors (HIFs) and p53 control the expression of important genes involved in cancer cell's energy metabolism and proliferation. Among these, PI3K signaling plays a major role (Cairns et al., 2011). Mounting evidences point out the fact that the PI3K/Akt signaling pathway plays central role in regulating glucose metabolism (Moench et al., 2016). This signaling pathway is also involved in cell proliferation and migration. Oncogenes which are expressed during the process of carcinogenesis activate PI3K and activated PI3K can induce cancer cell proliferation and metastasis. Akt is a central signaling molecule in the PI3K pathway and Akt signaling is activated by growth factors and other extracellular stimuli. Akt stimulates glucose uptake and glycolysis by upregulating the expression of glucose transporter proteins and activating glycolytic enzymes (Elstrom et al., 2004). Also, Akt is found to be involve in the phosphorylation and modulation of several downstream targets like GSK-3β and Bad, which are implicated in cell proliferation and metastasis (Vara et al., 2004).

The Akt signaling pathway is activated in a large number of human cancers including colorectal cancer and Akt inhibition is associated with reduced cell growth and decreased migration of cancer cells (Sarkar and Li, 2004). It is also evidenced that in non-neoplastic

normal intestinal mucosal cells, the PI3K/Akt signaling activity always low. Therefore, a low concentration of anticancer agents might not kill non-neoplastic cells or normal intestinal mucosal cells, suggesting that Akt inhibition may be an attractive methodology for preventing or treating human malignancies (Sarkar and Li, 2004).

1.3.2. Inflammation and CRC

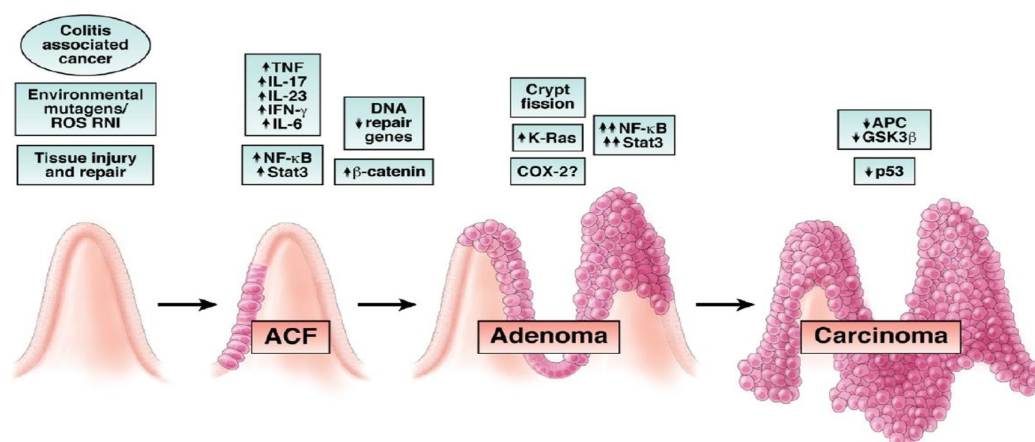

Figure 1.6. Mechanisms of colitis-associated cancer (CAC) development

(https://goo.gl/images/ix8dfF)

In normal cells inflammation is regarded as a biological response of the host tissue to protect itself against harmful stimuli such as pathogenic infections and irritants, and inflammation is important in the healing process. The link between inflammation and tumorigenesis is well-established and in the last decade many reports are available from genetic, pharmacological and epidemiological data further supporting this. Numerous studies provide evidence that chronic inflammation increases the risk of cancer, promotes tumour progression and supports metastatic spread (**Figure 1.6**) (Aggarwal and Gehlot, 2009). Currently it is accepted that human malignancies up to 25% of are associated to chronic inflammation and to bacterial and viral infections (Perwez Hussain and Harris, 2007). Such chronic inflammation associated with cancer enables unlimited replicative potential of cancer cells, independent of growth factors, resistance to growth inhibition,

escape of programmed cell death, enhanced angiogenesis, tumour extravasation and metastasis (Multhoff et al., 2012). Cancer-related inflammation denotes the seventh hallmark in the development of cancer (Colotta et al., 2009).

A clear link exits between inflammation and CRC and chronic sustained inflammation, triggered by high fat diet, obesity or consumption of less amount of vegetable are related with the CRC development (Cho et al., 2016). Inflammatory bowel disease is a major risk factor for the development of colon cancer. It is also observed that inflammation is also be involved with other forms of sporadic as well as heritable colon cancer. Furthermore, CRC patients having inflammatory responses have a tendency to show higher rate of tumour recurrence and more side effects to medical and surgical treatment than those without inflammatory responses (Yoon, 2012).

CRC, that is linked to genetic mutations shows a contribution from inflammation to tumour development, as shown by the diminished CRC mortality with regular use of anti-inflammatory drugs. These data further strongly support a pro-tumorigenic role of inflammation in colon cancer. Several factors can influence the initiation of inflammation and establishment of CRC. It is believed that chronic inflammation is a driving force for the growth of colorectal cancer as evident in patients with ulcerative colitis (UC) and patients with Crohn's disease (CD), the two most important forms of inflammatory bowel disease (IBD) in humans. Indeed, the natural history of inflammatory bowel disease, particularly UC can be marked by the development of colorectal cancer (CRC). There exists evidence that clearly shows that the risk of IBD-associated CRC is strictly linked to the duration and extension of inflammation (Eaden et al., 2001, Ekbom et al., 1990)

1.3.2.1. NF-κB signaling

NF-κB is a transcription factor activated by a variety of stimuli and regulates diverse gene expression and biological responses. NF-κB, a latent cytoplasmic transcriptional factor

complexed with an inhibitor of κB (IκB), is composed of relA (p65) and p50 subunits, while IκB-a, IκB-b and IκB-g are the most abundant inhibitors of NF-κB (Baeuerle and Baltimore, 1996). After stimulation by a variety of inflammatory mediators, it is phosphorylated and degraded and the free NF-κB thus produced will translocate into the nucleus to regulate the expression of multiple NF-κB-dependent genes, like acute-phase response proteins and inflammatory enzymes, including inducible nitric oxide synthase (iNOS) in that cells. The promoter of iNOS contains two consensus NF-κB binding sites that mediate further inflammatory responses (Viatour et al., 2005, Campbell and Perkins, 2006, Xie et al., 1993).

NF-κB imparts a prominent role in colitis associated tumorigenesis and colorectal cancer. In 50% of colorectal and colitis-associated tumours, the aberrant NF-κB activation has been detected and animal studies have established a role for NF-κB in colitis associated colon cancer development (Karin and Greten, 2005). The pro-tumorigenic role of NF-κB can be achieved through classical or alternative activation route. NF-κB activation results in tumorigenesis by promoting cell proliferation and angiogenesis, increasing cell invasion and metastasis and inhibiting cell death (Naugler and Karin, 2008). The anti-apoptotic action of NF-κB is mediated via its activation of Bcl 2 family genes like Bcl-xL, Bcl 2 along with other genes. Inhibition of NF-κB activity greatly increases cell sensitivity to chemotherapeutics and ionizing radiation and cancer cells with activated NF-κB shows resistance to these treatment approaches (Meylan et al., 2009).

1.3.2.2. MAPK (Mitogen-activated protein kinases) signaling

Inflammation in cells can also be mediated through MAPK signaling pathway and inhibition of these signaling pathways can reduce the inflammatory status of the cancer cells. Once the activation of the MAP kinases takes place, it will result in the activation

of transcription factors present in the cytoplasm or nucleus by phosphorylation, leading to expression of target genes resulting in a biological response (Meylan et al., 2009). Three main groups of particularly regulated MAP kinase cascades are recognized in humans that lead to altered gene expression are Extra cellular signal-regulated kinase (ERK1/2), Jun N-terminus kinase (JNK), and p38 MAP kinase. Amplified activity of MAPK, in particular p38 MAPK and their involvement in the regulation of the synthesis of inflammation mediators at the level of transcription and translation, make them potential targets for anti-inflammatory therapeutics. Researchers have developed inhibitors targeting p38 MAPK and JNK pathways and the results of preclinical studies using them reports that, they exhibit anti-inflammatory activity (Kaminska, 2005). So now it is widely accepted that the MEK/ERK pathway plays a significant role in CRC formation and progression.

The inflammatory mediators produced during the activation of inflammatory signaling include cytokines secreted by the activated tumour stroma that modulate tumour growth and enhance invasiveness of tumour cells by activation of oncogenic signaling pathways in tumour cells, including activation of NF-κB by Tumor necrosis factor-α (TNFα) and Interleukin-1β (IL-1β) and activation of signal transducer and activator of transcription 3 (STAT3) by Interleukin-6 (IL-6) (Lin and Karin, 2007). In addition, activation of oncogenes, such as k-Ras, has been shown to activate NF-κB signaling in tumour cells and to trigger the production of several proinflammatory mediators (Sparmann and Bar-Sagi, 2005).

1.3.2.3. Role of inducible NOS (iNOS) and cyclooxigenase-2 (COX-2)

Amplified expression of inducible NOS (iNOS) has been observed in colorectal tumours leading to the modification in the cell signaling and rise in the levels of pro-inflammatory and angiogenic signals of the cells and thus intensify the cancer. A 50% rise in the level

of cyclooxigenase-2 (COX-2) is observed in adenomas and 85% increase in adenocarcinoma and COX-2 is expressed in epithelial and stromal cells in human intestinal tumours. Pro-inflammatory cytokines, such as IL-1β and TNFα and hypoxic environment (Kaidi et al., 2006) are potent inducer of COX-2 expression. NF-κB and Wnt signaling have both been shown to regulate the expression of COX-2. Overexpression of COX-2 increased azoxymethane (AOM) induced tumour formation (Al-Salihi et al., 2009). So, inhibitors that can selectively inhibit iNOS and combination strategies to inhibit both iNOS and cyclooxygenase-2 may have a preventive role in colon cancer.

1.3.3. Colorectal cancer metastasis

As metastasis is one of the most important challenges against successful cancer treatment, its inhibition is an important strategy in anticancer drugs development. Metastasis of cancer cells is a process by which cancer cells migrate from point of origin to other parts of the body and is a complex processes involving the degradation of the extracellular matrix (ECM) (Chen et al., 2013). Nearly 20% of patients with CRC already have metastases at diagnosis and this figure has been constant over the past two decades (van der Geest et al., 2015). In the case of colorectal cancer, metastasis of CRC causes thousands of deaths every year worldwide (Dziki et al., 2011). The most common site of spread for colon or rectal cancer is the liver. Colorectal cancer cells may also metastases to the lungs, bones, brain or to spinal cord (**Figure 1.7**).

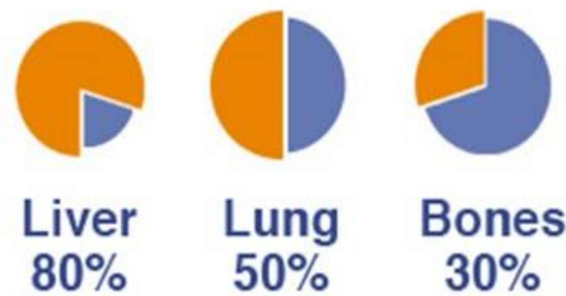

Figure 1.7. Organs involved most often in metastatic colon cancer

(*https://goo.gl/images/Vxj3yM*)

1.3.3.1. Role of Matrix metalloproteinase (MMPs) in CRC metastasis

Matrix metalloproteinase (MMPs), highly conserved zinc ion dependent proteolytic enzymes, are able to degrade the epithelial basement membrane or extracellular matrix and thus promote the invasion and metastasis of tumour cells. MMPs especially, MMP-2/−9 play significant roles in degrading ECM and its contribution is complex in the process of cancer metastasis leading to the invasion of cancer cells into the circulatory system and invade distant organs or tissues.

Actin is a conservative protein and the actin cytoskeleton is required for various biological functions like formation and maintenance of tight junctions, adherence junctions, focal adhesions and cell contraction and is involved in many different processes, such as muscle contraction, cell motility, vesicle trafficking, cell migration, adhesion and maintenance of shape of cells (Khaitlina, 2007, Simiczyjew et al., 2017). The alterations in these cellular compartments can affect cell adhesion dynamics. The organisation of the actin cytoskeleton is controlled by numerous actin-binding proteins (ABPs), which control the dynamic balance between monomeric and polymerized (filamentous) form of actin. This proportion is found to be disturbed in cancer cells (Gross, 2013). Throughout metastasis, alterations in shape and cell movement necessitate restructuring of the cell cytoskeleton. Studies have been reported that various approaches

that block the actin cytoskeleton alteration can bring about great therapeutic potential against tumour cell migration, invasion and metastasis.

1.3.3.2. Role of Wnt/β-catenin signaling pathway in CRC metastasis

The Wnt/β-catenin signaling pathway is associated with celll cycle regulating genes (c-myc & cyclin D1) & genes related to metastasis & invasion (MMP-2, MMP-7 and uPA) and has been seemed to be deregulated in colon cancer (Scholer-Dahirel et al., 2011). As MMP-2, MMP 7 and MMP-9 are the main targets of Wnt/β-catenin signaling pathway, it plays a significant role in cancer metastasis (78). The cytoplasmic protein, β-catenin plays a central role in the origin and progress of tumours, in the Wnt signaling pathway. When β-catenin phosphorylation and ubiquitin dependent degradation are inhibited, β-catenin accumulates in the cytoplasm and forms a complex with the transcription factor, lymphoid enhancing factor 1 (LEF1)/T cell factor (TCF), which is successively transported into cell nuclei. This transcription complex stimulates the expression of downstream target genes, resulting in abnormal cell proliferation and carcinogenesis (Scholer-Dahirel et al., 2011).

1.4. Treatment strategies available for colorectal cancer

The type of treatment will rely on numerous factors like the location, size, stage of the cancer, whether or not it is recurrent and the existing overall state of health of the patient. At present five important approaches are used for the treatment of CRC; surgery, chemotherapy, radiotherapy, targeted therapies and ablation.

Surgery is the most common way of treatment. The affected malignant tumours and any nearby lymph nodes will be removed, to lessen the risk of the cancer spreading. If the cancer is diagnosed at the early stage (stage 0 or I), surgery is found to be a successful tool (Kobayashi et al., 2011). Chemotherapy involves usage of a medicine or chemical to destroy the cancerous cells. As in the case of other cancers, chemotherapy is generally

adopted as a treatment for colon cancer. In certain cases, chemotherapy is used before surgery, for shrinking the tumour volume. Targeted therapy is a type of chemotherapy that specifically targets the proteins that involved in the development of some cancers and has lesser side effects than other types of chemotherapy.

Drugs that may be used for colorectal cancer include bevacizumab (Avastin), ramucirumab (Cyramza), drugs 5-fluorouracil (5-FU), irinotecan, oxaliplatin, cetuximab, panitumumab and capecitabine. Different combinations of these drugs, such as the FOLFIRI regimen (leucovorin, 5-FU and irinotecan) the FOLFOX regimen (leucovorin, 5-FU and oxaliplatin) and the XELOX regimen (oxaliplatin and capecitabine), with or without a monoclonal antibody agent, have been shown to improve outcomes in CRC (Edwards et al., 2012). Radiation therapy involves usage of high energy radiation beams to abolish the cancer cells and to inhibit them from multiplying. This is more frequently used for rectal cancer treatment. In some cases, radiation therapy and chemotherapy are given following surgery to reduce the chances of recurrence. During ablation tumour is destroyed without removing it. It can be performed using radiofrequency, ethanol or cryosurgery. A probe or needle guided by ultrasound or Computed Tomography (CT) scanning technology is used for this.

1.5. Prevention and management of CRC through modulation of diet

It is evident from a large number of studies that CRC cells resistant against actions of various anticancer agents by several mechanisms as well as they have high metastatic potential and have self-sufficiency in growth signals and exhibits intrinsic chemo resistance (Sillars-Hardebol et al., 2010). Out of the currently applied treatment approaches for of CRC, surgery is considered as the most effective treatment approach in the early stage of colorectal cancer and for patients diagnosed with colorectal cancer at advanced stage, chemotherapy also remains as an important strategy. Due to the

increased unfavourable side effects as well as ineffectiveness of currently available cancer chemotherapy, research has been focusing on the discovery of new anticancer agents derived from nature, especially from plants, which are safe and inexpensive and can exhibit excellent value in diminishing the morbidity and mortality of CRC.

Many studies have shown that alteration in lifestyle factors such as food intake, nutrient content and physical activity are closely associated with increased cancer incidence rates (Vijayvergia and Denlinger, 2015). Epidemiological data suggests that diet plays a key role in the prevention and management of CRC (Bishehsari et al., 2014). It is reported in 1981 itself that 90% of the CRC mortality is attributed to the dietary factors (Doll and Peto, 1981). The plant-derived dietary substances can play a vital role in cancer prevention by hindering the action of carcinogens on target tissue thereby quashing the cancer development. These phytochemicals are also reported to play a significant role in the secondary prevention, by reduction of cell growth or enhancement of differentiation and induction of apoptosis in tumour initiated cells (Surh, 2003).

It is reported that daily intake of > 400 g/day of vegetables and fruits diminishes the risk of CRC by 40% (Willett, 1995). There is growing evidence from both *in vivo* and *in vitro* studies that dietary components play protective role against cancer and that include spices, fruits, vegetables, cereals, pulses, nuts etc. The specific bioactive compounds present in them have been shown to be the candidates for their chemotherapeutic potential (Marmot et al., 2007).

1.5.1. Phytochemicals as anticancer agents

Phytochemical based research has explored the anticancer potential of extracts and phytochemicals from a large number of vegetables and fruits (Karikas, 2010, Saunders and Wallace, 2010). Protective elements in them include vitamins, selenium, food polyphenols such as flavonoids, phytoalexins, indoles, phenolic acids, carotenoids etc.

(Surh, 2003, Russo, 2007). Quercetin, curcumin, kaempferol, epigallocatechin gallate, catechin, lycopene, resveratrol and naringin are few compounds coming under this group and studied in depth (Hwang and Lee, 2006, Wong and Fiscus, 2015). Various synthetic derivatives of these compounds from plants, due to their specific structure and wide spectrum of biological activities, are being suggested for treating various types of cancers. Research conducted on these bioactive compounds revealed their capability to exert their antineoplastic activities (Tarapore et al., 2011) and led to the emergence of alternate forms of cancer treatment approach known as nutrition therapy, to battle against cancer through a healthy diet while presenting none of the side-effects which are usually encountered by patients undergoing treatment. 25% to 48% of Food and Drug Administration (FDA) approved anticancer agents are derived from plants (Harvey et al., 2015). Thus, recognizing these bioactive molecules, assessing their broad range pharmaceutical activity, evaluating their precise mechanism of action could aid in the treatment of cancer.

1.5.2. CRC and dietary fibre

Burkitt (1970) hypothesised that dietary fibre reduces the risk of colorectal cancer, based on the observation among rural Africans who ate a diet with a high fibre content are of low rates of colorectal cancer. Many explanations were put forward to explain the mechanism like, it is due to the bulking of stool and dilution of carcinogens in the colonic lumen, due to reduction in transit time and bacterial fermentation in the colonic lumen (Burkitt, 1971).

Even though a many definitions exist for the term dietary fibre, the most accepted definition put forward by American Association for Clinical Chemistry (AACC) in 2001 (Report, 2001) and it defines dietary fibre as the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine

and undergoes complete or partial fermentation in the large intestine. It includes polysaccharides, lignin, oligosaccharides and associated plant substances. Dietary fibre is mainly classified as water soluble/well fermented fibres: pectin, gums and mucilages and water insoluble/less fermented fibres: cellulose, hemicellulose, lignin (Antia, 1973). Now it is suggested from a huge number of research including epidemiologic, clinical metabolic and experimental studies, that colon cancer risk can be minimised by high intake of dietary fibre or a diet high in vegetables, grains or fruit. Fruits and vegetables contain 1.5–2.5 g/100 g of dry weight fibre, non-starch food contains up to 20–35 g of fibre/100 g dry weight and food containing starch provide about 10 g/100 g of dry weight. Whole grains are a main source of dietary fibre and in western countries cereals contribute to about 50% of the fibre intake, vegetables contribute 30–40% dietary fibre intake and fruits contribute about 16% of fibre intake. It is advised that healthy adults should consume 20 g to 35 g of dietary fibre in each day (Selvendran and Robertson, 1994, Lambo et al., 2005).

Fermentation of dietary fibre by the intestinal microflora resulting in the production of fermentation metabolites which can impart benefits to the host (Slavin, 2013). This intestinal microflora is called as probiotics and are defined by Fuller in 1989 as are live microbial supplements which beneficially affect the host animal by improving microbial balance. Currently it is redefined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Hotel and Cordoba, 2001). Out of the large number of microbial species, *Lactocbacillus* and *Bifidobacterium* are most important ones.

Inverse relationship is reported between dietary fibre intake and colorectal cancer risk from a large set of case-control and ecological studies. The underlying anti-carcinogenic mechanisms include: decrease in intestinal transit time and increase of faecal bulk, the

formation of short-chain fatty acids from fermentation by colonic bacteria, the reduction in the production of secondary bile acid (De Preter et al., 2011) and a reduction in insulin resistance (Weickert and Pfeiffer, 2018). Together with the gut immune system, colonic microflora helps in preventing the invasion of pathogenic bacteria to gastrointestinal tract, they competitively exclude the pathogens, prevent adhesion and colonization of pathogen (Buffie and Pamer, 2013). Probiotic bacteria bio-transforms pro-carcinogens and carcinogens into less toxic metabolites and hence, aid in their detoxification; thus, preventing CRC (Pool-Zobel et al., 2005). Studies showed that probiotics may induce apoptosis through different signaling pathways such as, activation of caspase mediated apoptotic signaling pathways, down-regulation of NF-κB, Mitogen-activated protein kinases (MAPK) signaling, COX-2 suppression, activation of cell death via autophagy, tyrosine kinase signaling pathway inhibition and inflammasome deactivation (Uccello et al., 2012). The encroachment in the gut microbiome research has proved that the gut microbes and its metabolome play a remarkable role in the differential modulation of the innate and acquired immunity at the systemic level and mucosal level (Cho et al., 2014). Among these, mechanisms the anticancer activity is more closely associated with the production of short chain fatty acids (SCFAs) in the fermentation metabolites. Acetic acid, propionic acid and butyric are the major short chain fatty acids produced and in these butyric acid is the major one responsible anticancer effect. SCFA production results in decline of intestinal pH, which inhibits growth and multiplication of several pathogenic bacteria in the colon. These acids have both local and systemic biological effects and can play a chief role in preventing cancer. Out of these SCFAs, butyrate a preferred energy source of colonocytes demonstrated direct effect. It enhances growth arrest, cell differentiation, modulates apoptosis of colon cancer cells, stimulates mucosal cell differentiation, promotes epithelial barrier function, detoxifies electrophiles associated

with oxidative stress and modulates glutathione S-transferase and histone acetylation pattern (Roy et al., 2006, Cho et al., 2014). A 45% reduction in aberrant crypt foci (ACF) was observed on laminar delivery of butyrate (Wong et al., 2005). The growth arrest induced by the SCFA was characterized by an increase in the expression of the p21 cell-cycle inhibitor and down-regulation of cyclin B1 (Hinnebusch et al., 2002).

Literatures clearly shows that unhealthy diet plays an important role behind the occurrence of majority of the non-communicable diseases including colorectal cancer. Studies showed a direct relationship between consumption of fruits and vegetables and delayed onset and progression of colorectal cancer. As the currently applied treatment for colorectal cancer possess various adverse effects, scientific world is focussing on plant derived anticancer agents with lesser side effects and lesser cost. As dietary fibre can play a significant role in the prevention and management of colorectal cancer, if it is possible to reach both phytochemical and the dietary fibre simultaneously, it can really take part in the fight against colorectal cancer in both prevention as well as management aspect.

1.6. Current study

With this background, for the current work, thorough literature search was done on phytochemicals and cancer. Based on search results, we selected a set of phytochemicals and the first part study is aimed for the screening of selected phytochemicals for their anticancer activity against colorectal cancer by a set of *in vitro* assays, in the second part, most potent phytochemical chosen will be encapsulated using soluble dietary fibre and in the last part will be mainly focussing on *in vivo* anticancer efficacy of the encapsulated product. Morin, fisetin, genistein, luteolin and zerumbone were the phytochemicals that we have chosen for the initial screening.

1.6.1. Morin

Morin, (2′,3,4′,5,7-Pentahydroxyflavone) (**Figure 1.8**) consisting of two aromatic rings which are linked by an oxygen-containing heterocycle (ring C), is a flavonoid mainly found in members of the Moraceae family (Ross and Kasum, 2002). It is found in mulberries, osage orange, almond, sweet chest nut, tea, wine as well as in many Chinese herbs used as herbal medicines (Basile et al., 2000).

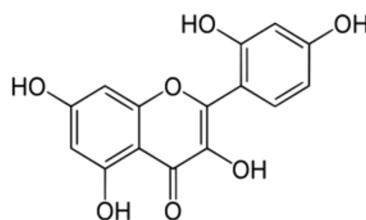

Figure 1.8. Chemical structure of morin

Morin is reported to possess various biological activities like cytoprotection, anti-oxidation, anti-inflammation, anti-diabetic, cardio-protective, anti-mutagenic and anti-cancer activities (Bartošíková et al., 2003). It is found that, morin significantly improved the status of kidney antioxidants and decreased the levels of markers that indicate kidney damage, induced by ammonium chloride (Subash and Subramanian, 2011). Study by Chandrakesan et al (Chandrakesan et al., 2018) showed that morin could prevent the photo damage of macromolecules such as lipids and the oxidation of proteins induced by Ultraviolet-B (UV-B) radiation. Morin can act as a chemopreventive agent against oral carcinogenesis in *in vitro* and *in vivo* condition as evident from the study of Kawabata et al., (Kawabata et al., 1999) morin exerted protective effect against chemically induced rat tongue carcinogenesis. Reports suggests morin can induce apoptosis in human leukemic cells. Hsiang et al., (Hsiang et al., 2005) has reported that morin repressed phorbol ester-induced transformation of hepatocytes (Subash and Subramanian, 2008, Kawabata et al., 1999, Hsiang et al., 2005).

1.6.2. Fisetin

Fisetin (3,7,3′,4′-tetrahydroxyflavone) (**Figure 1.9)** is a plant polyphenol from the flavonoid group. It is found in many plants, such as persimmons, apples, strawberries, onions, kiwis, grapes and cucumbers (Sahu et al., 2014, Maher et al., 2011). The highest levels of fisetin (160 μg/g wet food) is reported in strawberries (Arai et al., 2000).

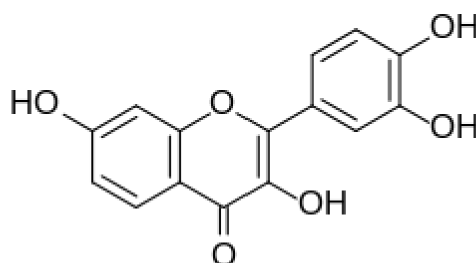

Figure 1.9. Chemical structure of fisetin

Fisetin is reported to have strong antioxidant (Hanneken et al., 2006) and anti-inflammatory (Higa et al., 2003) activity. When mice were administered with fisetin, it was found that the melonama growth was suppressed by disrupting Wnt/β-catenin signaling (Syed et al., 2011). Kang et al. (Kang et al., 2015) reported that fisetin treatment resulted in the down regulation of Bcl 2 expression leading to apoptosis in human non-small cell lung cancer cells. Fisetin aslo reported to induce apoptosis in LNCaP human prostate cancer cells (Khan et al., 2008). Furthermore, fisetin has been described as an inhibitor of, NF-κB, PI3K/AKT, PI3K/AKT/mTOR pathway (N Syed et al., 2013) In vitro studies it also has been reported that fisetin can interfere with the cell cycle in several ways (Gupta et al., 2014).

1.6.3. Genistein

Genistein (4',5,7-Trihydroxyisoflavone) (**Figure 1.10)** is most abundant isoflavones in soy and also found in a number of plants including kudzu, fava beans, lupin and coffee (Coward et al., 1993, Kaufman et al., 1997).

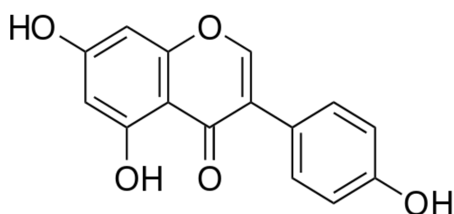

Figure 1.10. Chemical structure of genistein

Studies have reported that genistein possess potent anti-inflammatory and anti-angiogenic properties. Anti-inflammatory effect of genistein is associated with its inhibitory potential towards the release and expression of pro-inflammatory cytokines induced by lipopolysaccharide. It can also inhibit the excretion as well as the expression of VEGF and thus can act as anti-angiogenic compound (Su et al., 2005). One of interesting attribute of genistein is that its intake is considered as nontoxic and safe following pharmacological administration in animals and humans (Roberts et al., 2000, Bloedon et al., 2002). Study conducted by Raynal et al. (Raynal et al., 2008) in rodents found that genistein is useful for the treatment of leukemia and it can improve the efficacy of conventional antileukemic drugs when used in combination.

1.6.4. Luteolin

Luteolin (3',4',5,7-tetrahydroxyflavone) (**Figure 1.11**) is one of the most prevalent flavones. Dietary sources include carrot, celery peppermint, broccoli, cabbage, rosemary, green pepper, apple parsley, carrots and olive oil.

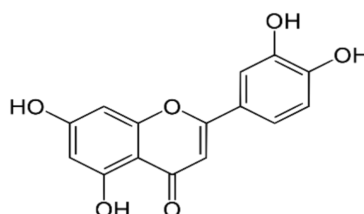

Figure 1.11. Chemical structure of luteolin

Luteolin is an active flavone with anti-inflammatory, anti-oxidant, anti-tumour and anti-apoptotic activities (Zhang et al., 2016, Sun et al., 2015). Luteolin exhibits strong cardiovascular protective activities through enhancing left ventricular ejection fraction, diminishing myocardial infarct size and decreasing myocardial apoptosis (Liao et al., 2011). The anticancer effect of luteolin is linked with inhibition of cancer cell proliferation, inducing DNA damage, apoptosis and suppressing angiogenesis and metastasis (Lin et al., 2008). Since luteolin is permeable to blood-brain barrier, it used for treating central nerve system diseases, including brain cancer (Wruck et al., 2007).

1.6.5. zerumbone

Zingiber zerumbet as known as shampoo ginger, belongs to the Zingeberaceae family, is extensively distributed throughout tropics predominantly in Southeast Asia (Eid et al., 2011). The rhizome is mostly used in the preparation of seasoning as a spice. It is rich in phytochemicals and has been used against many disorders in traditional medicine. *Z. zerumbet* has been established to have various biological properties including antiulcer (Yob et al., 2011), anti-inflammatory (Murakami et al., 2002), and anticancer (Aggarwal et al., 2008) activities. Zerumbone (**Figure 1.12),** an 11-membered cyclic sesquiterpene, is a key compound in this plant having a significant pharmacological profile (Sung et al., 2012) with minimal side effects on normal cells (Prasannan et al., 2012).

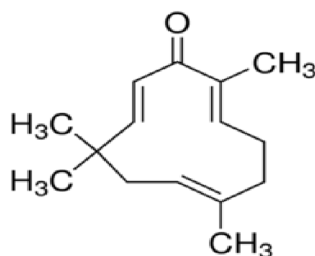

Figure 1.12. Chemical structure of zerumbone

1.7. Objectives of the study

The main objectives the present study are summarized below

1. Screening of selected phytochemicals for their anticancer activity against colorectal cancer
2. Evaluation of effect of most active phytochemical on glucose metabolism (Warburg effect) on SW480 colorectal cancer cells
3. Valuation of impact of most active phytochemical on inflammatory status of SW480 colorectal cancer cells
4. Assessment of effect of most active phytochemical on metastasis of SW480 colorectal cancer cells
5. Encapsulation of most active phytochemical with dietary fiber (Inulin) & evaluation of anticancer efficacy of encapsulated product

1.8. Work flow

Schematic illustration of the work is as follows:

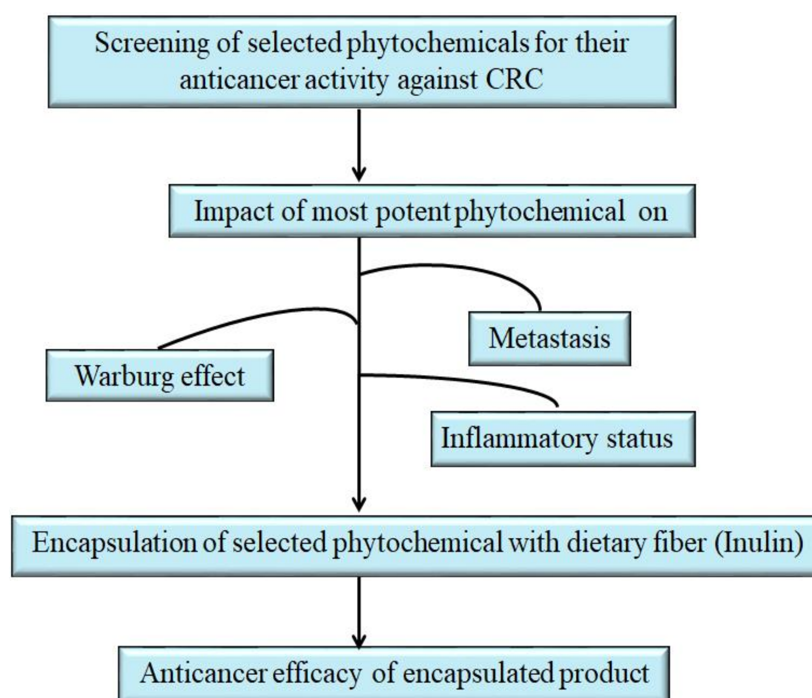

Figure 1.13. Outline of the work

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CHAPTER 2

Screening of selected phytochemicals for their anticancer activity against colorectal cancer

2.1 INTRODUCTION

The incidents of cancer is increasing globally with high mortality rate and is a prominent cause of death just after cardiovascular diseases amongst the population in both developed and developing countries (Jemal et al., 2011, Wang et al., 2016). Epidemiological data indicate that colorectal cancer (CRC) is the third most frequently diagnosed cancer, after lung and breast cancers (Spanos et al., 2008). Numerous studies have found that diet plays a key role in the prevention and management of CRC (Bishehsari et al., 2014) and 90% of the CRC mortality is ascribed to the dietary factors (Doll and Peto, 1981). About 40% of CRC risk can be reduced by daily consumption of >400 g of vegetables and fruits (Willett, 1995). At present, the main treatment approaches for cancer include chemotherapy, radiotherapy and surgery. Chemotherapy drugs used include DNA-interactive agents like doxorubicin; antimetabolites like methotrexate, cisplatin; anti-tubulin agents like taxanes; hormones and molecular targeting agents. Clinical uses of these drugs are associated with many side effects like hair loss, suppression of bone marrow, gastrointestinal lesions, drug resistance, cardiac toxicity and neurologic dysfunction (Nussbaumer et al., 2011).

Epidemiological studies indicated a defending effect of diets rich in fruits and vegetables against various diseases while biologically active plant compounds and secondary metabolites from plants have been reported to play the major role in this. Plants are good sources for the development of new medications for different diseases. A large number of medicinal plants and herbal ingredients have been known for exhibiting anticancer activities. A number of compounds isolated from medicinal plants have been shown to decrease cell proliferation, retard metastasis, induce apoptosis and inhibit angiogenesis. This huge group of compounds, now collectively known as ‘phytochemicals’, provides much of the colour and flavour of edible plants

and the beverages derived from them. Many of these compounds also exert anti-carcinogenic effects in animal models and much progress has been made in defining their biological activities at the molecular level (Fimognari, 2017). It has been stated that 25% to 48% of Food and Drug Administration (FDA) permitted anticancer agents are derived from plants. Protective elements in plants include selenium, vitamins, food polyphenols like carotenoids, flavonoids, phenolic acids, phytoalexins, indoles etc. (Surh, 2003, Russo, 2007). Curcumin, kaempferol, epigallocatechin gallate, catechin, quercetin, lycopene, naringin and resveratrol are few among them studied broadly (Aggarwal et al., 2004, Bishayee, 2009, Wong and Fiscus, 2015). In-depth research on these bioactive compounds discovered their capability to exert antineoplastic actions (Tarapore et al., 2011) and led to the emergence of alternate forms of cancer treatment method called nutrition therapy, to fight against cancer through a healthy diet with avoiding the side-effects which are often associated with routine anticancer treatment. Thus, recognizing these bioactive molecules, assessing their broad range of pharmaceutical activity, evaluating their precise mechanism of action could help to fight against cancer in much effective way.

With this background the present study focussed on evaluating anticancer activities of few selected phytochemicals against colorectal cancer using cell line models (SW480 and SW620).

2.2. MATERIALS AND METHODS

2.2.1. Materials

Dulbecco's modified eagle's media (DMEM), antibiotic antimycotic mix, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), ethidium bromide, acridine orange, 2',7'-dichlorofluorescindiacetate (DCFH-DA), Rhodamine 123 (Rh123), JC1 kit, Hoechst 33342, morin, genistein, fisetin and luteolin were purchased

from Sigma–Aldrich Chemicals (St Louis, MO, United States). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Auckland, New Zealand). Annexin V – FITC assay kit (600300) was purchased from Cayman chemicals. Glutathione colorimetric assay kit (K261-100) and catalase activity assay kit (K773-100) were purchased from Biovision. BCA protein assay kit was procured from Pierce Biotechnology, Rockford, IL, United States. Primary antibodies (β actin, cleaved PARP, cleaved caspase 3, cleaved caspase 8, cleaved caspase 9, Bcl 2 and Bax) and corresponding secondary antibodies for western blot analysis were purchased from Santa Cruz Biotechnology, United States. Clarity Western ECL substrate was purchased from Bio-rad, United States. All other chemicals used were of the standard analytical grade.

Isolation of Zerumbone: Fresh *Z. zerumbet* rhizomes were collected from the hills near Trivandrum, Kerala, India and identified by Dr. Mathew Dan, taxonomist, Jawaharlal Nehru Tropical Botanical Garden & Research Institute (JNTBGRI), Palode, Trivandrum, India. The voucher specimen (No. TBGRI 60680) of this collection has been maintained at herbarium of JNTGBRI, Palode Trivandrum, Kerala, India for future use. It was then dried, ground into powder and extracted using acetone at room temperature. Solvent when removed under reduced pressure gave crude extract. This crude extract was subjected to silica gel column chromatography and the active component in 3% ethyl acetate:hexane was crystallized and identified as zerumbone.

2.2.2. Cell Culture and Treatment

The human colon cancer cells (SW480 and SW620) and human hepatoma cell line (Hep G2) were obtained from ATCC (American Type Culture Collection, Manassas, United States) were maintained in DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic mix at 37°C under a humidified 5% CO_2 and 95% air atmosphere. Cells were exposed to 0.25% trypsin-EDTA and harvested cells were

seeded at a density of 1×10^4 cells/well on 24 well-plates, 6-well plates (Costar, United States) and 96-well black plates (BD Biosciences, Franklin Lakes, NJ, United States) for different assays.

2.2.3. Experimental design

The study has been divided in to two parts as follows

a) Evaluation of anticancer activity of selected phytochemicals in early stage of colorectal cancer (SW480 cells)

b) Evaluation of anticancer activity of selected phytochemicals in advanced stage of colorectal cancer (SW620 cells)

Schematic representation of work flow of this chapter 2 is depicted in **Figure 2.1**

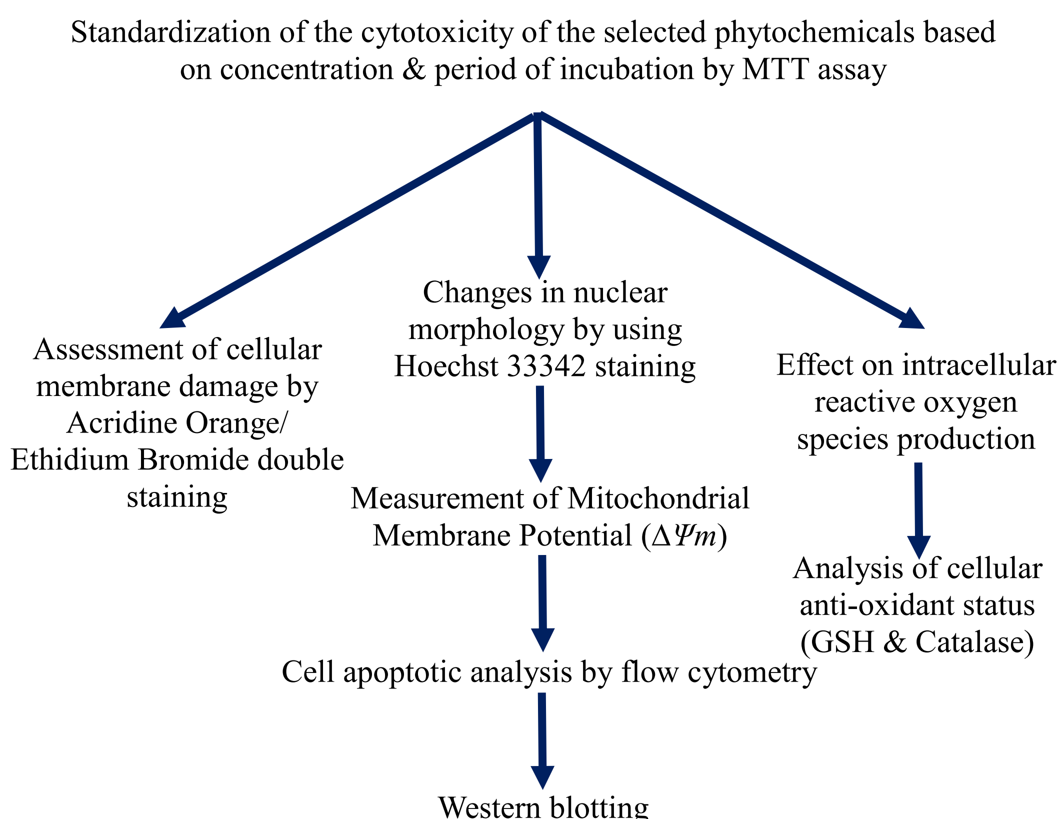

Figure 2.1. Outline of Chapter 2

2.2.4.1. Cell viability by MTT assay

The viability of SW480 cells was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) reduction assay as described previously (Mosmann, 1983). Briefly, after treating the cells with different concentrations of selected phytochemicals (morin, zerumbone, genistein, fisetin and luteolin) at various concentrations for 24 and 48 h, washed and MTT (0.5g/L) was added to each well, incubated at 37°C in a CO_2 incubator. After 4 h incubation, 10% SDS (sodium dodecyl sulfate) in DMSO (dimethyl sulfoxide) was added to each well, and the absorbance of solubilized MTT formazan products was measured at 570 nm after 45 min using a microplate reader (BIOTEK-USA). Percentage of cytotoxicity was calculated using the following equation. Results were expressed as % cell viability.

$$\text{Percentage of Toxicity} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of control}} \times 100$$

Cell viability (%) = 100 - percentage toxicity.

The same MTT assay was done for performing cell viability assays in SW620 cells and Hep G2 cell lines also.

2.2.4.2. Acridine Orange – Ethidium Bromide Double Staining (AO/EB staining)

Membrane damage during apoptosis was studied using acridine orange/ethidium bromide staining (Jimenez et al., 2008) with a slight modification of the original procedure. SW480 cells were treated with the desired concentration of selected phytochemicals at selected concentrations for 48 h, washed with PBS and trypsinized. Cell suspension (25 µL) was taken and incubated with 1 µL of acridine orange/ethidium bromide (one part each of 100 µg/mL of acridine orange and 100 µg/mL of ethidium bromide in PBS) just prior to microscopy. Suspension (10 µL, of gently mixed) was placed under a microscope and images were taken using a

fluorescent microscope (Pathway 855, BD Bioscience, USA). Camptothecin (50 μM) was used as positive control.

2.2.4.3. Nuclear staining using Hoechst 33342 stain

The changes in nuclear morphology of the cells, on treatment with selected phytochemicals at selected concentrations and camptothecin (50 μM) for 48 h were examined using the cell-permeable DNA dye, Hoechst 33342 (Hickman, 1992). After incubation, cells were stained with Hoechst 33342 (10 μg/mL) for 20 min at 37ºC followed by washing with PBS for 3 times and the nuclei were observed under fluorescent microscope (Pathway 855, BD Bioscience, USA) equipped with filters in the excitation of 350 nm and emission of 460 nm.

2.2.4.4. Cell apoptotic analysis by flow cytometry

Cell apoptosis was measured using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide staining. Briefly, cells were incubated with varying concentrations selected phytochemicals at selected concentrations camptothecin for 48 h. Treated cells were collected by trypsinization and centrifuged at 2300 rpm for 5 min, washed with cold PBS and re-suspended in diluted binding buffer. It was mixed well, centrifuged at 400 x g for 5 minutes. The supernatant was discarded and the cells were stained with a mixture of FITC-Annexin-V (10 μl) and propidium iodide solution (10 μl) in binding buffer (5 ml), from the Annexin-V apoptosis detection kit (Cayman Chemical Company, USA) and incubated for 10 min at room temperature in the dark condition, centrifuged at 400 x g for 5 min and then re-suspended in 1 ml assay binding buffer and analysed by Fluorescence Activated Cell Sorting (BD FACS Aria II, BD Biosciences, USA) within 1 h following the staining. The data acquisition and analysis were performed using BD FACSDiva™ Software v6.1.2, and a minimum of 10000

cells was analysed in each group. The ratio of apoptotic cells was measured by flow cytometry as described by manufacturer's instructions.

2.2.4.5. Assay for mitochondrial membrane potential by JC-1 staining

Mitochondrial membrane potential was measured using mitochondrial staining kit, JC1. The experiment was done as per the protocol provided with the kit (JC1 kit, Sigma). The kit uses the cationic, lipophilic dye, JC-1(5,5',6,6'-tetrachloro1,1',3,3'tetraethyl benzimidazolo carbocyanine iodide (JC-1). In normal cells, due to the existence of electrochemical potential gradient, the dye concentrates in the mitochondrial matrix, where it forms red fluorescent aggregates (JC-1 aggregates). Change in mitochondrial membrane potential prevents the accumulation of the JC-1 dye in the mitochondria and thus, the dye is dispersed throughout the entire cell leading to a shift from red (JC-1 aggregates) to green fluorescence (JC-1 monomers). The cells after treatments were incubated with JC-1 staining solution for 20 minutes at 37ºC. The stain was washed off with PBS and examined under fluorescent microscope (Pathway 855, BD Bioscience, USA) and images were collected and fluorescence intensity was also measured. For JC-1 monomers, the fluorescence was measured at 490 nm excitation and 530 nm emission wavelengths, for JC-1 aggregates, the fluorescence was measured at 525 nm excitation and 590 nm emission wavelengths.

2.2.4.6. Measurement of mitochondrial membrane potential (*ΔΨm*)

The effect of selected phytochemicals on the mitochondrial membrane potential was measured by staining with Rhodamine 123, a cationic fluorescent indicator which selectively accumulates within the mitochondria in a membrane potential dependent way (Zhang et al., 2008). Cells grown in 6 well plates were treated with indicated concentration of phytochemicals for 24 h and 48 h and positive control, H_2O_2 (200

μM) for 2 h. The harvested cells were rinsed twice with PBS, re-suspended in Rh123 (0.625 mg/ml) and incubated at 37°C for 25 min in the dark followed by rinsing with several changes of PBS. The fluorescence was detected by Fluorescence Activated Cell Sorting (BD FACS Aria II, BD Biosciences, USA). A reduction in rhodamine 123 fluorescence indicates reduced $\Delta\Psi m$. The data acquisition and analysis were performed using BD FACSDiva™ Software v6.1.2, and a minimum of 10000 cells was analysed from each group.

2.2.4.7. Measurement of intracellular Reactive Oxygen Species (ROS) generation

The effect of the selected phytochemicals on intracellular ROS level was assessed using Fluorescent probe 2’, 7’ - dichlorofluorescein-diacetate (DCFH-DA) staining as described by Cathcart et al., (Cathcart et al., 1983). Initially cells were incubated with different concentrations of selected phytochemicals for 48 h, washed with phosphate buffer saline (PBS, pH-7.4) then treated with DCFH-DA (20 μM) for 20 min and observed under fluorescent microscope (Pathway 855, BD Bioscience, USA) equipped with filters in the FITC range (Excitation, 490 nm; and Emission, 525 nm). For the exact quantification of ROS production cells were grown in 6 well plates and treated with indicated concentration of selected phytochemicals for 24 h and 48 h. The harvested cells were rinsed twice with PBS, re-suspended in 20 μM DCFH-DA and incubated at 37°C for 20 min in the dark. Data analysis was performed using Fluorescence Activated Cell Sorting (BD FACS Aria II, BD Biosciences, USA). The data acquisition and analysis were performed using BD FACSDiva™ Software v6.1.2, and a minimum of 10000 cells was analysed from each group.

2.2.4.8. Antioxidant assays

SW480 cells were pretreated with different concentrations of selected phytochemicals for 48 h and positive control, H_2O_2 (200 μM) for 2 h. After incubation, cells were

washed with PBS and lysed using respective enzyme specific buffer and the lysed cells were used to determine the antioxidant activity. Cells without treatment were used as the control. The intracellular catalase activity was determined using catalase activity colorimetric assay kit according to the manufacturer instructions (K773-100) and the glutathione level was tested using glutathione assay kit according to the manufacturer instructions (K261-100).

2.2.4.9. Immunoblot analysis

Following incubation of cells with morin (150, 200 & 250μM), zerumbone (50, 75 & 100 μM) and camptothecin (50 μM) for 48 h, cells were washed twice with ice cold PBS, lysed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 0.5 mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100 and protease inhibitor cocktail, pH 8.0) for 30 min on ice and were centrifuged at 12000 x g for 10 min. The protein content of the lysate was measured using BCA protein assay kit. Lysates were diluted to an equal concentration of total protein and supernatants were then stored at -80ºC until analysis. These samples were boiled for 10 min at 75°C in reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). The lysate containing 50 µg of protein was subjected to SDS-PAGE on 12% gel and transferred onto a polyvinylidene difluoride membrane (Immobilon P™, Millipore®, USA) by using Trans-Blot Turbo™ transfer system (Bio-Rad Laboratories, Germany). The membranes were blocked by incubating in blocking buffer (5% skim milk in PBST, PBST-PBS buffer containing 0.1% Tween 20), for 1 h at room temperature, washed three times with PBST and probed over night at 4°C with primary antibodies (β actin, cleaved PARP, cleaved caspase 3, cleaved caspase 8, cleaved caspase 9, Bcl 2 and Bax at 1:500 dilution). After washing three times with PBST for 5 min each, the membrane was incubated with horseradish

peroxidase (HRP) conjugated secondary antibody at 1:1000 dilution and again washed three times in PBST. The bound antibodies were detected using an enhanced chemiluminescence substrate (Biorad, USA) and measured by densitometry using a Chemi Doc XRS digital imaging system and the Multi-Analyst software from Bio-Rad Laboratories (USA).

2.2.4.10. Statistical analysis

Results were expressed as mean ± SD (standard deviation) from three independent experiments. The differences between treatments in comparison with control were assessed using one-way ANOVA and the significance of differences between mean was calculated by Duncan's multiple range test, using SPSS for Windows, standard version 16 (SPSS, Inc.), and significance was accepted at $p \leq 0.05$.

2.3. RESULTS

2.3.1. Cytotoxic effect of selected phytochemicals

To test the effect of selected phytochemicals namely, morin, genistein, fisetin, luteolin and zerumbone on viability of SW480 colon cancer cells, we treated the cells with various concentrations of these phytochemicals (50 to 500 μM) for 24 or 48 h. The effect of these phytochemicals on the viability of SW480 cells was analysed using the MTT assay. Although a decrease in cell viability was observed after incubation for both 24 and 48 h, we could observe much greater reduction in cell viability after 48 h of incubation with comparatively lower concentrations of these phytochemicals. So the treatment time of 48 h was selected for the further assays. These results suggest that these phytochemicals reduced the viability of SW480 cell lines in a dose and time dependent manner (**Figure 2.2, Table 2.1**).

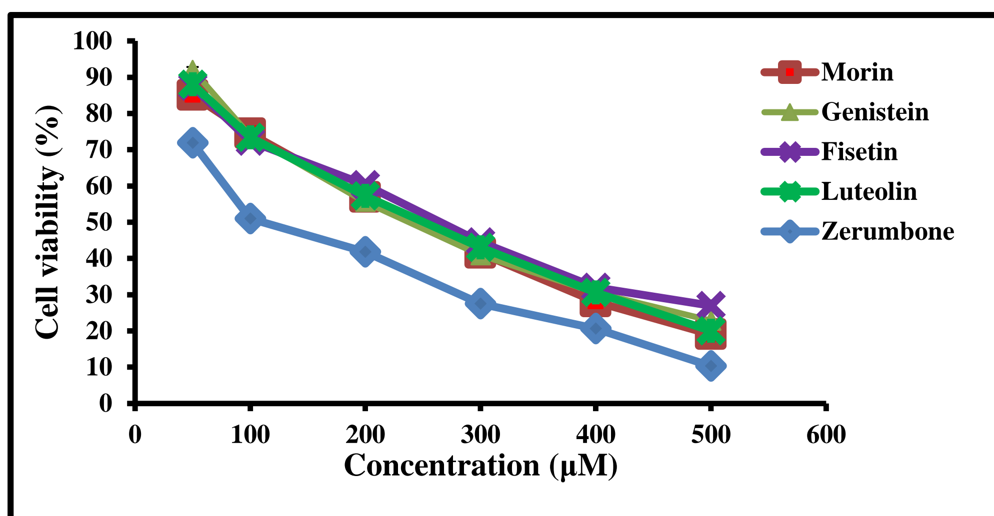

Figure 2.2. Cytotoxic effect of selected phytochemicals on SW480 cells after 48 h treatment. SW480 cells were treated with various concentrations of phytochemicals (50–500 mM) for 48 h. Cell viability was measured sing MTT assay. Results are shown as percentage reduction in cell viability of treated cells compared to untreated control cells. Values shown are the means ± SD obtained from three independent experiments.

Table 2.1. **The IC_{50} values of selected phytochemicals after 48 h treatment on SW480 cells**

Sr No	**Compound**	**IC_{50} value (µM)**
1	Zerumbone	102
2	Morin	227
3	Genistein	242
4	Luteolin	234
5	Fisetin	262

2.3.2. Effects of selected phytochemicals on plasma membrane integrity

Acridine orange/ethidium bromide staining was used to check membrane blebbing and leakage on treatment with selected phytochemicals. When stained with acridine orange/ethidium bromide, the live cells showed green fluorescence as they are permeable only to acridine orange and the dead cells appeared in red in fluorescence as they are permeable to both acridine orange and ethidium bromide (Saraste and Pulkki, 2000). On treatment with these phytochemicals cells appeared orange due to co-staining of acridine orange and ethidium bromide (indicated by white arrows), whereas the control cells exhibited only green fluorescence (**Figure 2.3a, 2.3b**). Similar results as treated group were demonstrated by the positive control, camptothecin also.

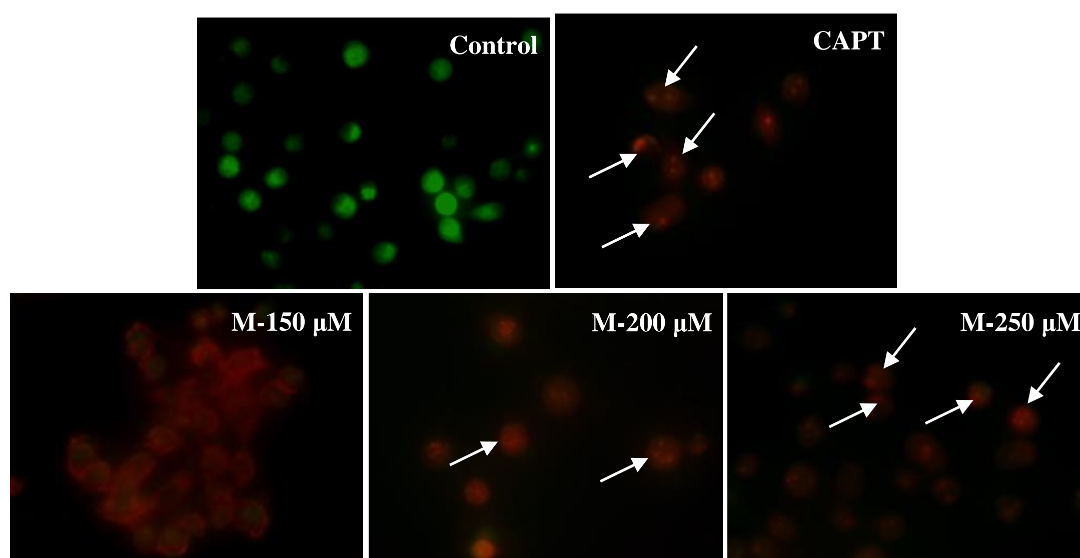

Figure 2.3a. Acridine orange/ethidium bromide staining. Images showing the effects of morin (150, 200 and 250 µM), and camptothecin (50 µM) treatment for 48 h on membrane damage associated with apoptosis. White arrows indicate the co-staining of ethidium bromide along with acridine orange due to membrane leakage (magnification 40×).

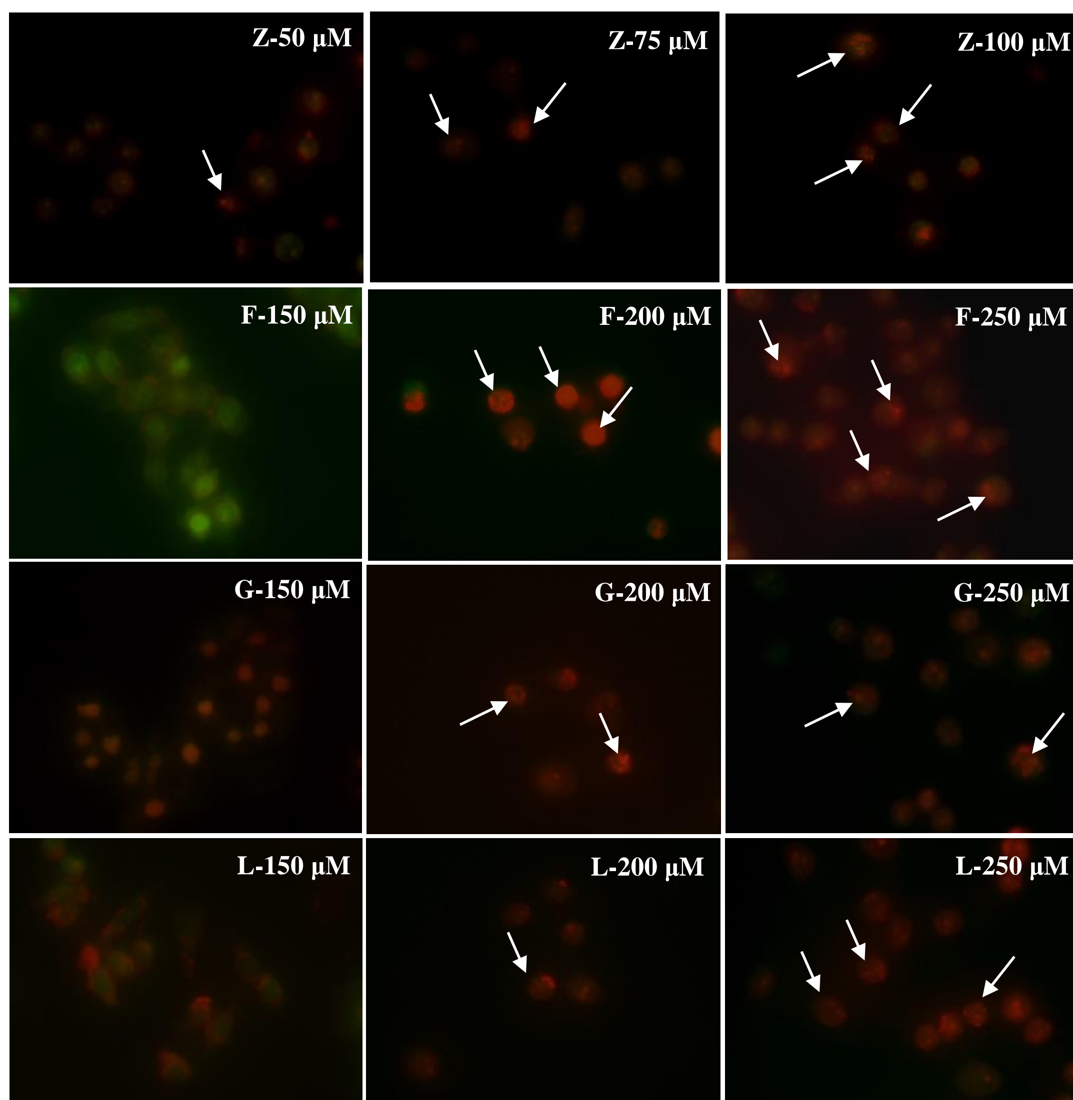

Figure 2.3b. Acridine orange/ethidium bromide staining. Images showing the effects of zerumbone (50, 75, and 100 µM), fisetin (150, 200 and 250 µM), genistein (150, 200 and 250 µM) and luteolin (150, 200 and 250 µM) treatment for 48 h on membrane damage associated with apoptosis. White arrows indicate the co-staining of ethidium bromide along with acridine orange due to membrane leakage (magnification 40×).

2.3.3. Hoechst 33342 staining

The antiproliferative activity shown by phytochemicals may be due to the induction of apoptosis and it can be observed by staining the cells with a fluorescent DNA-binding dye (DiBartolomeis and Moné, 2003). In the current study the nuclear variations like chromatin condensation and DNA fragmentation (hallmarks of apoptosis) were examined by Hoechst 33342 staining. From the figure (**Figure 2.4a & 2.4b**), untreated control cells exhibited uniform staining and emitted a blue fluorescence with consistent intensity, demonstrating that the chromatin was homogeneously distributed in the nuclei. The fluorescence light was denser and brighter in cells treated with phytochemicals and exhibited remarkable nuclear changes of apoptosis such as chromatin condensation, the formation of apoptotic bodies and nuclear fragmentations. Further confirmation of apoptosis in the cells was carried out by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide staining and flow cytometry analysis.

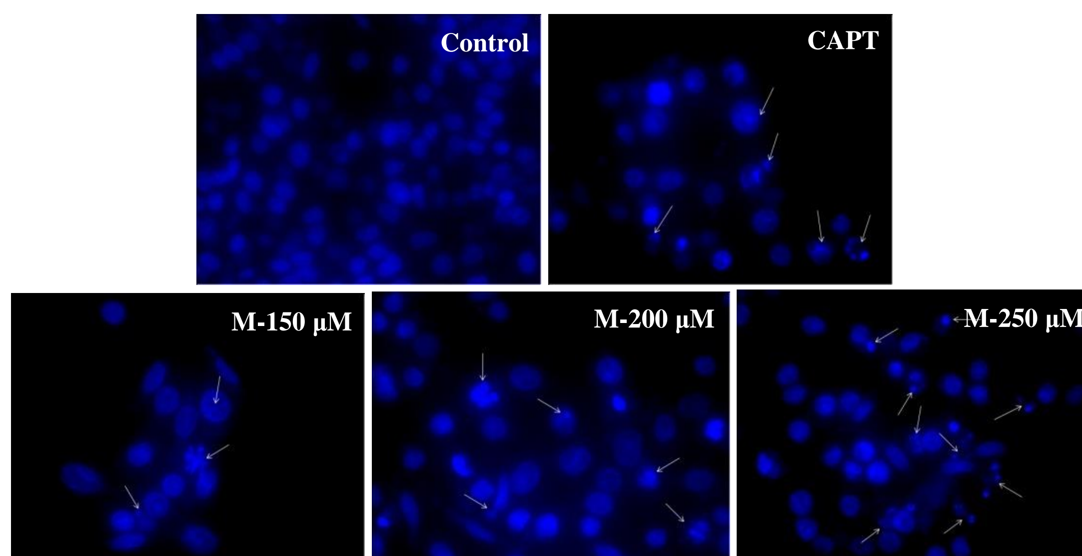

Figure 2.4a. Chromatin condensation and nuclear fragmentation observed using Hoechst 33342 staining. Images showing the effects of morin (150, 200 and 250 µM), and camptothecin (50 µM) treatment for 48 h on nuclear morphology. White arrows represent cells with chromatin condensation inside the nucleus or fragmentation of the nucleus. (magnification 40 ×).

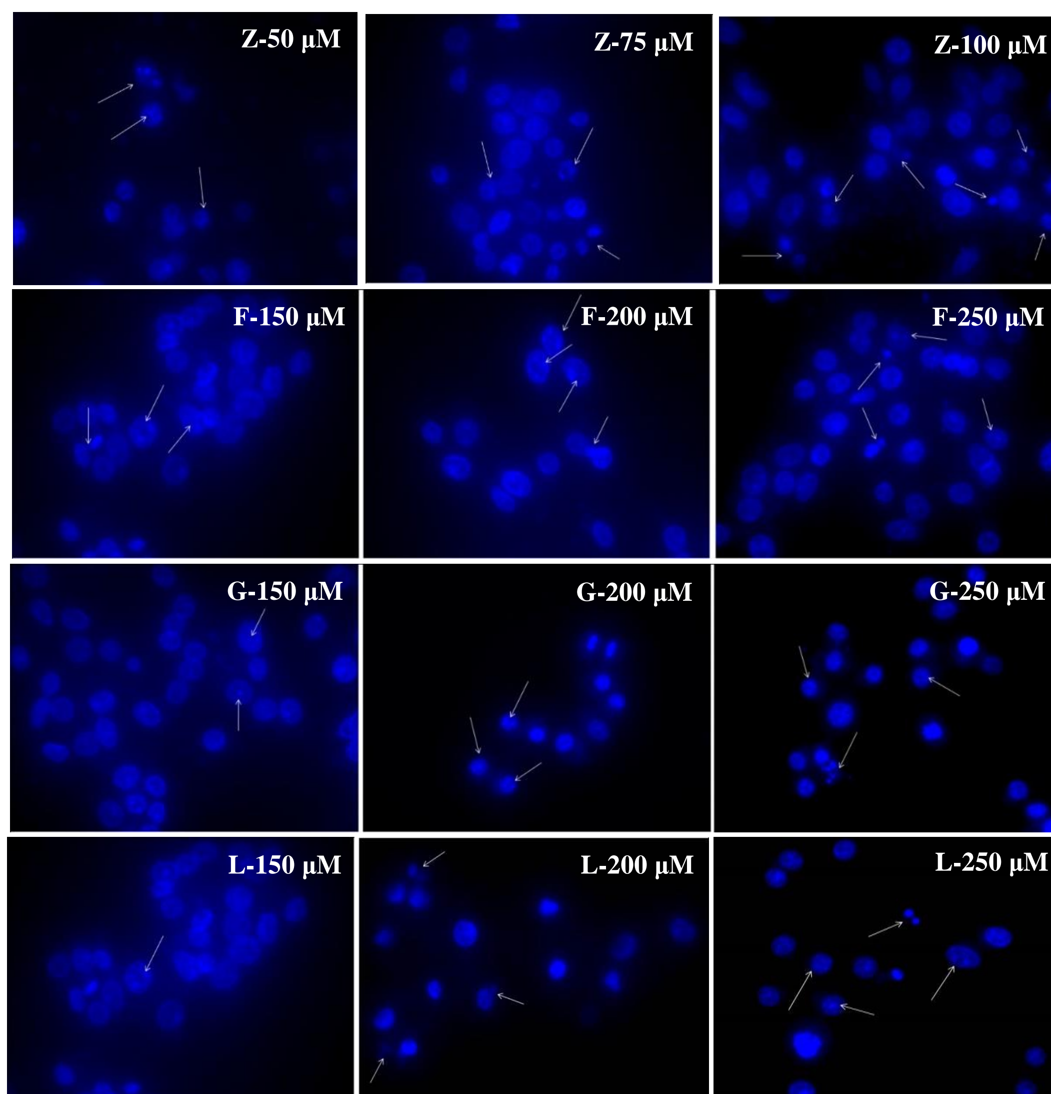

Figure 2.4b. Chromatin condensation and nuclear fragmentation observed using Hoechst 33342 staining. Images showing the effects of zerumbone (50, 75, and 100 µM), fisetin (150, 200 and 250 µM), genistein (150, 200 and 250 µM) and luteolin (150, 200 and 250 µM) treatment for 48 h on nuclear morphology. White arrows represent cells with chromatin condensation inside the nucleus or fragmentation of the nucleus. (magnification 40×).

2.3.4. Detection of cell apoptosis using flow cytometry

Cells were stained with Annexin- V/FITC and propidium iodide (PI) followed by flow cytometry analysis for detecting externalization of phosphatidylserine (PS) during apoptosis. Viable cells are negative for both fluorescence-conjugated annexin- V binding and of propidium iodide uptake as there is no externalization of phosphatidylserine (PS) and cell membrane damage, early apoptotic cells are positive for fluorescence-conjugated annexin- V binding but negative for propidium iodide uptake because there is only externalization of phosphatidylserine (PS), late apoptotic cells are positive for both fluorescence-conjugated annexin- V binding and of propidium iodide uptake as there is both externalization of phosphatidylserine (PS) and cell membrane damage while necrotic cells are Annexin- V/FITC negative but PI positive because there is no externalization of phosphatidylserine (PS) but there is the cell membrane damage.

It was observed that a significant increase in the number of apoptotic cells with the increase in the concentration of phytochemicals. The mean percentage of cells in the early and late apoptotic population on treatment with 150, 200, 250 μM morin for 48 h was 17.26 ± 0.75, 9.26 ± 0.40, 11.56 ± 0.37 and 15.83 ± 0.41, 36.3 ± 1.35, 48.76 ± 0.2 respectively. The mean percentage of cells in the early and late apoptotic population on treatment with 50, 75, 100 μM zerumbone for 48 h was 9.4 ± 0.43, 54.43 ± 0.66, 62.3 ± 0.36 and 32.2 ± 0.91, 24.5 ± 0.55, 17.26 ± 0.35. It was observed that mean percentage of cells in the early and late apoptotic population on treatment with 150, 200, 250 μM fisetin for 48 h was 15.33 ± 0.35, 19.4 ± 0.5, 37.3 ± 0.96 and 0.66 ± 0.05, 6.16 ± 0.45, 6.86 ± 0.15 respectively. It was observed that mean percentage of cells in the early and late apoptotic population on treatment with 150, 200, 250 μM genistein for 48 h was 19.83 ± 0.4, 17.66 ± 0.65, 23.7 ± 0.7 and 4.63 ± 0.41, 24.4

±0.45, 22.3 ± 0.6 respectively. While it was found that mean percentage of cells in the early and late apoptotic population on treatment with 150, 200, 250 μM luteolin for 48 h was 32.26 ± 0.86, 22.2 ± 1.05, 20.56 ± 0.61 and 5. 23 ± 0.61, 25.23 ±0.63, 43.5 ± 0.88 respectively. All these values were significantly different from control cell population (early apoptotic: 1.4 ± 0.2 and late apoptotic: 0.3 ± 0.1). The typical histogram representing cells in the early and late stage of apoptosis is illustrated in the figure (**Figure 2.5a, 2.5b**).

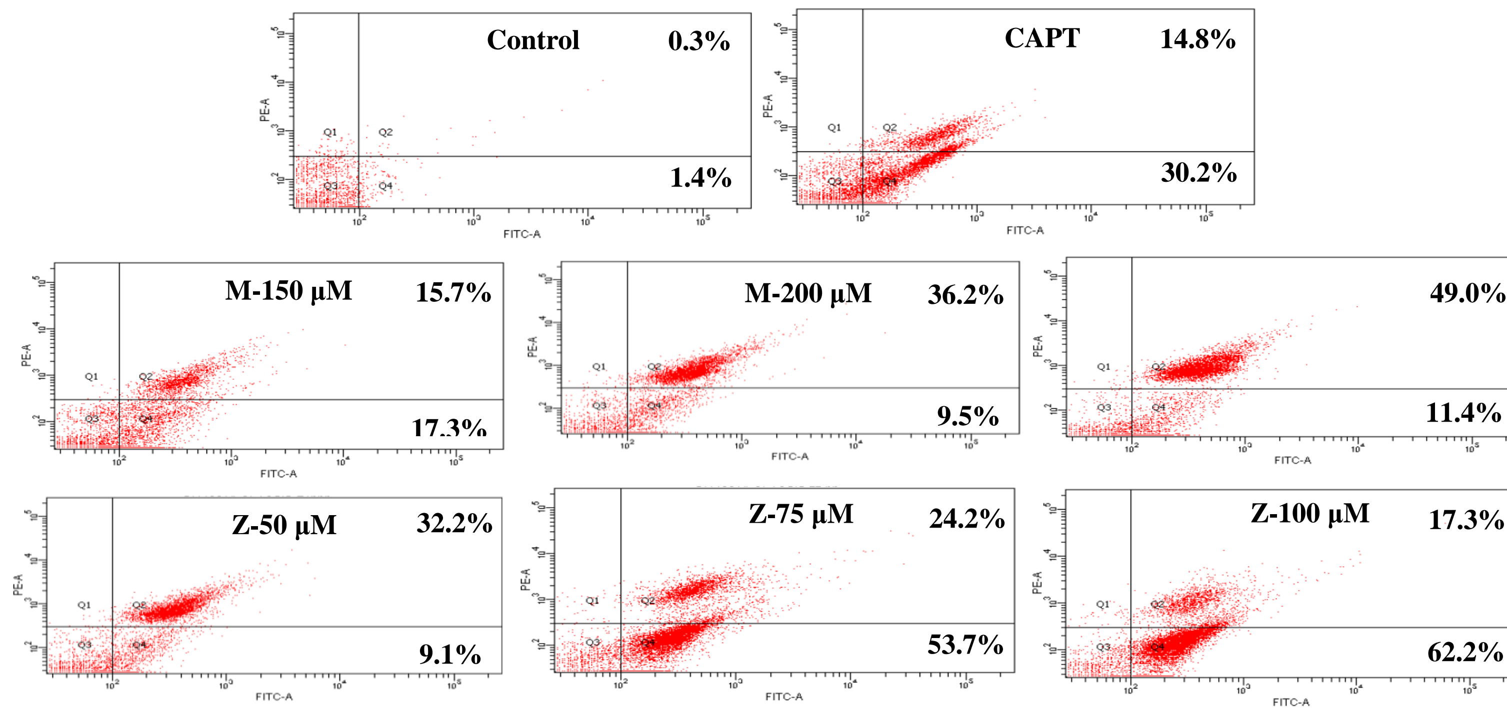

Figure 2.5a. Flow cytometry analysis of the percentage of apoptotic cells in each group by Annexin- V/FITC and PI staining. Images showing the effects of morin (150, 200 and 250 µM) and camptothecin (50 µM) treatment for 48 h on induction of apoptosis in SW480 cells.

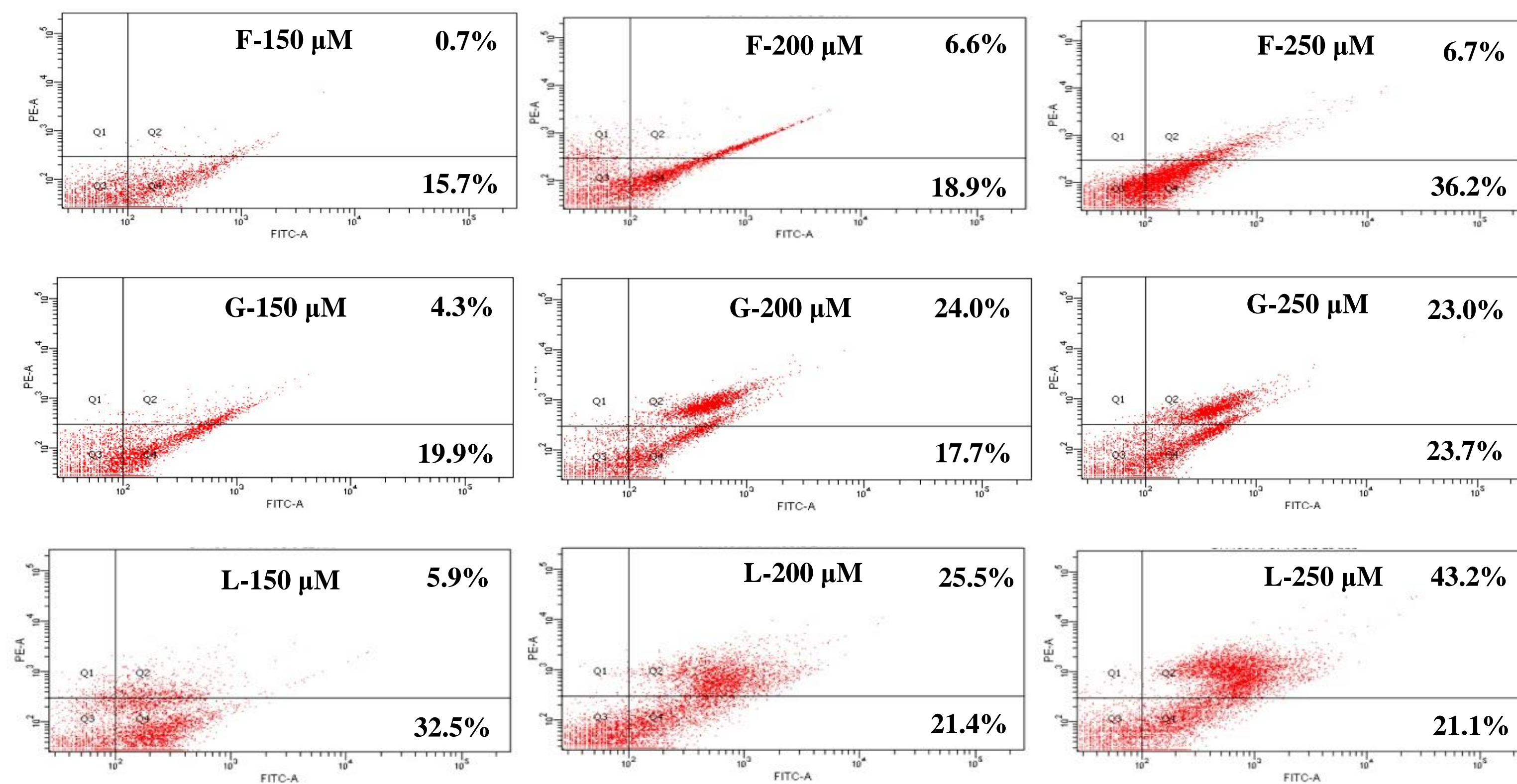

Figure 2.5b. Flow cytometry analysis of the percentage of apoptotic cells in each group by Annexin- V/FITC and PI staining. Images showing the effects of fisetin (150, 200 and 250 µM), genistein (150, 200 and 250 µM) and luteolin (150, 200 and 250 µM) treatment for 48 h on induction of apoptosis in SW480 cells.

2.3.5. Mitochondrial membrane potential

It is clear from the above studies that apoptosis is induced in the SW480 cells on treatment with selected phytochemicals. Mitochondria serve as a convergent centre of apoptotic signals for both intrinsic and extrinsic pathways and changes induced in the mitochondrial membrane potential represent a determinant in the execution of cell death (Kim et al., 2006). In order to assess the role of mitochondria in inducing apoptosis in SW480 cells, the change in mitochondrial membrane potential was examined. A variation in mitochondrial depolarization patterns of cells indicated by a shift from red fluorescence to green fluorescence was examined using fluorescent microscope (Pathway 855, BD Bioscience, United States). A decrease in red/green ratio was taken as indicative of alteration in mitochondrial membrane potential and apoptosis (**Figure 2.6a, 2.6b, 2.6c**).

From the results, it was clear that all the selected phytochemicals could alter mitochondrial membrane potential and the extent of mitochondrial membrane depolarization was quantified using flow cytometry after rhodanine 123 staining. Rh123 enters only to mitochondria with an intact membrane potential and is retained there. Once the membrane potential is lost, the dye is leached out of the mitochondria and therefore a drop in the fluorescence, which is correlated with the mitochondrial membrane potential. The results indicated that the mitochondrial membrane potential of SW480 colon cancer cells depleted when pre-treated with selected phytochemicals in a dose and time-dependent manner (**Figure 2.7a, 2.7b, 2.7c, 2.7d, 2.7e**) All the results were statistically significant compared to the corresponding untreated control groups ($p \leq 0.05$).

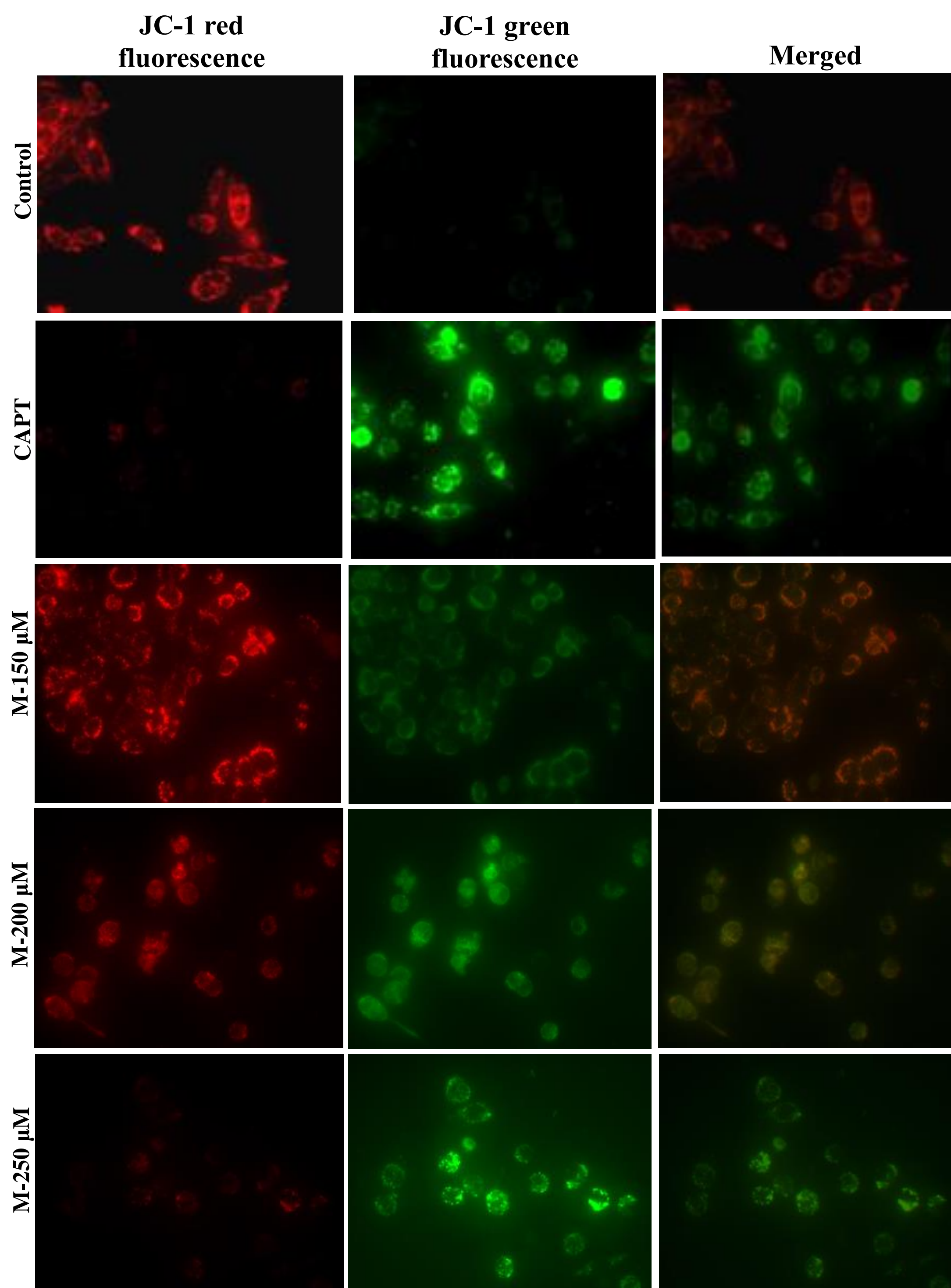

Figure 2.6.1. Change in mitochondrial membrane potential by JC-1 staining. Images showing the effects of morin (150, 200 and 250 µM) and camptothecin (50 µM) treatment for 48 h on mitochondrial membrane potential of SW480 cells. (magnification 40×).

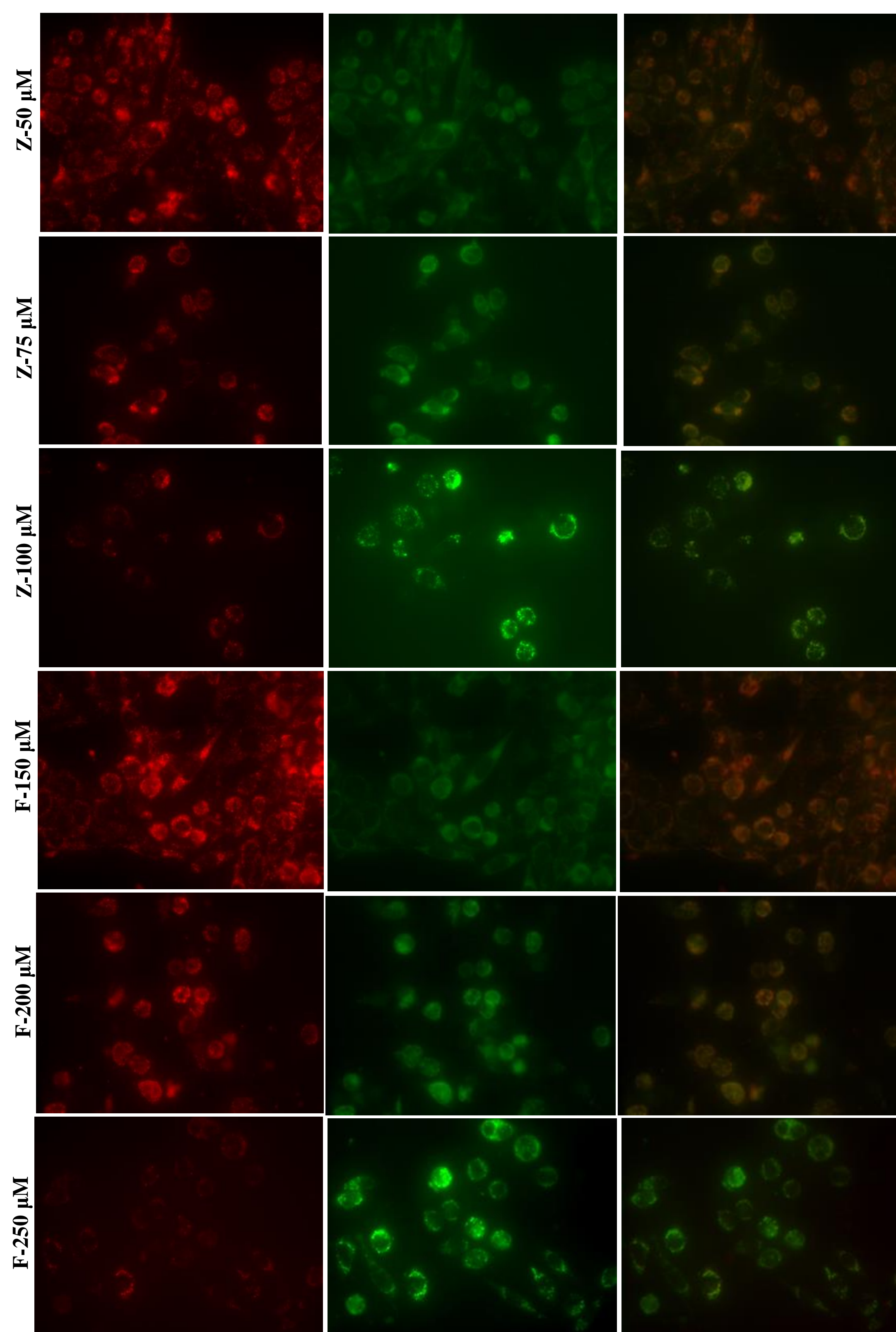

Figure 2.6b. Change in mitochondrial membrane potential by JC-1 staining. Images showing the effects of zerumbone (50, 75, and 100 µM) and fisetin (150, 200 and 250 µM) treatment for 48 h on mitochondrial membrane potential of SW480 cells. (magnification 40×).

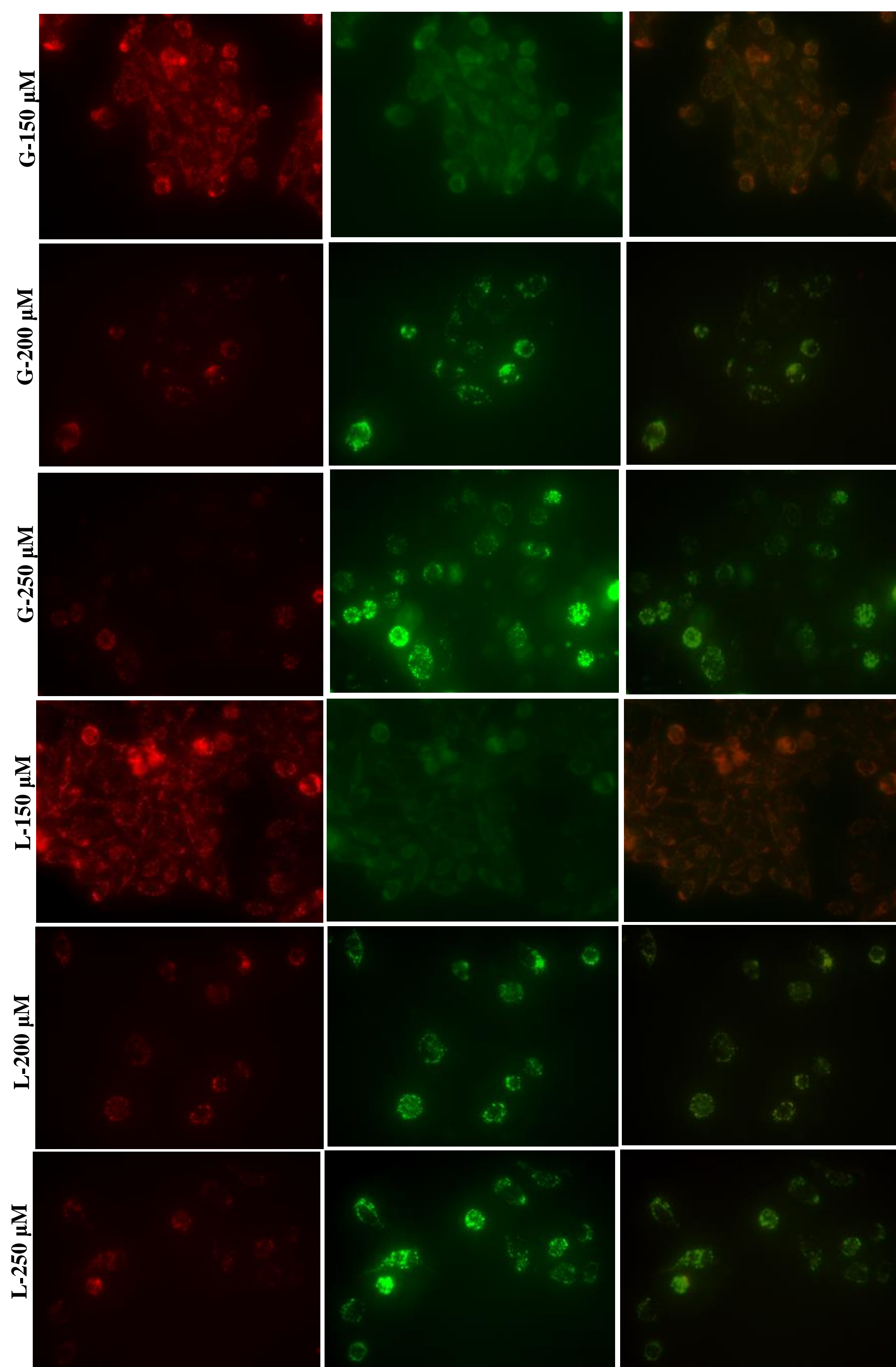

Figure 2.6c. Change in mitochondrial membrane potential by JC-1 staining. Images showing the effects of genistein (150, 200 and 250 µM) and luteolin (150, 200 and 250 µM) treatment for 48 h on mitochondrial membrane potential of SW480 cells. (magnification 40×).

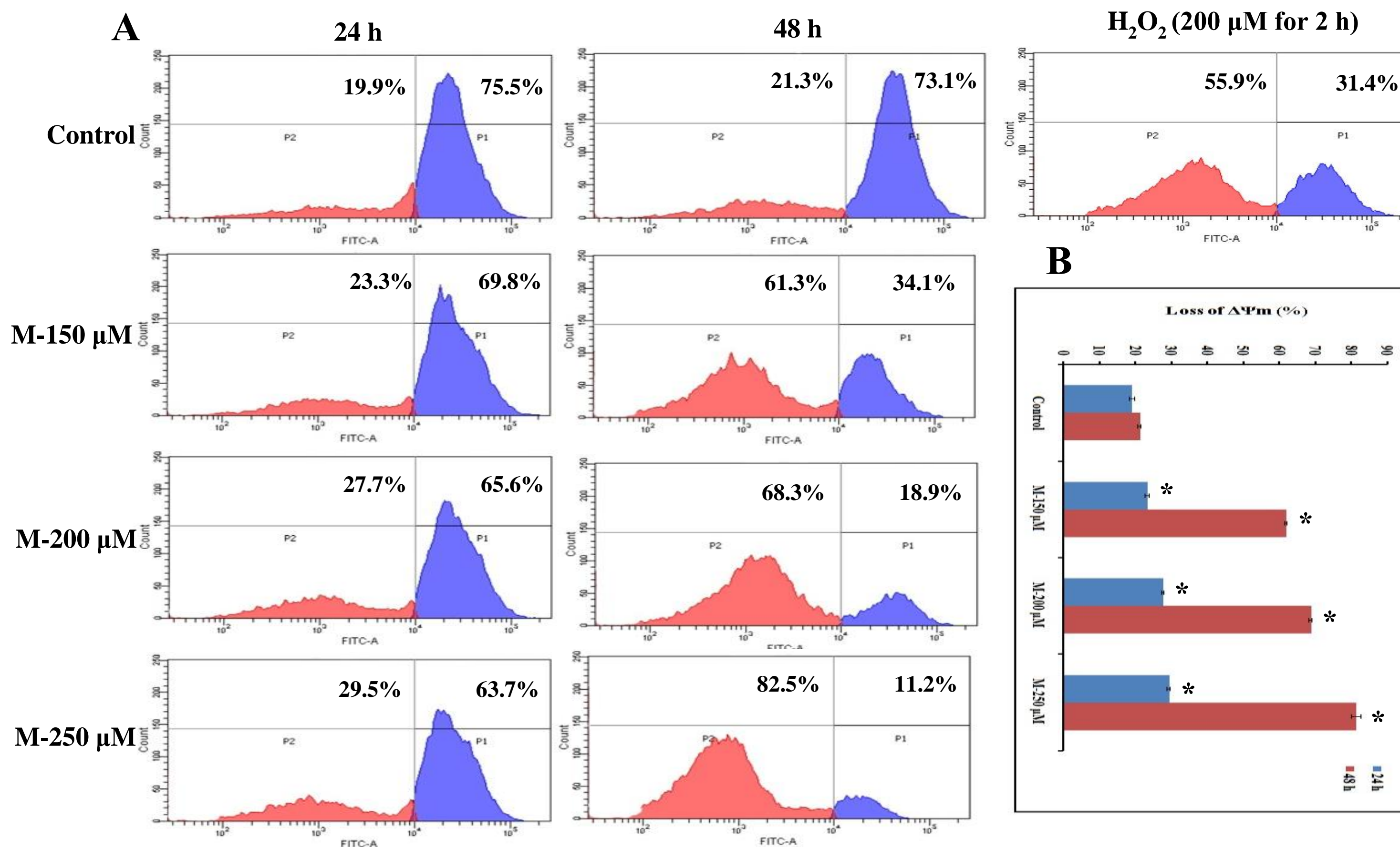

Figure 2.7a. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining. SW480 cells were treated with various concentrations of morin (150, 200 and 250 µM) for 24 and 48 h and H_2O_2 (200 µM) for 2 h. After incubation, cells were stained with Rhodamine 123 and analyzed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

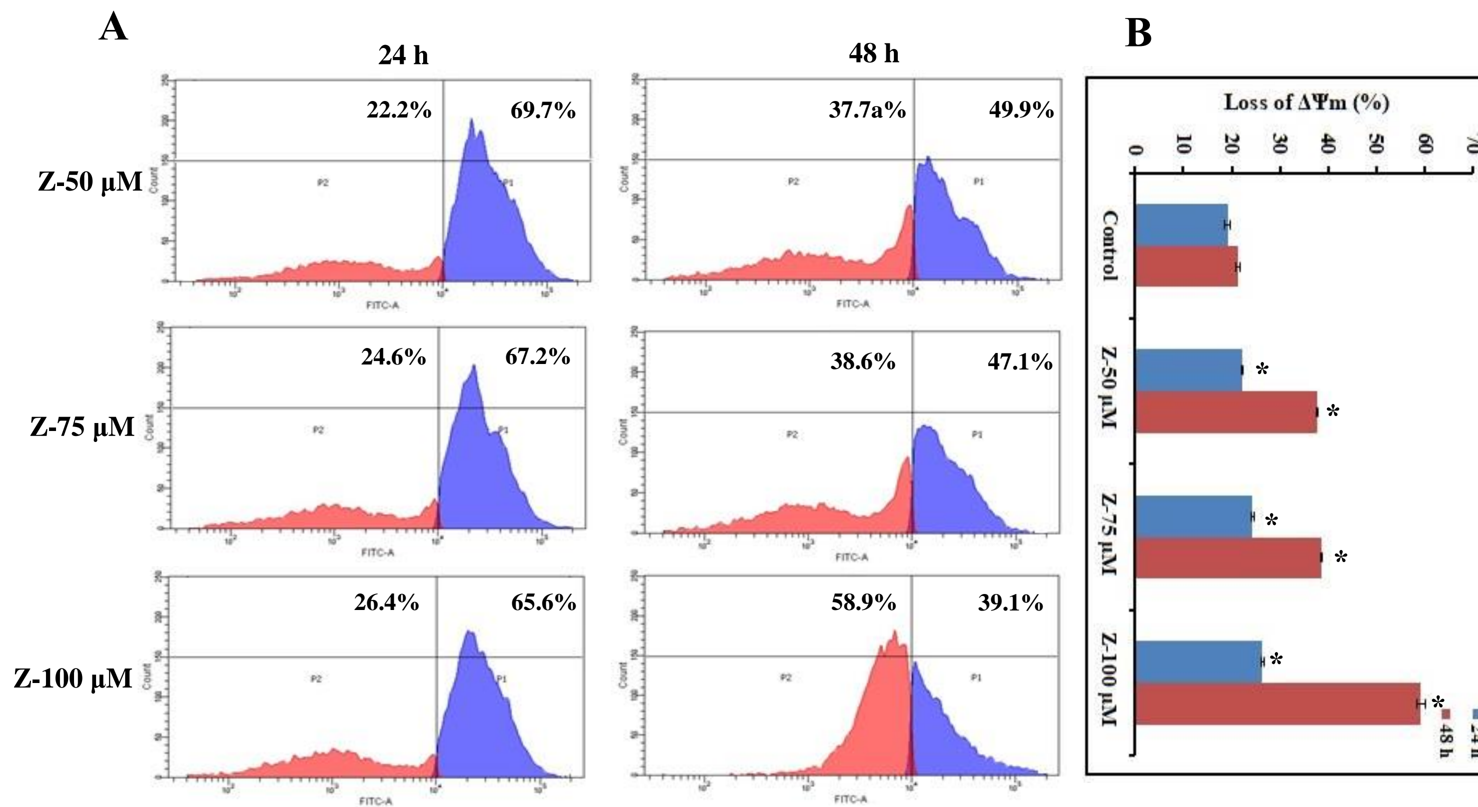

Figure 2.7b. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining. SW480 cells were treated with various concentrations of zerumbone (50, 75, and 100 μM) for 24 and 48 h. After incubation, cells were stained with Rhodamine 123 and analyzed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

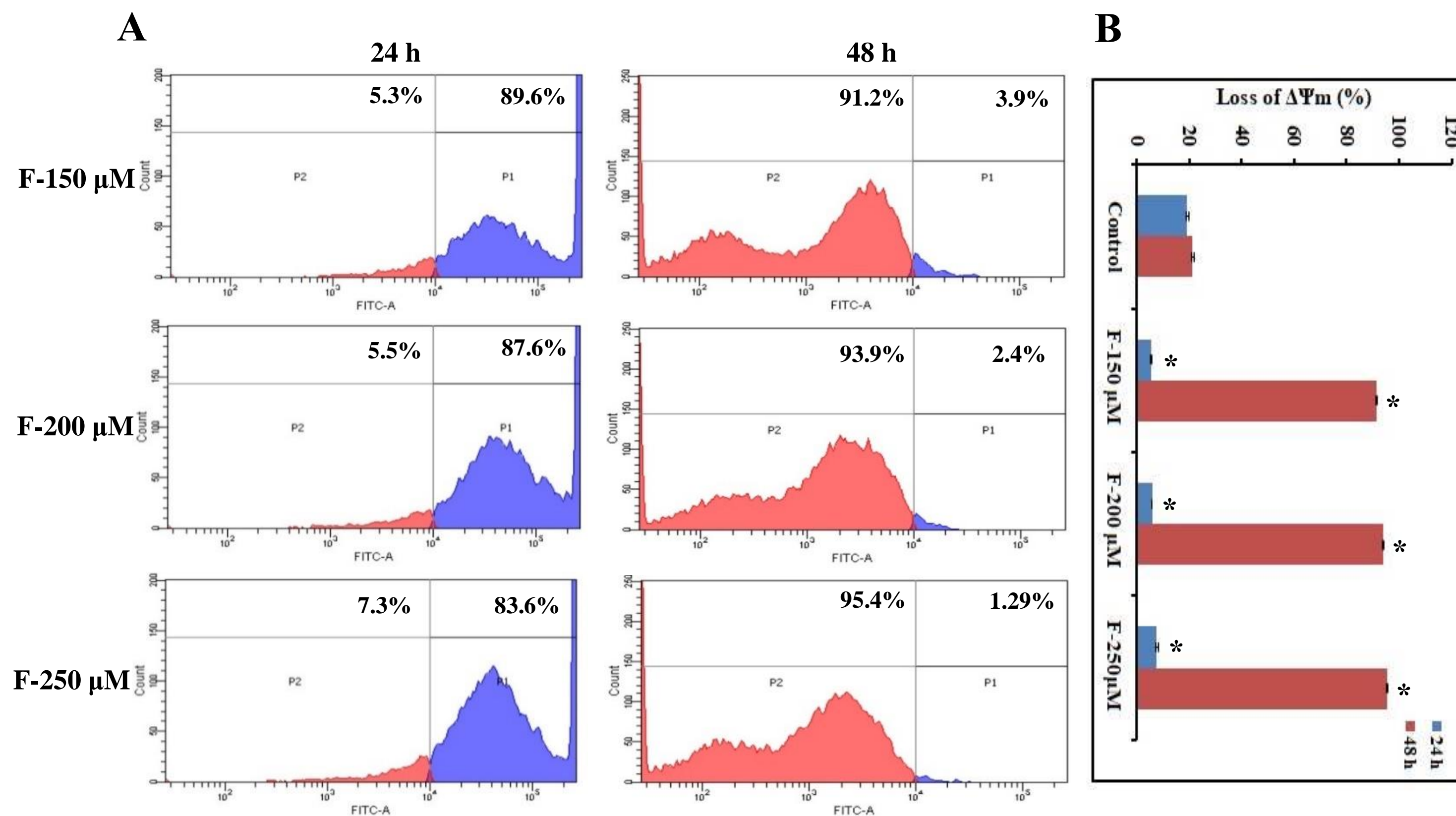

Figure 2.7c. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining. SW480 cells were treated with various concentrations of fisetin (150, 200 and 250 µM) for 24 and 48 h. After incubation, cells were stained with Rhodamine 123 and analyzed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

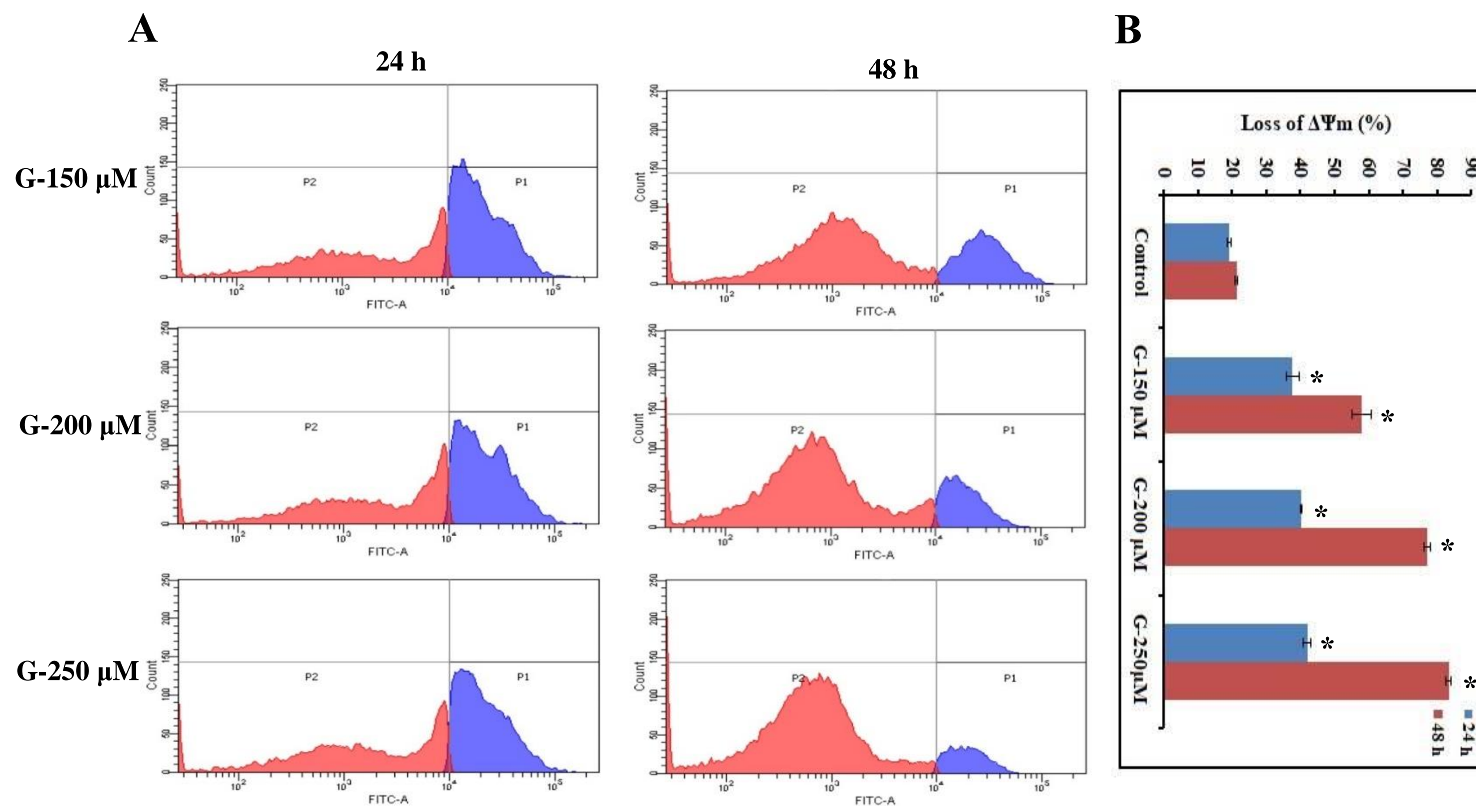

Figure 2.7d. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining. SW480 cells were treated with various concentrations of genistein (150, 200 and 250 µM) for 24 and 48 h. After incubation, cells were stained with Rhodamine 123 and analyzed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

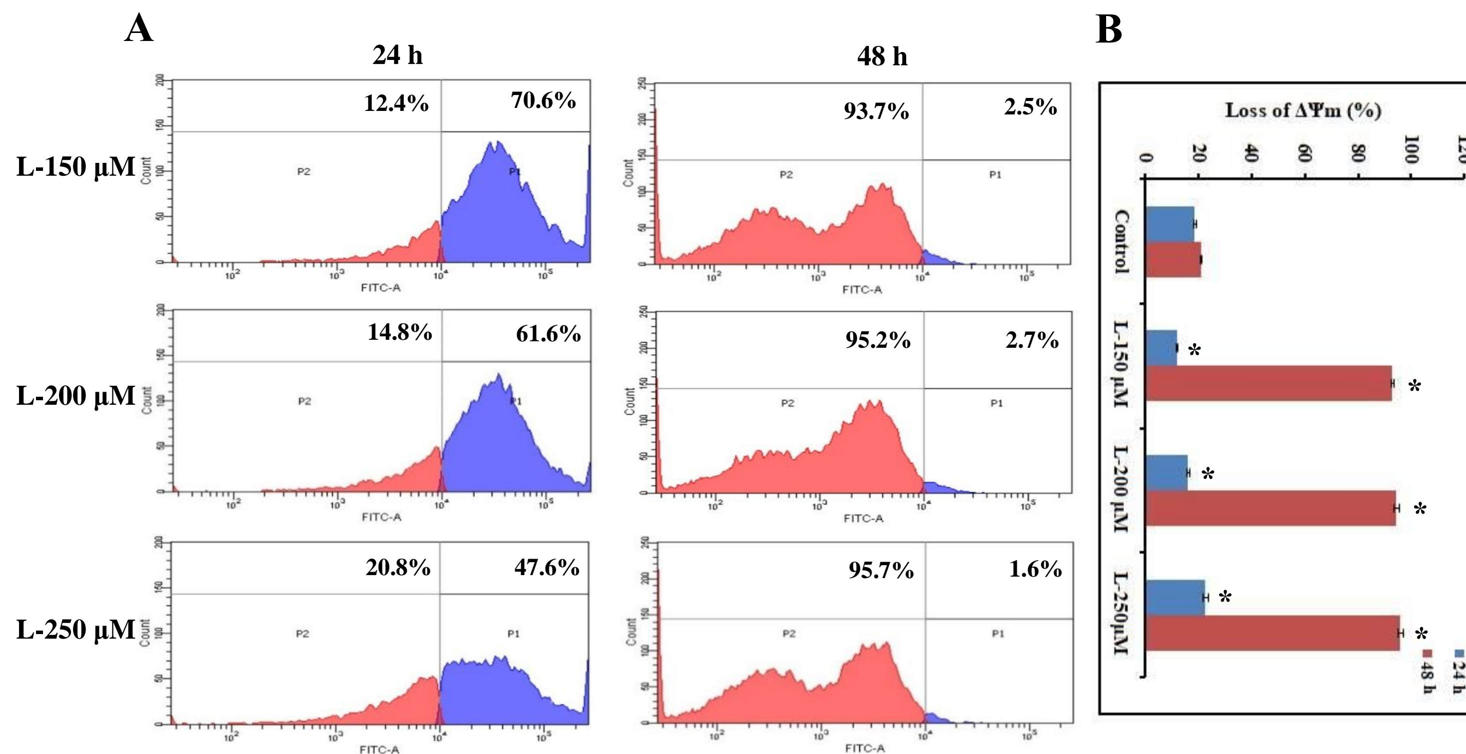

Figure 2.7e. Quantification of loss of mitochondrial membrane potential by Rhodamine 123 staining. SW480 cells were treated with various concentrations of luteolin (150, 200 and 250 µM) for 24 and 48 h. After incubation, cells were stained with Rhodamine 123 and analyzed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

2.3.6. Intracellular ROS production by DCFH-DA staining

It is reported that cancer cells are having increased oxidative stress and are more susceptible and sensitive to exogenous agents induced rapid surge in ROS levels (Nogueira and Hay, 2013) and several phytochemicals having potent anticancer activity act via altering the ROS status in cells and thereby induce a mitochondrial-mediated apoptosis (Paul et al., 2012). In order to understand further, whether selected phytochemicals induced apoptosis is associated with alteration in ROS level or not, ROS level in the cells after treatment was determined by measuring the intracellular ROS levels, by detecting dichlorofluorescein (DCF), derived from the oxidation of H2DCFDA by ROS, using fluorescent microscope (**Figure 2.8a, 2.8b**) as well as the flow cytometry (**Figure 2.9a. 2.9b, 2,9c, 2.9d, 2.9d**). The results indicated a dose and time dependent increase in ROS production in the cells after treatment.

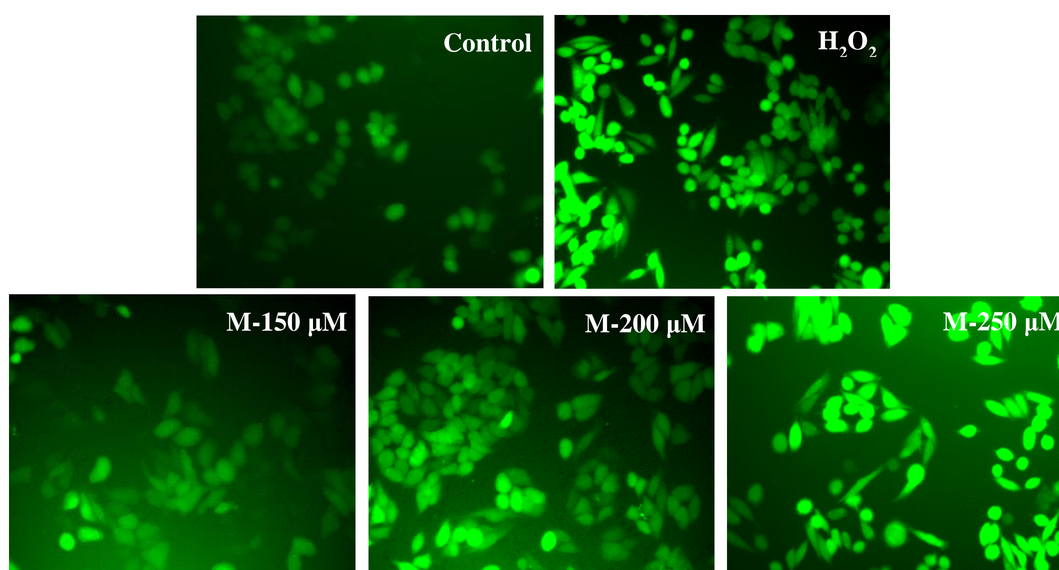

Figure 2.8a. Detection of ROS formation by DCFH-DA staining. SW480 cells were treated with various concentrations of morin (150, 200 and 250 µM and H_2O_2 (200 µM) for 2 h. After incubation, cells were stained with DCFH-DA and imaged using fluorescent microscope. (magnification 40×).

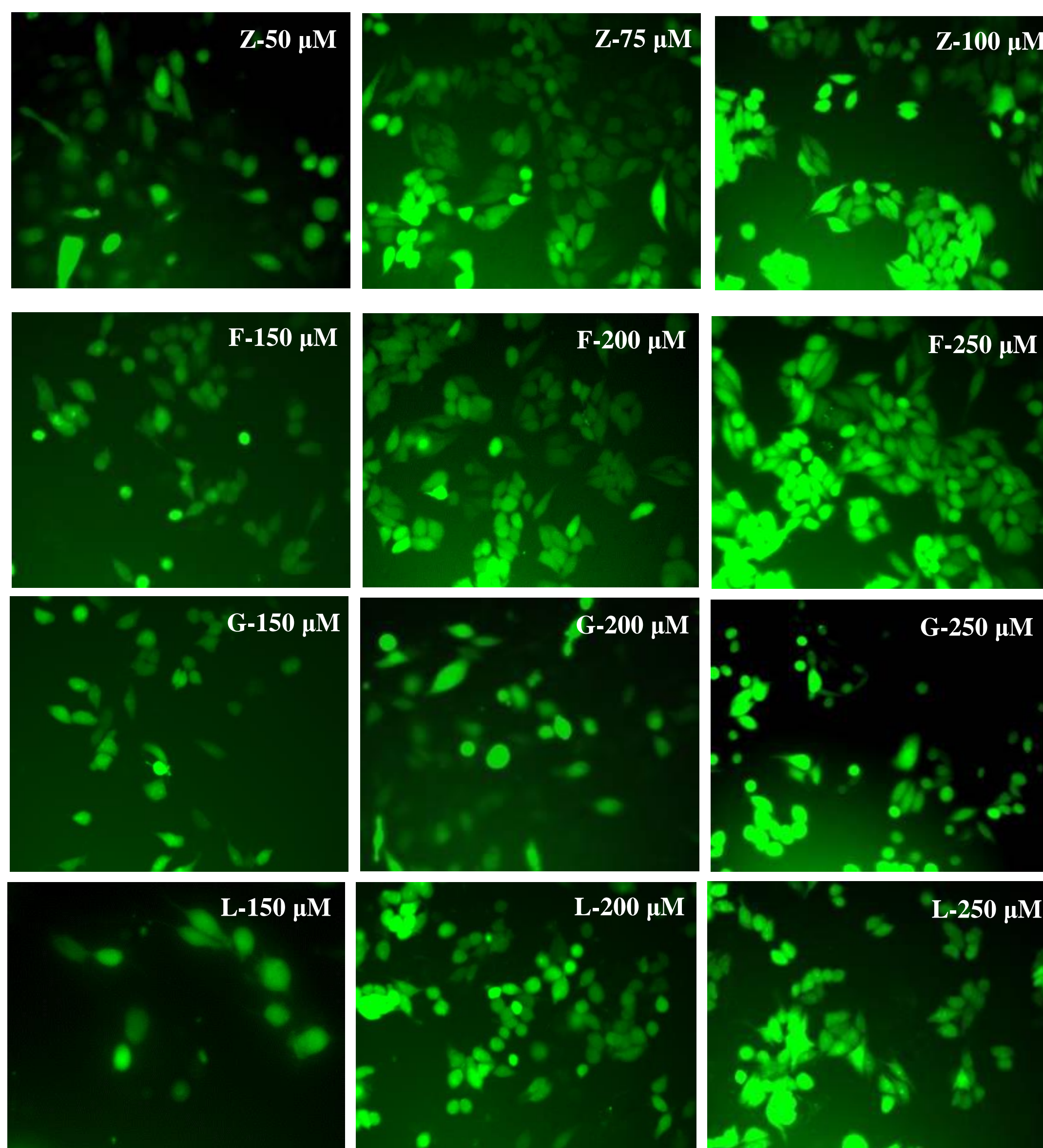

Figure 2.8b. Detection of ROS formation by DCFH-DA staining. SW480 cells were treated with various concentrations of zerumbone (50, 75, and 100 µM), fisetin (150, 200 and 250 µM), genistein (150, 200 and 250 µM) and luteolin (150, 200 and 250 µM) for 48 h. After incubation, cells were stained with DCFH-DA and imaged using fluorescent microscope. (magnification 40×).

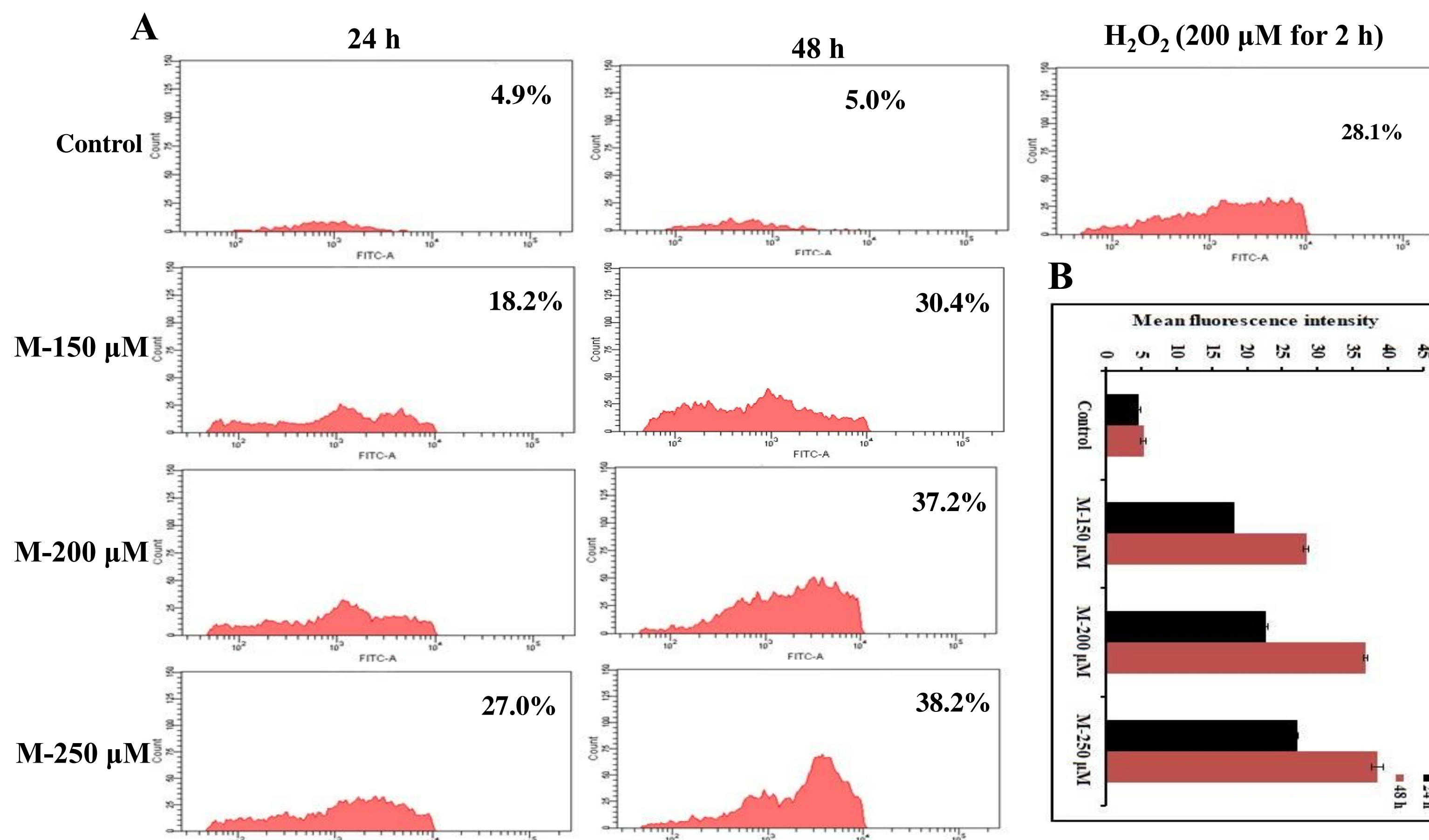

Figure 2.9a. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis. SW480 cells were treated with various concentrations of morin (150, 200 and 250 µM for 48 h and H_2O_2 (200 µM) for 2 h. After incubation, cells were stained with DCFH-DA and analysed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Significance levels between different groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

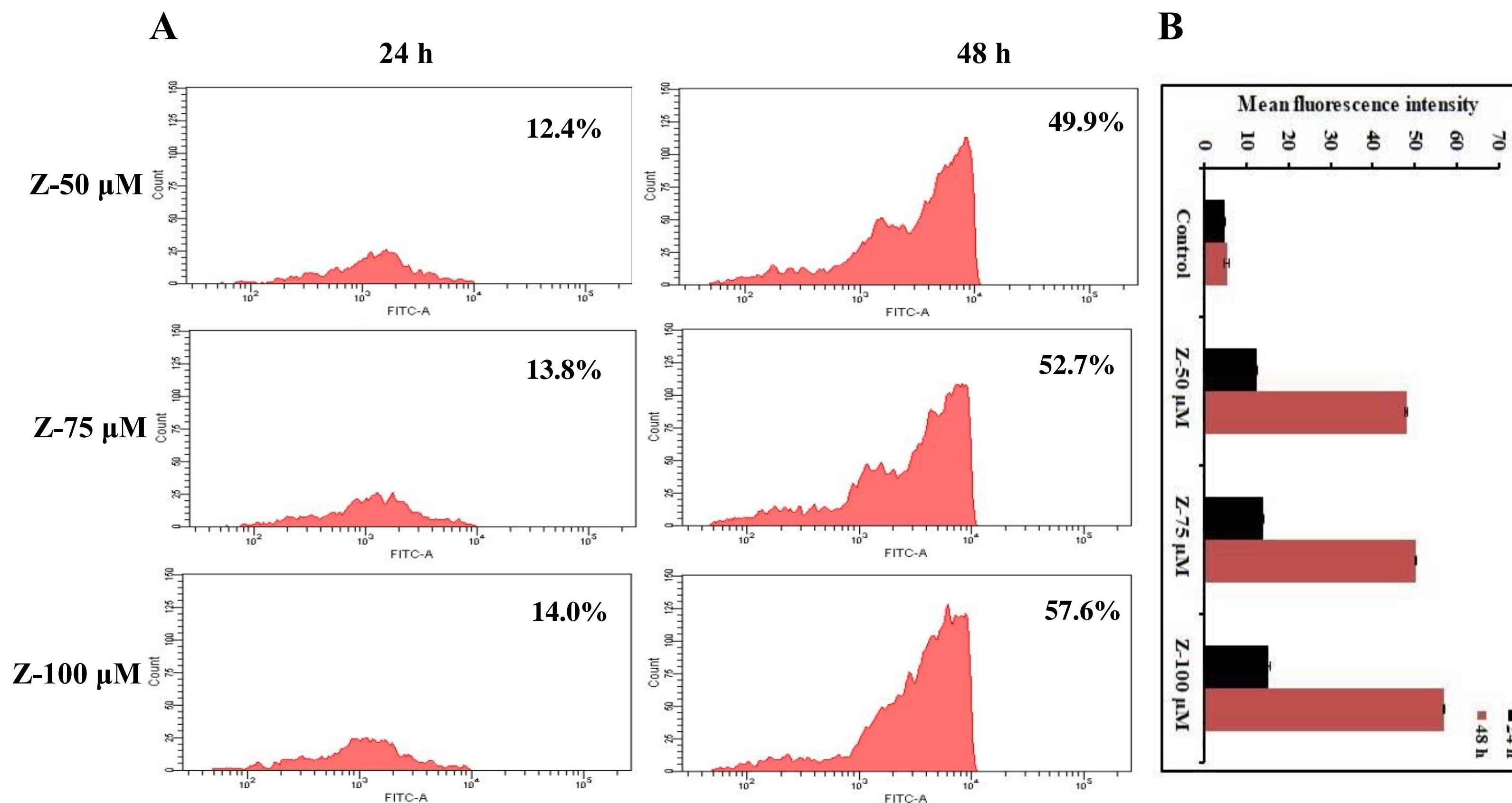

Figure 2.9b. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis. SW480 cells were treated with various concentrations of zerumbone (50, 75, and 100 µM). After incubation, cells were stained with DCFH-DA and analysed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Significance levels between different groups were determined by using one way ANOVA, followed by Duncan's multiple range test. $*p \leq 0.05$ versus control.

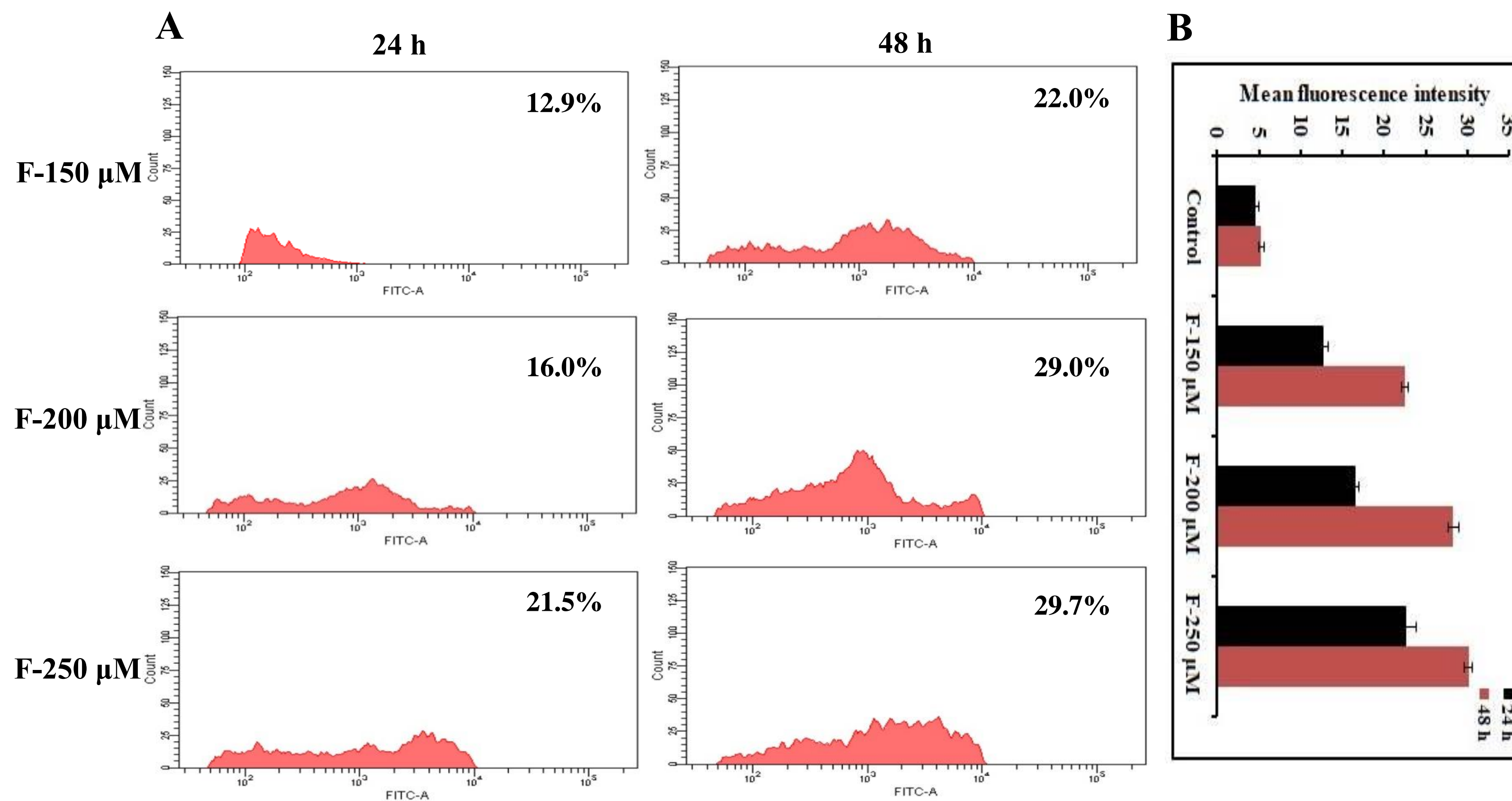

Figure 2.9c. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis. SW480 cells were treated with various concentrations of fisetin (150, 200 and 250 µM). After incubation, cells were stained with DCFH-DA and analysed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Significance levels between different groups were determined by using one way ANOVA, followed by Duncan's multiple range

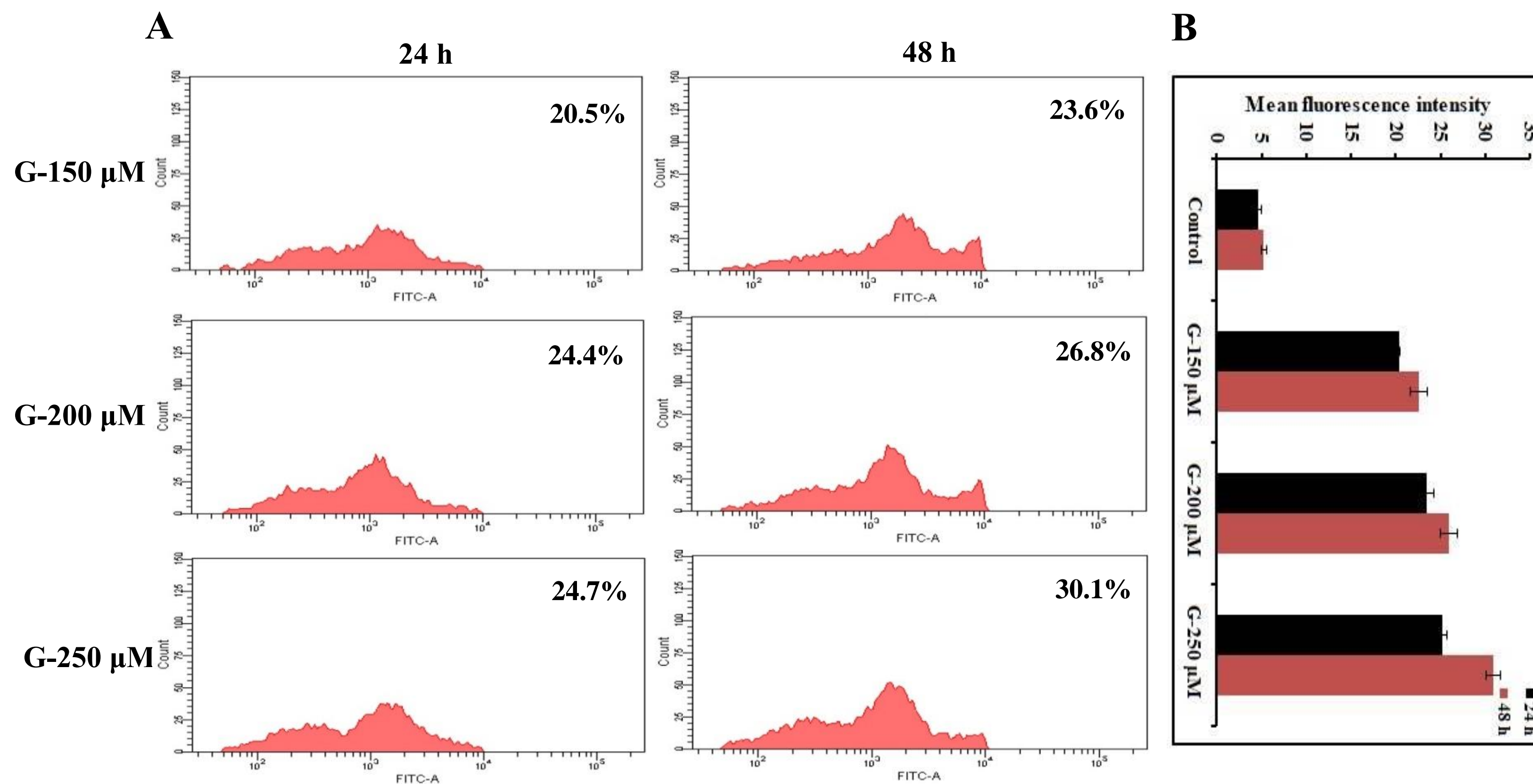

Figure 2.9d. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis. SW480 cells were treated with various concentrations of genistein (150, 200 and 250 µM). After incubation, cells were stained with DCFH-DA and analysed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Significance levels between different groups were determined by using one way ANOVA, followed by Duncan's multiple range test. $*p \leq 0.05$ versus control.

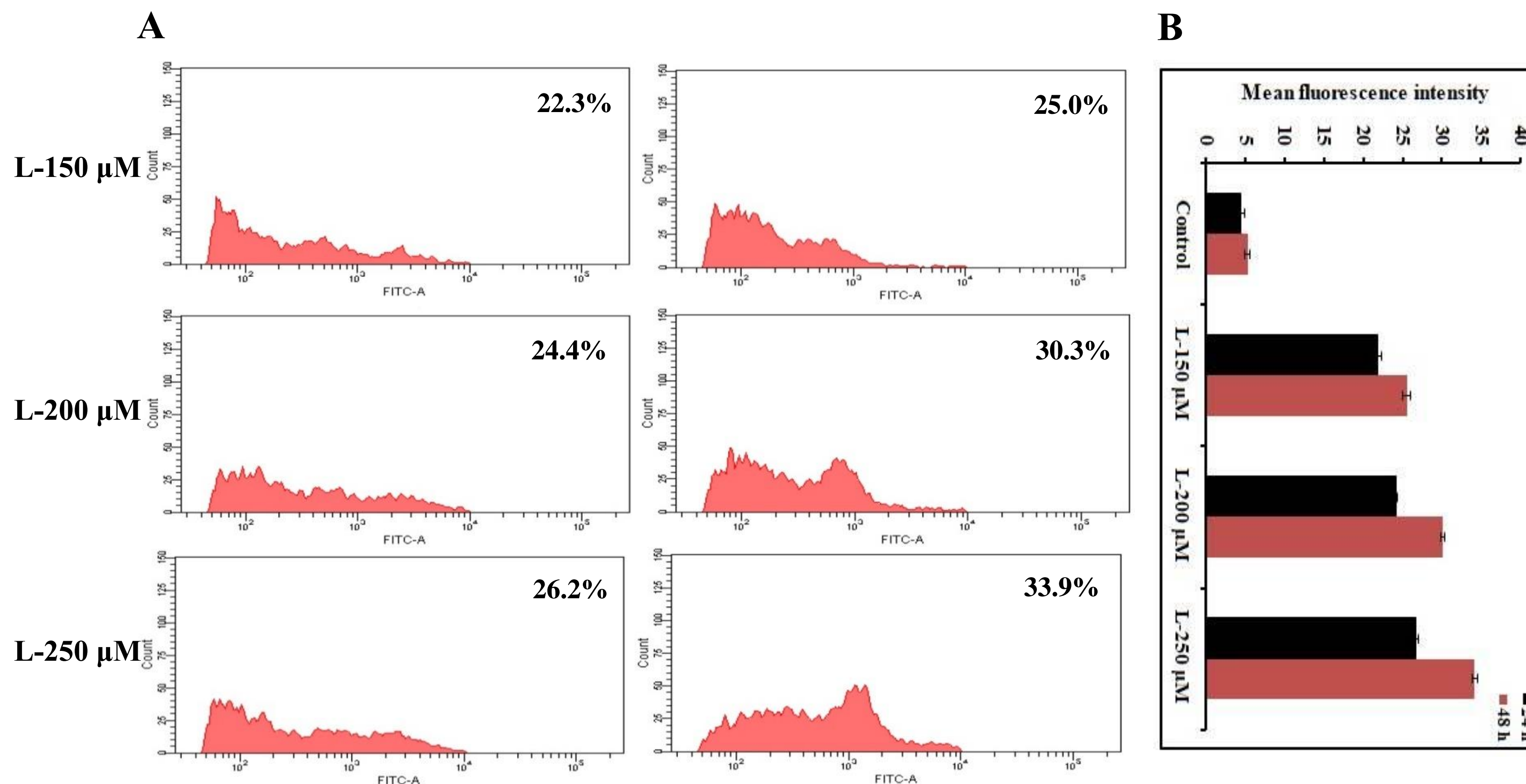

Figure 2.9e. Quantification of ROS level by DCFH-DA staining followed by flow cytometry analysis. SW480 cells were treated with various concentrations of luteolin (150, 200 and 250 µM) for 48 h. After incubation, cells were stained with DCFH-DA and analysed using flow cytometer. (A) Representative results. (B) Data analyzing fluorescence intensity from triplicate measurements. Significance levels between different groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *p ≤ 0.05 versus control.

2.3.7. Antioxidant level in SW480 cells (catalase activity and glutathione levels)

To check the changes in the antioxidant status in SW480 cell after treatment with selected phytochemicals, catalase activity and glutathione levels were estimated. It is reported that ROS-mediated apoptotic signaling is linked with inactivation of intracellular catalase that can contribute to the efficiency of ROS mediated induction of apoptosis. ROS-dependent apoptotic effect is also linked with decreased cellular GSH levels either by ROS-induced GSH oxidation or by GSH export from cells (Lu and Armstrong, 2007). The results showed a significant diminution in the levels of both catalase and glutathione in the cells, in a dose-dependent manner when compared with untreated control cells ($p \leq 0.05$). (**Figure 2.10**).

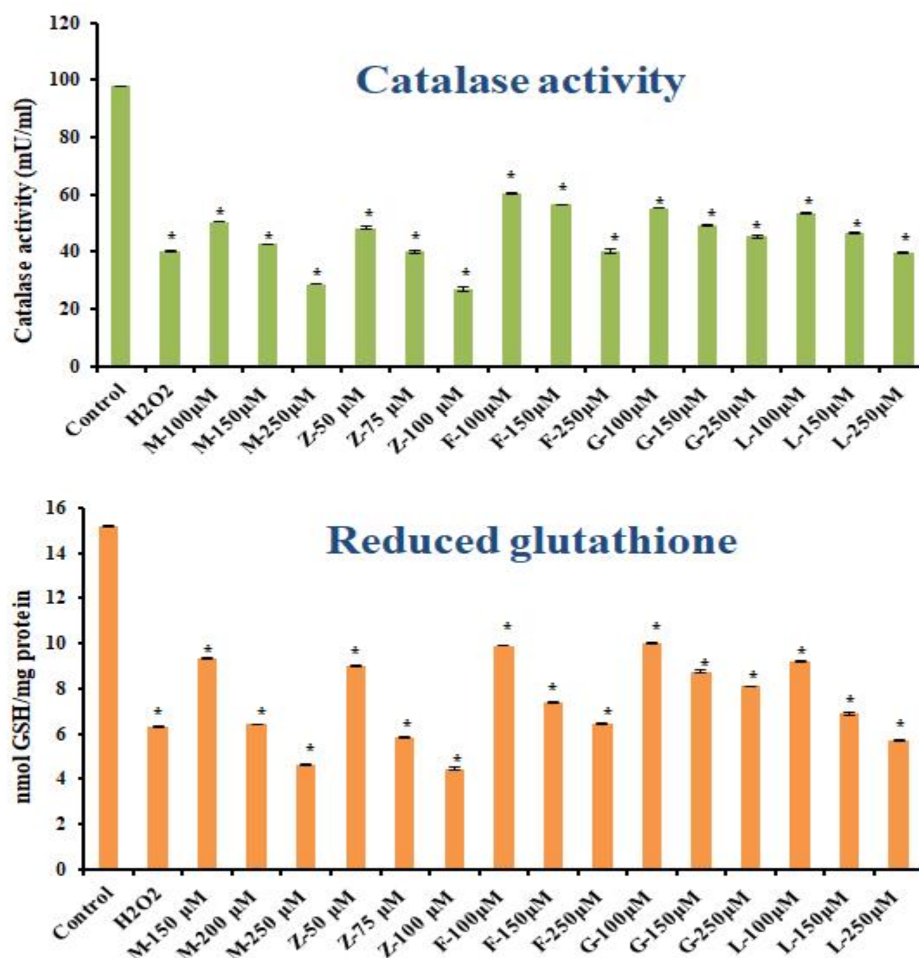

Figure 2.10. Treatment with selected phytochemicals resulted in a declined level of cellular reduced glutathione and catalase activity. The levels of reduced glutathione and catalase activity were assayed after treatment with morin (150, 200 and 250 μM), zerumbone (50, 75, and 100 μM), fisetin (150, 200 and 250 μM), genistein (150, 200 and 250 μM), luteolin (150, 200 and 250 μM) for 48 h and H_2O_2 (200 μM) for 2 h. Each value represents mean ± SD (standard deviation) from triplicate measurements (n = 3). Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *p ≤ 0.05 versus control. #p ≤ 0.05 versus camptothecin.

2.3.8. Western blot analysis

Among various proteins involved in the regulation of apoptosis, Bcl 2 family of proteins are regarded as the most important ones in the regulation of apoptotic cell death. It includes anti-apoptotic members like Bcl 2, Bcl xL and pro-apoptotic members like Bax, Bad, Bim, Bak etc. The anti-apoptotic members of this family inhibit apoptosis either by preventing the release of mitochondrial apoptogenic factors

into the cytoplasm or by sequestering caspases. On the other hand, pro-apoptotic members of this family mediated a cascade of events leading to caspase activation. Caspases play important roles in the induction of apoptosis and are classified as either initiator (Caspase 8 and Caspase 9) or effecter caspases (Caspase 3 and Caspase 7) (Vishchuk et al., 2013). Activated initiator caspases can cleave effecter caspases leading to its activation and which in turn cleave PARP (Poly ADP-Ribose Polymerase, a nuclear enzyme having a significant role in DNA repair by caspases) and its cleavage has been regarded as a hallmark of apoptosis (Jin and El-Deiry, 2005). In the present study, from above mentioned assays, it is clear that among the various phytochemicals studied, morin and zerumbone were showing better activity. Therefore, these two phytochemicals were investigated further for their pathway through which they induced apoptosis. Western blot results indicated a significant variation in the expression of Bcl 2 and Bax on treatment of SW480 cells with morin and zerumbone for 48 h. It was also observed that the expression of Bcl 2 was decreased while Bax was increased, leading to a rise in the Bax/Bcl2 ratio favouring induction of apoptosis. The level of cleaved caspases (3, 8 and 9 in the cells were significantly increased on treating with morin and zerumbone for 48 h and a raise in the level of cleaved PARP was also observed as illustrated in **Figure 2.11a, 2.11b,** indicating induction of both intrinsic as well as extrinsic pathways of apoptosis.

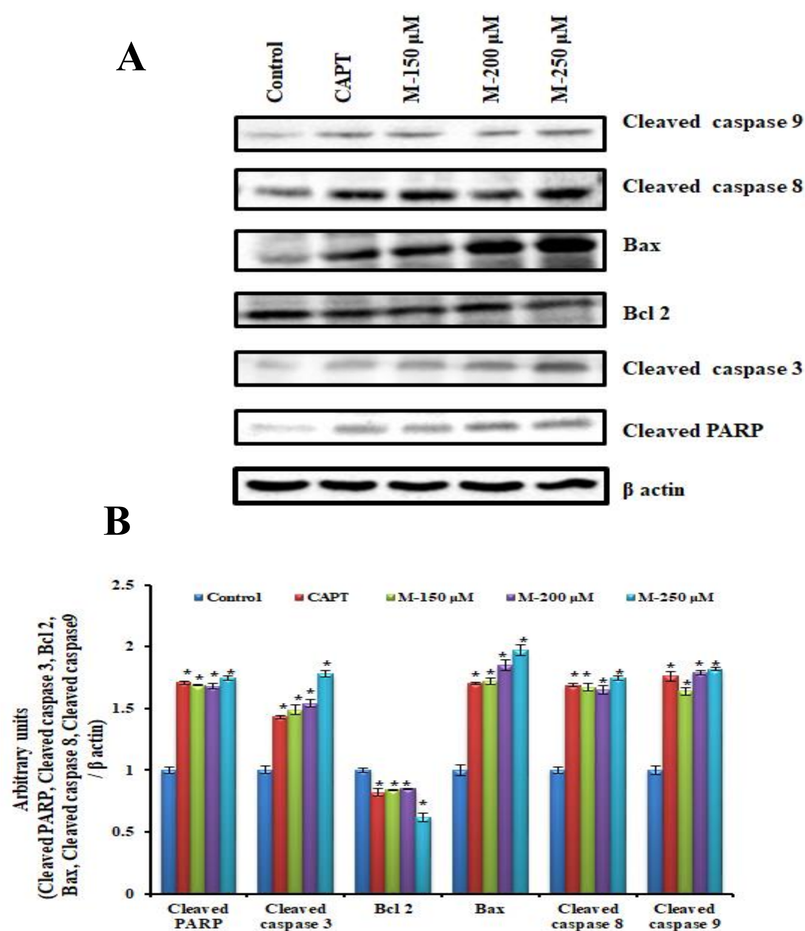

Figure 2.11a. Effect of morin on the expression of apoptosis related proteins. SW480 cells were treated with various concentrations of morin (150, 200 and 250 μM) and camptothecin (50 μM) for 48 h. Western blot analysis for expression of cleaved PARP, cleaved caspase 3, Bcl 2, Bax, cleaved caspase 8, and cleaved caspase 9 was carried out. (A) Representative results. (B) The levels of different proteins were quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

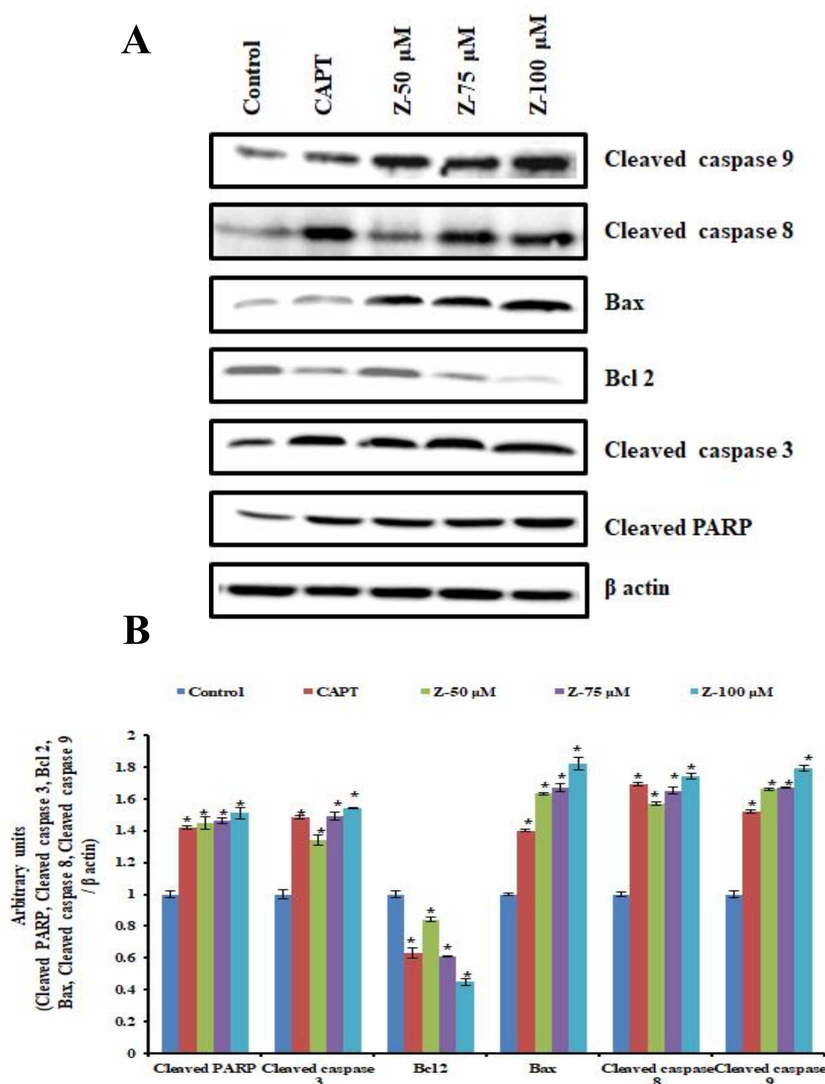

Figure 2.11b. Effect of morin on the expression of apoptosis related proteins. SW480 cells were treated with various concentrations of zerumbone (50, 75 and 100 µM) and camptothecin (50 µM) for 48 h. Western blot analysis for expression of cleaved PARP, cleaved caspase 3, Bcl 2, Bax, cleaved caspase 8, and cleaved caspase 9 was carried out. (A) Representative results. (B) The levels of different proteins were quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *p ≤ 0.05 versus control.

2.3.9. Effect of morin and zerumbone on viability of SW620 cells by MTT assay

Till this we have used SW480 cells, that are the cells derived from Dukes' type B colorectal adenocarcinoma and as the next step, we further evaluated the anticancer potential of morin and zerumbone on more advanced stage of colorectal cancer using SW620 cells, that are the colon cancer cells derived from Dukes' type C colorectal

adenocarcinoma. For this, we treated the cells with various concentrations of morin and zerumbone for 48 h. The obtained results suggest that these phytochemicals reduced the viability of SW620 cell lines in a dose dependent manner (**Figure 2.12**). The IC_{50} values of selected phytochemicals after 48 h treatment on SW620 cells were found to be 146 µM and 235 µM for zerumbone and morin respectively.

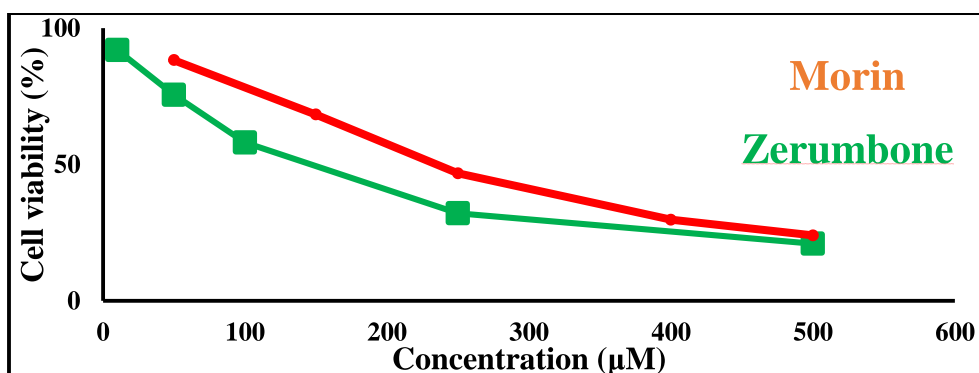

Figure 2.12. Cytotoxic effect of morin and zerumbone on SW620 cells after 48 h treatment. SW620 cells were treated with various concentrations (50-500 µM) of morin and zerumbone for 48 h. Cell viability was measured using MTT assay. Results are shown as percentage reduction in cell viability of treated cells compared to untreated control cells. Values shown are the mean ± SD obtained from three independent experiments.

2.3.10. Detection of SW620 cell apoptosis by Annexin V-FITC staining and flow cytometry

The effect of morin and zerumbone on apoptosis of SW620 cells were analysed by Annexin V-FITC staining followed by flow cytometry. A significant increase in the number of apoptotic cells was observed with the increase in the concentration of morin. But in the case of cells treated with zerumbone increment in apoptotic opulation was not significant as the concentration of zerumbone was increased (**Figure 2.13).**

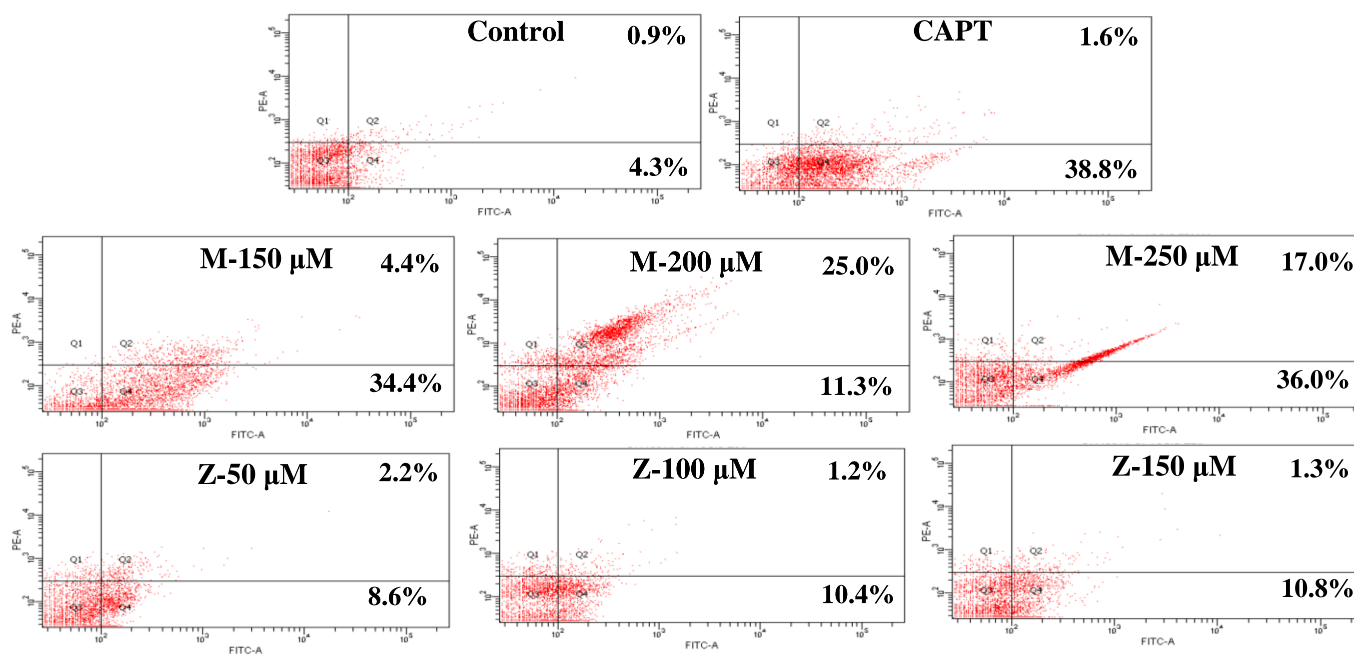

Figure 2.13. Flow cytometry analysis of the percentage of apoptotic cells in each group by Annexin- V/FITC and propidium iodide (PI) staining. Images showing the effects of morin (150, 200 and 250 µM), zerumbone (50, 100, and 150 µM), and camptothecin (50 µM) treatment for 48 h on induction of apoptosis in SW620 cells.

2.3.11. Western blot analysis

The level of cleaved caspase 3 and cleaved PARP levels in SW620 cells after treatment with morin and zerrumbone were evaluated by western blotting. The results demonstrated that treatment of SW620 cells with morin resulted in significant elevation in both cleaved caspase 3 and cleaved PARP in a concentration dependant manner compared to untreated SW620 control cells. But increase in the levels of both cleaved caspase 3 and cleaved PARP in SW620 cells were not significant in the case of cells treated with zerumbone (**Figure 2.14**)

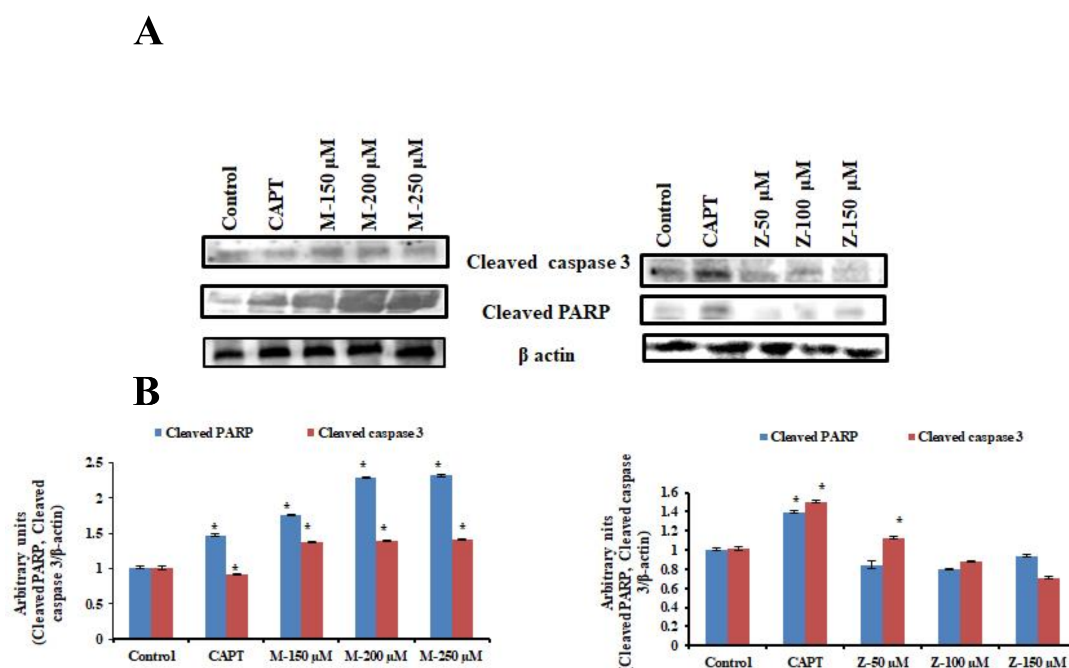

Figure 2.14. Effect of morin and zerumbone on apoptosis of SW620 cells. SW620 cells were treated with various concentrations of morin (150, 200 and 250 µM) and zerumbone (50, 75, and 100 µM) and camptothecin (50 µM) for 48 h. Western blot analysis for expression of cleaved PARP and cleaved caspase 3 was carried out. (A) Representative results. (B) The levels of different proteins were quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

2.3.12. Evaluation of cytotoxicity of morin on human hepatoma cell line (Hep G2)

Liver is the organ involved in the detoxification reactions. Drugs that are toxic to liver can bring about a large number of health issues and hence screening of drugs for liver toxicity is an unavoidable thing. A number of human hepatoma cell lines, including Hep G2, HuH 7, Hep 3B and Hepa RG, are generally used for drug metabolism and hepatotoxicity studies. Even though the metabolic functions of hepatoma cells are more limited than those of primary hepatocytes, they offer advantages for *in vitro* studies, like easily available, easy to handle, nearly unlimited life-span etc. Among them Hep G2 is the most commonly used human hepatoma cell line in pharmaco-toxicological research. Hence, in the present study we have evaluated effect of morin on viability of Hep G2 cells by MTT assay and found that the concentrations of morin selected for screening the anticancer potential studies were not toxic to Hep G2 cells (**Figure 2.15**).

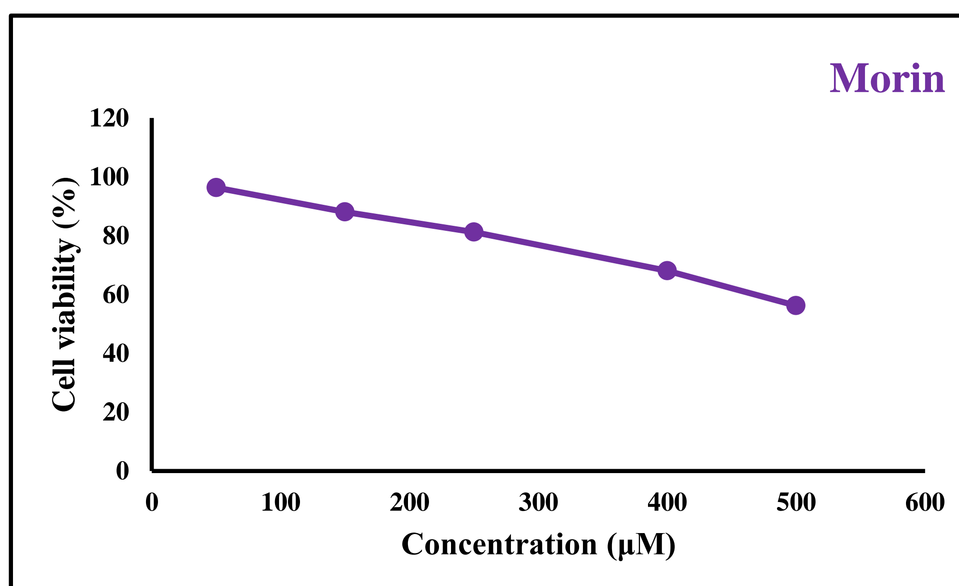

Figure 2.15. Effect of morin on viability of Hep G2 cells. Hep G2 cells were treated with various concentrations of morin for 48 h. Cell viability was measured using MTT assay. Results are shown as percentage reduction in cell viability of treated cells compared to untreated control cells.

2.4. DISCUSSION

Plants are the storehouse of a large number of phytochemicals and hence many plants are being used either directly as such or indirectly by isolating and purifying specific bioactive compounds from them, against numerous diseases. In the case of cancer research, promising results have been obtained for both prevention and management of cancer using bioactive compounds, especially in the case of colorectal cancer as it is having a close link with food habits and scientists are focussing more on colorectal cancer. In the study under investigation, we have done thorough literature search and selected few phytochemicals that have shown anticancer activity against other types of cancers but, haven’t studied in detail for their mechanism of action.

Results from cell viability assay showed that selected phytochemicals are exhibiting antiproliferative effect on SW480 cells, colon cancer cells from Dukes' type B, colorectal adenocarcinoma in a time and dose dependent manner. Apoptosis is regarded as the fundamental mechanism by which cells undergo death to control over proliferation of the cells. Disturbance in the innate apoptotic activity is regarded as a hallmark of neoplastic transformation and tumour formation. Modulation of the apoptotic cascade has been projected as pioneering approach towards cancer treatment (Kaufmann and Earnshaw, 2000, Ghobrial et al., 2005). In anticancer therapy, compounds that can trigger apoptosis are believed to be possessing anticancer potential (Strasser et al., 2011, Kelly and Strasser, 2011). In the present study, characteristic apoptotic features such as fragmented chromatin or highly condensed nucleus that was homogenously fluorescent as observed by fluorescence microscopy in cells treated with selected phytochemicals after Hoechst 33342 staining point out that these selected phytochemicals could induce apoptosis. The phosphatidyl serine externalization

during apoptosis was established with Annexin- V/FITC and PI staining followed by flow cytometry analysis.

Further studies were done to look over the mechanism by which the selected phytochemicals induced apoptosis in SW480 cells. In the recent times mitochondria are regarded as a novel target for chemotherapy-induced apoptosis. Loss of mitochondrial membrane potential due to opening of mitochondrial permeability transition pores is an evincive of apoptosis (Hengartner, 2000, Kim et al., 2007) and in the current study, a noteworthy loss of mitochondrial membrane potential was observed in SW480 cells on selected phytochemicals treatment. In comparison with the normal cells, cancer cells are more sensitive to rapid increases in ROS levels and ROS released during several established treatments can mediate proapoptotic effects in cancer cells (Engel and Evens, 2006, Gallego et al., 2008). Cancer cells with increased levels of antioxidant systems and mitochondrial suppressor of ROS (e.g., uncoupling protein-2), have been found to induce chemoresistance in them (Ramanathan et al., 2005, Derdak et al., 2008). Hence the tempering of oxidative stress in tumour cells has been suggested as a noteworthy approach to sensitize tumours to cytotoxic drugs. The balance between intracellular ROS levels and ROS scavenging antioxidant systems is keeping the redox homeostasis in cells. During oxidative stress, this balance is disquieted as seen in most of the cancer cells. This make cancer cells more depend on their antioxidant defense system to preserve redox balance and hence they are more vulnerable to further oxidative stress. Hence, any agent that enhances intracellular ROS level in cancer cells to a toxic level can lead to mitochondrial damage and thereby cell death (Pramanik et al., 2011). In the current study, a raised ROS level along with decreased antioxidant status in selected phytochemicals treated

cells point out that they are inducing ROS-mediated apoptosis in SW480 colon cancer cells.

Out of the five selected phytochemicals, morin and zerumbone is found to be having more activity than the other three. Hence morin and zerumbone were studied in detail for evaluating the apoptosis pathway involved. The very complex cascade of molecular events in apoptosis is mainly mediated via two apoptotic pathways, the extrinsic apoptotic pathway and the intrinsic apoptotic pathway. The extrinsic apoptotic pathway is accompanied by the stimulation of initiator caspase 8 through cell surface death receptors. The intrinsic apoptotic pathway is via apoptotic cascades happening in mitochondrion, resulting in the amendment of the membrane potential and the initiator caspase 9 activation (Chowdhury et al., 2006). These two activated initiator caspases can further activate downstream caspase 3 and results in the cleavage of the PARP protein, which force the cells to undergo apoptosis (Tewari et al., 1995, Woo et al., 2011). In this study, treatment of SW480 cells with both morin and zerumbone resulted in the activation of both extrinsic as well as intrinsic pathways of apoptosis and caspase 3 mediated cleavage of PARP.

The Bcl 2 family members are functioning as main regulators of mitochondrial response towards apoptotic signals. Augmented ratio of antiapoptotic to proapoptotic Bcl 2 proteins is found in most of the neoplastic cells and this facilitates them to survive under adverse conditions. A latest approach for cancer therapy is by directly triggering of the apoptotic pathway by reducing the activity of antiapoptotic Bcl 2 proteins and enhancing the function of proapoptotic Bcl 2 proteins (Certo et al., 2006, Vogler et al., 2009). In this study when SW480 cells were treated with morin and zerumbone an upregulation of Bax protein level and downregulation of Bcl 2 protein levels were found, which further confirms their anticancer potential.

As the next step we further evaluated the anticancer potential of morin and zerumbone on advanced stage colorectal cancer cells using SW620 cells, colon cancer cells from colon; derived from metastatic site: lymph node, Dukes' type C, colorectal adenocarcinoma. MTT analysis was used for determining the treatment doses of morin and zerumbone for detailed analysis. The effect of morin and zerumbone on apoptosis was done using Annexin- V/FITC and PI staining followed by flow cytometry analysis. From the observed results it was clear that morin could induce apoptosis in SW620 cells very effectively and effect of zerumbone on apoptosis of SW620 cells were quite negligible. To further confirm these results, we have performed evaluation of the levels of cleaved caspase 3 and cleaved PARP levels by western blotting. The obtained blot results were in agreement with the flow cytometry results and it was found that, there was significant increment in the levels of both cleaved caspase 3 and cleaved PARP on treatment with morin compared with untreated control SW620 cells, while the levels of cleaved caspase 3 and cleaved PARP remained unchanged on treating with zerumbone, indicating that morin is active against colorectal cancer both in early and advanced stage and zerumbone is active only on the early stage of colorectal cancer. So for the further analysis morin was selected as the lead compound.

2.5. CONCLUSION

Based on literature survey we had selected five phytochemicals (morin, genistein, fisetin, luteolin and zerumbone) for the evaluation of anti-colorectal cancer potential using SW480 cells, Dukes' type B, colorectal adenocarcinoma cells. All the five compounds were found to be inducing DNA damage as evidenced from Hoechst 33342 staining. Flow cytometry analysis after propidium iodide and annexin V-FITC staining showed that the nuclear damage induced cellular apoptosis. This nuclear damage and apoptosis was accompanied by the alteration in mitochondrial membrane potential as

evidenced from JC 1 staining followed by fluorescent microscope images as well as flow cytometry analysis after rhodamine 123 staining. Further analysis of intracellular ROS status and antioxidant status of the cells revealed that all of these phytochemicals are inducing ROS mediated apoptosis. Among the five selected phytochemicals morin and zerumbone exhibiting more activity and were studied for apoptotic pathway by which these two are inducing apoptosis and found that both are inducing apoptosis through both extrinsic and intrinsic pathway of apoptosis.

In next step, we have evaluated anticancer efficacy of morin and zerumbone on more advanced stage of colorectal cancer using SW620 cells. Even though both could reduce cell viability, zerumbone failed to induce apoptosis in SW620 cells as evidenced from the results of flow cytometric analysis of apoptosis after propidium iodide and FITC labelled annexin V staining and western blotting. Meanwhile morin could induce apoptosis in a concentration dependent manner and it was associated with increased levels of cleaved caspase 3 and cleaved PARP. While considering chemotherapeutic drugs, it is regarded as mandatory that a good anticancer agent should be effective against cancer cells and at the same time have minimal cytotoxic effects in normal cells. Hence in the present study we used Hep G2 cells, which are the most widely used human hepatoma cell line in pharmaco-toxicological research and found that the concentrations of morin that was used for various assays were nontoxic to Hep G2 cells. So, morin was selected for further studies to see the mechanism by which it exhibits anticancer potential.

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CHAPTER 3

Effect of Morin on Warburg Effect

(SW480 Colorectal Cancer Cells)

3.1. INTRODUCTION

Cellular metabolism in cancer cells is different from the normal cells. Cancer cells show unusually high rates of glucose uptake and lactate production compared with normal tissues, even in the presence of oxygen, rather than metabolizing pyruvate through the TCA cycle. This first and best-known cancer metabolic abnormality was described in the 1920s by Otto Warburg. Dr. Warburg stated that, even in the presence of oxygen, tumours displayed unusually high rates of glucose uptake and lactate production compared with normal tissues. This up taken glucose will undergo glycolysis in tumour cells.

Glycolysis results in the production of ATP, but its efficacy is less compared with other glucose metabolising pathway but the advantage is that, it happens at a faster rate than oxidative phosphorylation. This quicker rate of ATP production helps in the rapid proliferation of cancer cells. It is also observed that along with the production of ATP, the high glycolytic rate may stimulate the growth of cancer cells through increasing biosynthesis of important molecules such as amino acids, nucleotides lipids and NADPH (Samuels et al., 2005). Glycolysis also results in the production of metabolic products like H^+ and lactate and their production will lead to steady acidification of the extracellular environment (Schornack and Gillies, 2003) and which in turn favour invasion and metastasis of cancer cells to other parts of the body (Smallbone et al., 2005). Hence, it is important to understand the molecular pathways that regulate aerobic glycolysis, to exploit the altered cancer metabolism for cancer therapy.

The greater dependency on glycolysis by cancer cells offers a number of potential therapeutic targets for killing cancer cells, very selectively and effectively. Since glycolysis is active in both normal cells and cancer cells, it is very hard to target cancer cell metabolism specifically without affecting normal cells. Scientific world has come

out with a solution for this that the key signalling molecules like G6PD (Glucose-6-phosphate dehydrogenase, LDH-A (lactate dehydrogenase-A), HK (Hexokinase), PK (pyruvate kinase) and GLUT 1 are over expressed in cancer cells than the normal cells (Zhang and Yang, 2013) and drugs targeting these molecules can effectively fight against cancer cells without affecting normal cells. In short, finding out a compound that can significantly reduce glucose uptake as well as the expression of associated molecules will definitely be a new light in the field of anticancer research.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Morin, antibiotic antimycotic mix, dulbecco's modified eagle's media (DMEM), SC79, ATP, ADP, AMP were purchased from Sigma–Aldrich Chemicals (St Louis, MO, United States). 2-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose (2-NBDG) was obtained from Molecular Probe (Invitrogen Life Technologies, Carlsbad, CA, United States). Foetal bovine serum (FBS) was purchased from Gibco-BRL (Auckland, New Zealand). Primary antibodies (β actin, GLUT 1, PI3K, PDK1, Akt, p-Akt, PIP5K) and corresponding secondary antibodies were purchased from Santa Cruz Biotechnology, United States. Clarity Western ECL substrate was purchased from Bio-rad, United States. All other chemicals used were of the standard analytical grade.

3.2.2. Cell culture and treatment

The human colon cancer cells (SW480) obtained from ATCC (American Type Culture Collection, Manassas, United States) were maintained in DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic mix at 37°C under a humidified 5% CO_2 and 95% air atmosphere. Cells were exposed to 0.25% trypsin-EDTA and harvested cells

were seeded at a density of 1×10^4 cells/well on 24 well-plates and 6-well plates (Costar, United States) for different assays.

3.2.3. Experimental design

Schematic representation of Chapter 3 is depicted in the flowchart (**Figure 3.1**).

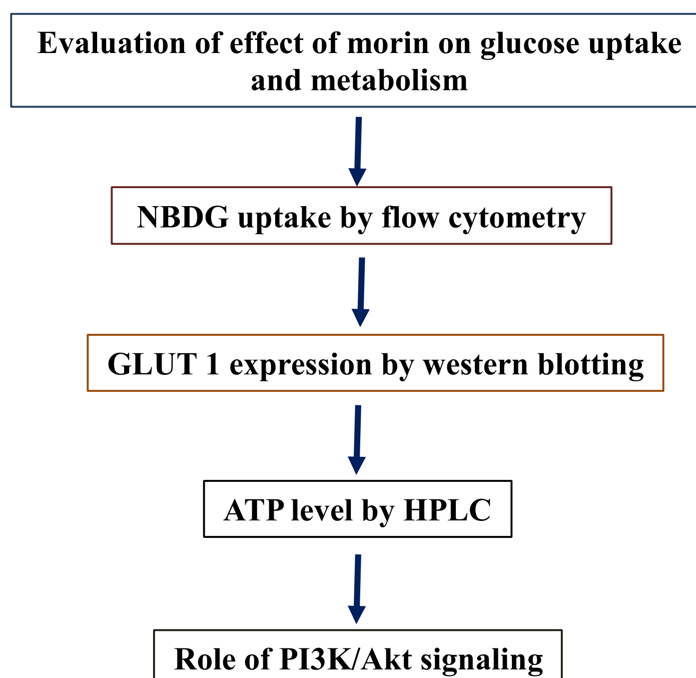


Figure 3.1. Outline of Chapter 3

3.2.4.1. Fluorescence analysis of 2-NBDG uptake by flow cytometry

The changes in glucose uptake on treatment with morin (150, 200 & 250 µM) and camptothecin (50 µM) for 48 h, were examined using fluorescent D-glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) and flow cytometric detection of fluorescence emitted by the cells (Chen et al., 2010). For this, cells were grown in 6 well plates and treated with indicated concentration of morin for 48 h, the culture medium was replaced with 100 µM fluorescent 2-NBDG in PBS for 30 min, washed two times with cold phosphate-buffered saline (PBS), trypsinized, resuspended in ice-cold PBS. Results were analysed using Fluorescence

Activated Cell Sorting (BD FACS Aria II, BD Biosciences, USA) at FITC range (excitation 490 nm, emission 525 nm band pass filter) and the mean fluorescence intensity of different groups were analysed BD FACSDiva^{TM} Software v6.1.2 and a minimum of 10000 cells were analysed from each group, corrected for auto fluorescence from unlabelled cells.

3.2.4.2. Extraction and measurement of Adenosine Tri Phosphate (ATP) levels

The extraction adenine nucleotide was performed as previously reported (Hahn-Windgassen et al., 2005) with slight modifications. After treatment, the cells were trypsinized and centrifuged at 800 × g for 3 min, and the pellets were suspended in 4% perchloric acid on ice for 30 min. The pH of the lysate was adjusted between 6 and 8 with 2 M KOH with in 1 h. Precipitated salt was separated from the liquid phase by centrifugation (KUBOTA 7780) at 13000 × g for 10 min at 4°C. The samples were stored at -80°C till further analysis.

ATP was quantified on a Prominence HPLC system (Shimadzu, Japan) containing LC-20 AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µL volume and a diode array detector (SPD-M20A). A buffer made with 20 mM KH2PO4 and 3.5 mM K2HPO4 3H2O (pH 6.1) was used as the mobile phase. The flow rate was 1 ml/min, the injection volume was 20 µl and the column was at 37°C. The fractions were monitored at 259 nm. Sample peaks were identified by comparing with retention times of standard peaks. LC LabSolutions software was used for data acquisition and analysis.

3.2.4.3. Immunoblot analysis

Cells were treated with 150, 200 and 250 µM concentrations of morin and 50 µM concentration of camptothecin for 48 h. After the incubation, cells were washed twice

with ice cold PBS, lysed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 0.5 mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100 and protease inhibitor cocktail, pH 8.0) for 30 min on ice and were centrifuged at 12000 x g for 10 min. BCA protein assay kit was used for determining the protein concentration of the lysate. Lysates were diluted to an equal concentration of total protein and supernatants were then stored at -80ºC until analysis. These samples in reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue) were boiled for 10 min at 75°C. The lysate containing 50 µg of protein was subjected to SDS-PAGE on 12% gel and transferred onto a polyvinylidene difluoride membrane (Immobilon PTM, Millipore®, USA) by using Trans-Blot TurboTM transfer system (Bio-Rad Laboratories, Germany). The blocking buffer (5% skim milk in PBST, PBST-PBS buffer containing 0.1% Tween 20), for 1 h at room temperature was used for blocking. The membranes were washed three times with PBST and probed over night at 4°C with primary antibodies (β actin, GLUT 1, PI3K, PDK1, p-Akt, PIP5K at 1:500 dilution). After washing three times with PBST for 5 min each, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody at 1:1000 dilution and again washed three times in PBST. The bound antibodies were sensed using an enhanced chemiluminescence substrate (Biorad, USA) and measured by densitometry using a Chemi Doc XRS digital imaging system and the Multi-Analyst software from Bio-Rad Laboratories (USA). To further confirm the action of morin through PI3K/Akt signalling, cells were treated with specific Akt activator, SC79 and blotting was performed for seeing the change in the level of p-Akt.

3.2.4.4. Molecular docking (*In silico* studies)

The preparation of the receptor and the ligands for binding affinity studies, ADME/T (Absorption, Distribution, Metabolism, Excretion and Toxicity) prediction and molecular docking studies were done by Schrodinger suite 2018-2 (https://www.schrodinger.com/suites/small-molecule-drug-discovery-suite). All the conformers of the two compounds (morin and PI3K inhibitor) were generated by LigPrep tool, then used for QikProp analysis to verify the pharmacokinetic parameters and the values are compared with the range obtained from QikProp manual (Sherin et al., 2019). The crystal structures of human phosphoinositide 3-kinase (PI3K) (PDB ID: 1E8Z) and the human glucose transporter Glut I (PDB ID: 4PYP) were retrieved from RCSB PDB (Protein Data Bank), (Walker et al., 2000, Deng et al., 2014).

The prepared PDB files were further used for grid generation around the centroid of the workspace ligand and the same binding site is used for docking. Molecular docking of ligands with the receptors were carried out using Glide programme of Schrodinger suite (Friesner et al., 2006, Halgren et al., 2004). The binding affinity of the ligands were predicted on the basis of scoring functions- G-score (Glide score) and D-score (Dock score) using XP-visualizer.

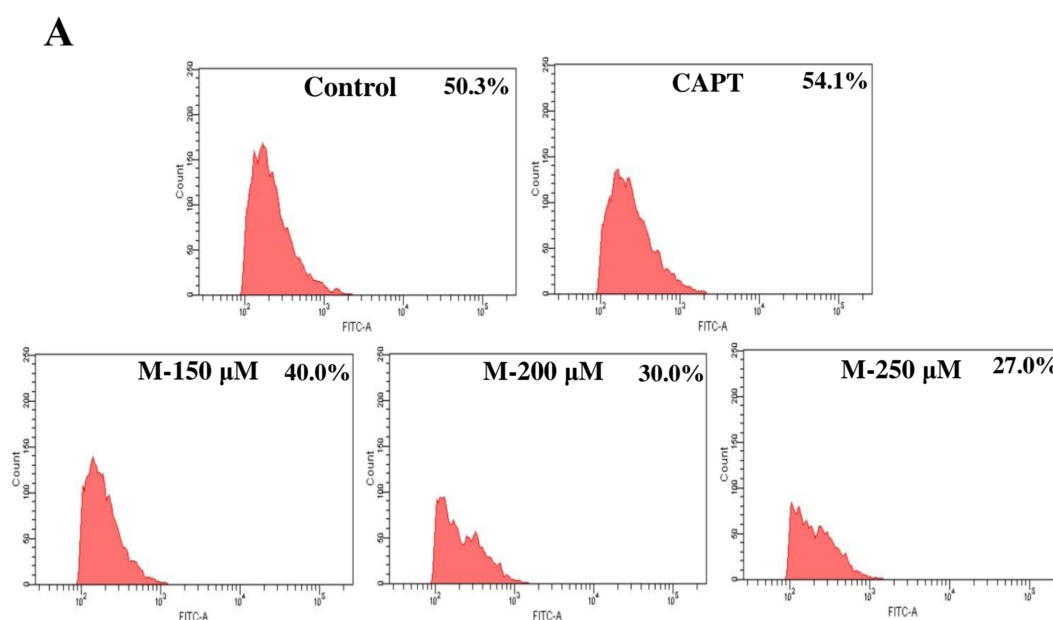
3.2.4.5. Statistical analysis

All the experiments were done at least in triplicate and results were expressed as mean ± SD (standard deviation). The differences between treatments in comparison with control were assessed using one-way ANOVA and the significance of differences between means was calculated by Duncan's multiple range test, using SPSS for Windows, standard version 16 (SPSS, Inc.) and significance was accepted at $p \leq 0.05$.

3.3. RESULTS

3.3.1. Effect of morin on 2-NBDG uptake by flow cytometry

It is reported that the cancer cells use glucose for aerobic glycolysis and hence their need for glucose is higher than the normal cells. This will further lead to augmentation of glycolytic metabolism and increase in the glucose transport across the plasma membrane (Macheda et al., 2005). Hence it is noted that compared to the normal cells, cancer cells demonstrate increased sensitivity to glucose deprivation-induced cytotoxicity (Aykin-Burns et al., 2009). GLUT 1 is the natural transporter of glucose and is required for the high glycolytic rate seen in colorectal tumours. In colorectal cancer cells with the help of this GLUT 1, glucose is more effectively transported in to the cells. In the present study, it was noticed that glucose uptake (**Figure 3.2)** and GLUT 1 expression was reduced significantly in the cells treated with morin when compared to the untreated control cells ($p \leq 0.05$).



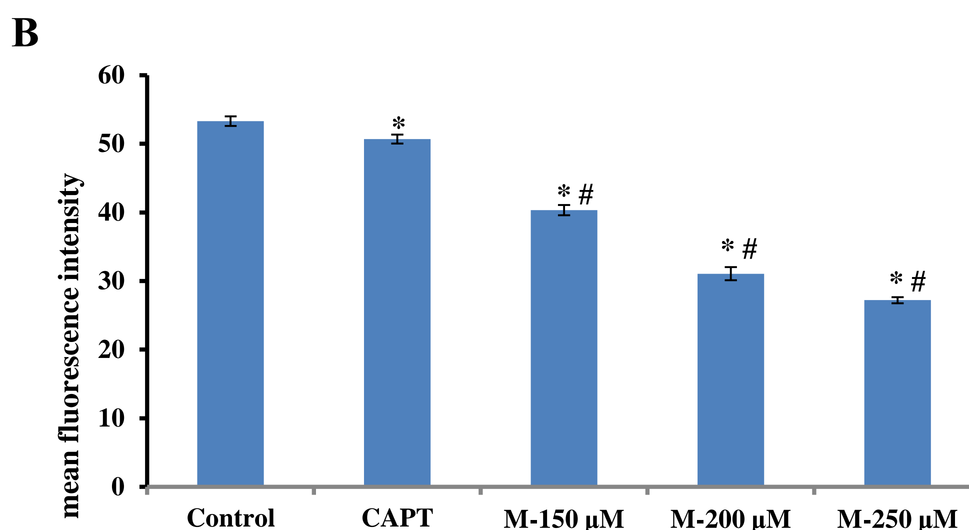


Figure 3.2. Flow cytometry analysis of 2-NBDG uptake. SW480 cells were treated with various concentrations of morin (150, 200, and 250 µM) and camptothecin (50 µM) for 48 h. (A) FACS analysis of 2-NBDG uptake in SW480 cells plotting cell count against FITC. (B) Graphical representation of percentage of glucose uptake by SW480 cells in different groups. Each value represents mean ± SD (standard deviation) from three independent experiments. Significance levels between different groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control, #$p \leq 0.05$ versus camptothecin.

3.3.3. Glucose transporter 1 (GLUT 1) level by western blotting

Glucose transporters are involved in the transport of glucose in to the cells and helps in regulating energy metabolism. Malignant cells require high energy levels through glycolytic generation of ATP for their proliferation and survival. Out of the 14 glucose transporters, GLUT 1 is overexpressed in many tumours, including colorectal cancer (Yamamoto et al., 1990). In cancer-induced starvation, overexpression of GLUT 1 regulates mechanisms that favour tumour growth at the expense of host tissues (Macheda et al., 2005). Thus, in the current study we examined GLUT 1 expression, by western blotting and found that the GLUT 1 expression was reduced significantly in the cells pretreated with morin when compared to the untreated control cells ($p \leq 0.05$). (**Figure 3.3**).

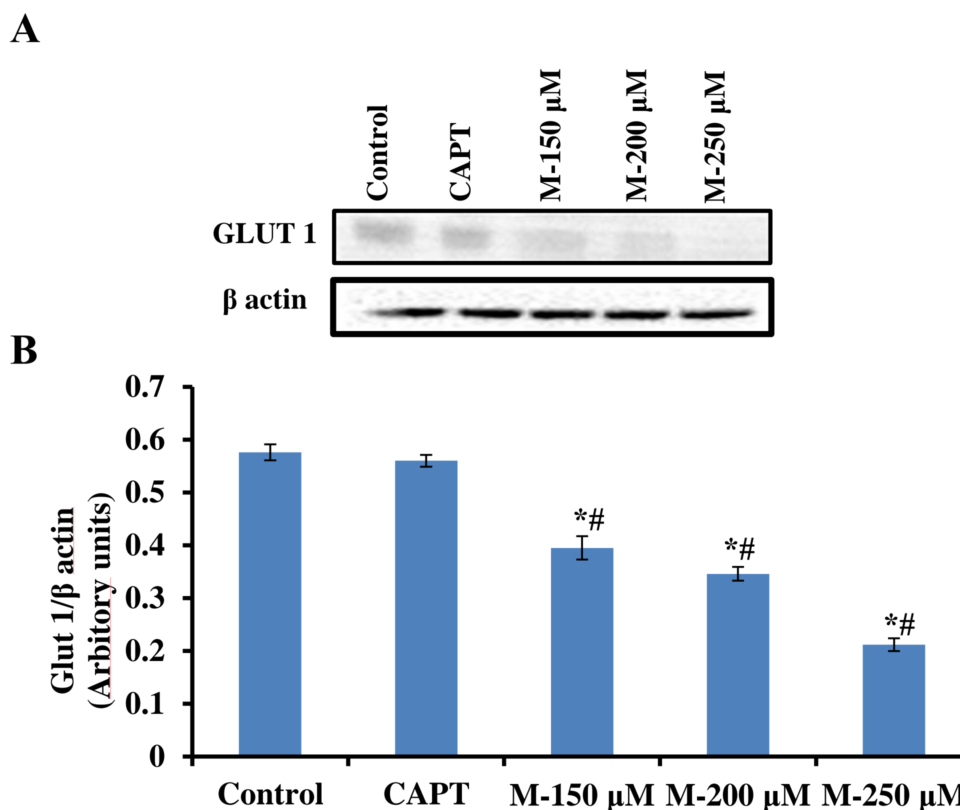


Figure 3.3. Effect of morin on GLUT 1 expression. SW480 cells were treated with various concentrations of morin (150, 200 and 250 µM) and camptothecin (50 µM) for 48 h. Western blot analysis for expression of GLUT 1 was carried out. (A) Representative results. (B) The level protein was quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control

3.3.3. Determination of ATP production

ATP is the primary energy currency of the cell and uncontrollably dividing cancer cells have a high demand for ATP. From the experiments in the previous chapter, it was clear that morin is inducing apoptosis in colorectal cancer cells with a significant alteration in mitochondrial membrane potential. Studies have reported that during late-stage apoptosis, ATP levels sharply drop, mostly because of the loss of mitochondrial function and consumption by ATP-dependent proteases. Since cancer cells rely more on glycolysis for ATP production, we evaluated the cellular levels of ATP after morin

treatment by HPLC method. From the **Figure 3.4**, it can be seen that the morin pre-treatment resulted in a significant decrease in ATP level with a simultaneous increase in ADP and AMP levels when compared with untreated control cells ($p \leq 0.05$).

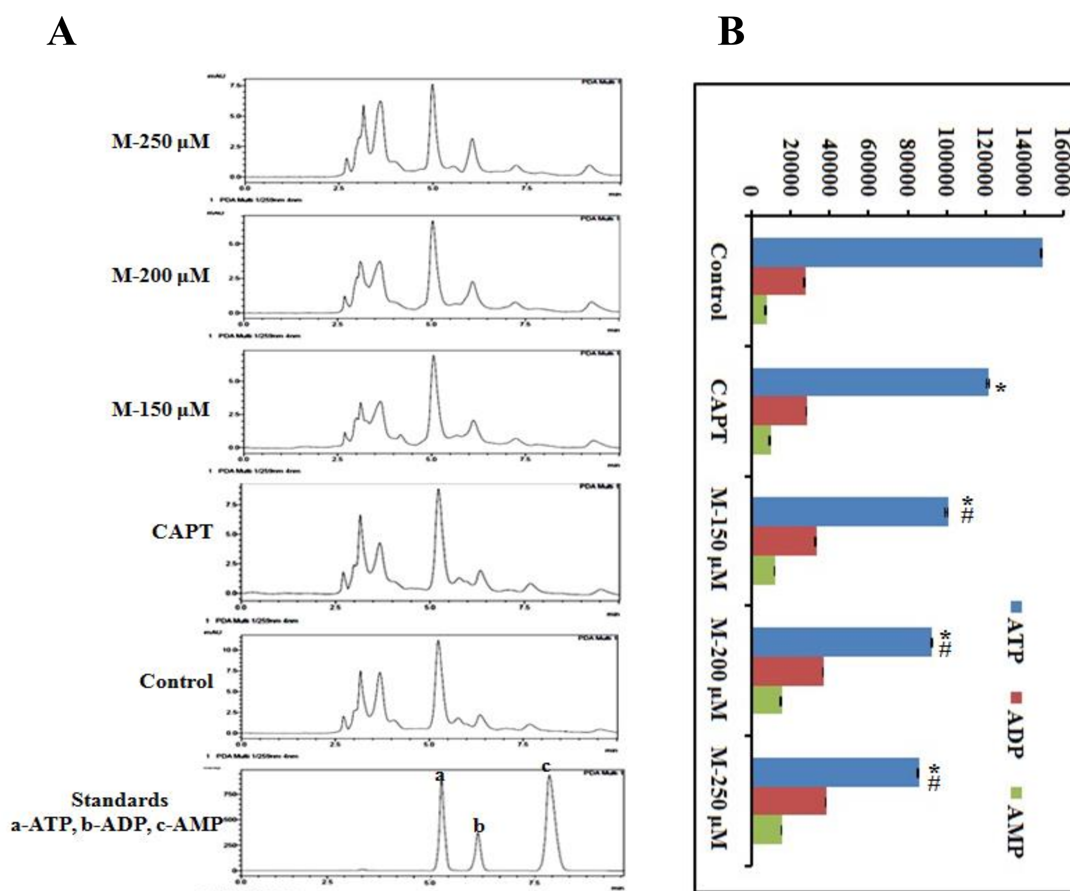


Figure 3.4. Quantification of ATP level by HPLC method. SW480 cells were treated with various concentrations of morin (150, 200, and 250 µM) and camptothecin (50 µM) for 48 h. Morin treatment significantly decreases mitochondrial capacity to produce ATP. (A) Representative HPLC chromatogram showing decrease in ATP production in SW480 cells on treatment morin. (B) Graphical representation of reduction in ATP production by morin in SW480 colon cancer cells. Each value represents mean ± SD from triplicate measurements and significance levels between different groups were determined by using one way ANOVA, $p \leq 0.05$ versus Control; #$p \leq 0.05$ versus camptothecin.

3.3.4. Effect of morin on PI3K/Akt signalling decreased glucose uptake through GLUT 1 mediated mechanism

The phosphoinositide 3-kinase (PI3K) pathway is the most frequently actuated signalling pathway in human cancer. Activation of this signalling pathway contributes to cell survival, proliferation and angiogenesis as well as motility, which are responsible for all important aspects of tumorigenesis (Liu et al., 2009). Hence, a number of academic laboratories and pharmaceutical companies are actively involved in research focussing on inhibitors targeting PI3K and other key components in this pathway. The PI3K enzymes activated by diverse stimuli results in converting PIP2 into PIP3 promoting the recruitment and activation of cytosolic proteins, PDK1 and Akt to the plasma membrane and beginning of PI3K/Akt signalling cascades. The activated Akt will further recruit and activate PIP5K and which in turn results in the glucose transport in to the cell (Bunney and Katan, 2010, Samuels et al., 2005).

From our afore said data it was clear that a significant reduction glucose uptake in SW480 colorectal cancer cells on treatment with morin and hence we evaluated impact of morin on PI3K/Akt signaling by checking the expression levels of key proteins involved in PI3K/Akt signaling by western blotting. The obtained results showed a significant reduction in the levels of PI3K, PDK1, p-Akt and PIP5K protein levels on treatment with morin when compared with untreated control cells (**Figure 3.5a, 3.5b, 3.5c, 3.5d**). To confirm the results further, we used specific activator of Akt, SC79. When SW480 cells were treated with SC79 alone, the levels of p-Akt was found to be increased significantly compared to untreated control cells. When SW480 cells were treated with morin along with SC79, we could observe masking of the action of SC79 by morin and resulting in a significant decline in the level of p-Akt (**Figure 3.5e**). This

further confirm that the action of morin is mediated through PI3K/Akt signalling pathway.

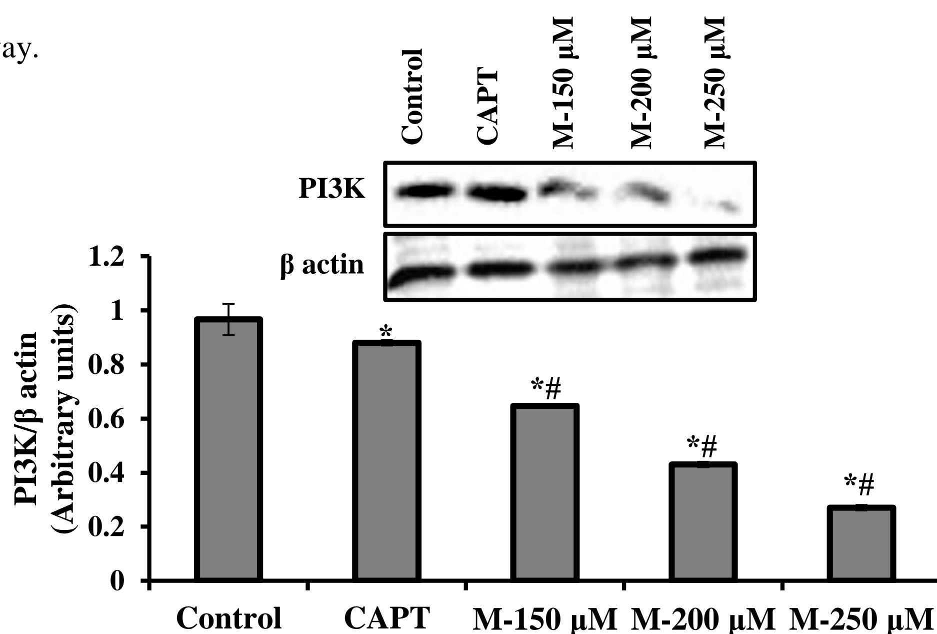


Figure 3.5a. **Effect of morin on PI3K level**. SW480 cells were treated with various concentrations of morin (150, 200 and 250 µM) and camptothecin (50 µM) for 48 h. Western blot analysis for expression of PI3K was carried out. (A) Representative results. (B) The level protein was quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. $*p \leq 0.05$ versus control.

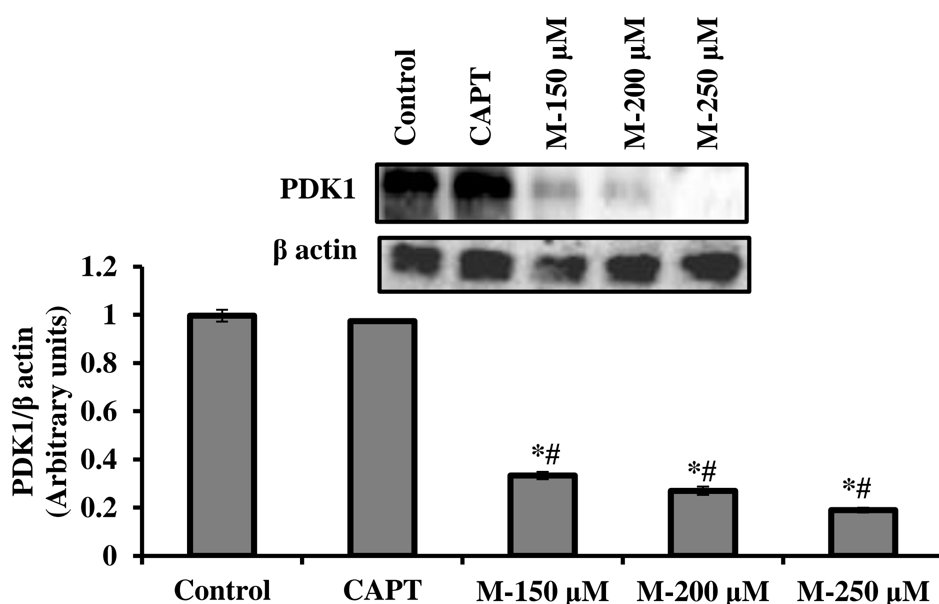


Figure 3.5b. Effect of morin on PDK1 level. SW480 cells were treated with various concentrations of morin (150, 200 and 250 µM) and camptothecin (50 µM) for 48 h. Western blot analysis for expression of PDK1 was carried out. (A) Representative results. (B) The level protein was quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. $*p \leq 0.05$ versus control.

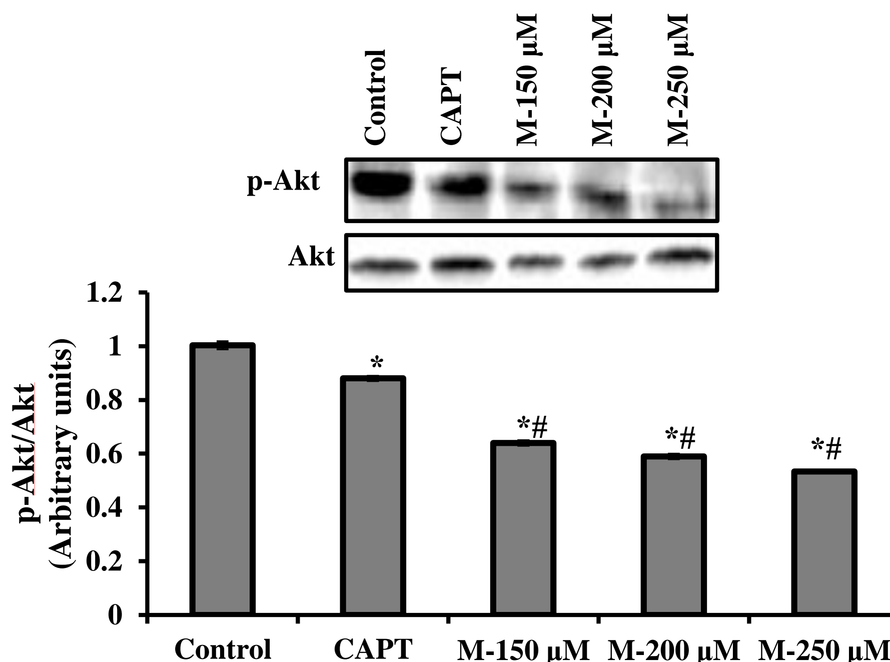


Figure 3.5c. Effect of morin on p-Akt level. SW480 cells were treated with various concentrations of morin (150, 200 and 250 μM) and camptothecin (50 μM) for 48 h. Western blot analysis for expression of p-Akt was carried out. (A) Representative results. (B) The level protein was quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *p ≤ 0.05 versus control.

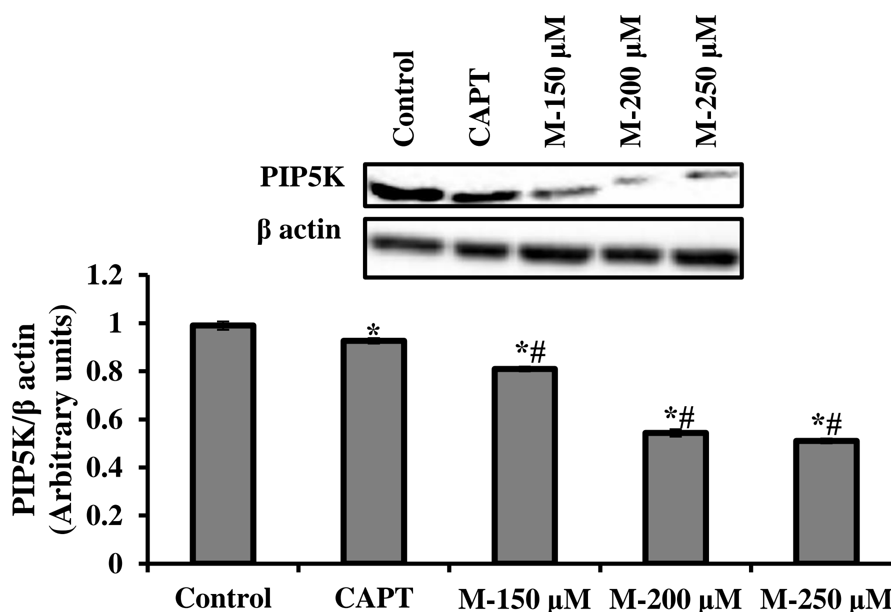


Figure 3.5d. Effect of morin on PIP5K level. SW480 cells were treated with various concentrations of morin (150, 200 and 250 μM) and camptothecin (50 μM) for 48 h. Western blot analysis for expression of PIP5K was carried out. (A) Representative results. (B) The level protein was quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *p ≤ 0.05 versus control.

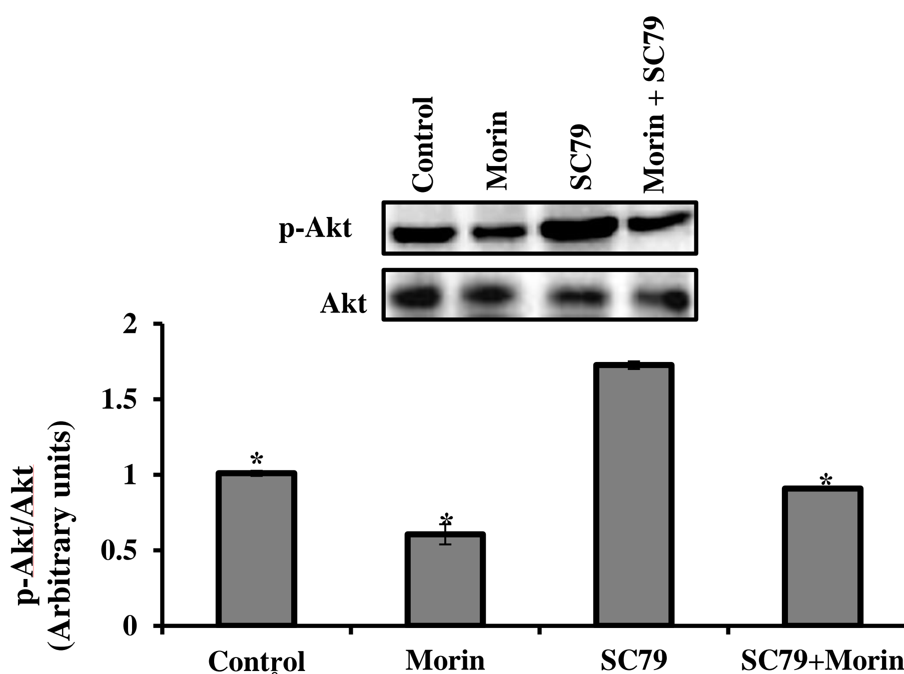


Figure 3.5e. Effect of morin and p-Akt activator (SC79) on p-Akt level. SW480 cells were treated with various concentrations of morin, SC79 and morin along with SC79 for 48 h. Western blot analysis for expression of p-Akt was carried out. (A) Representative results. (B) The level protein was quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

3.3.5. Molecular docking studies

To predict the druggability of morin, we carried out the QikProp analysis (**Table 3.1**) and it was found that morin was more drug like with few #stars value. The molecular weight of morin is below 310 with tolerable number of non-trivial and non-hindered rotatable bonds (#rotor, 0 15). Morin is less toxic to central nervous system and having appreciable range of hydrogen bond donors and acceptors. The human oral absorption value indicates their oral absorptivity and binding to human serum albumin (QPlogKhsa, -1.5 to 1.5) increases its bioavailability, which is further recognized by octanol/water partition coefficient, QPlogPo/w (-2.0 to 6.50) and aqueous solubility, QPlogS (-6.5 to 0.5). Morin is predicted as good candidate as drug, based on suitable ADME/T with minimum violation from Lipinske Rule of Five (Ro5).

Table 3.1. Pharmacokinetic parameters of morin based on QikProp analysis

M.W	#stars	#rotor	CNS	HBA	HBD	QPlogKhsa	HOA	QPlogP o/w	QPlogS	Ro5
302.24	0	5	-2	5.25	4	-0.345	2	0.412	-2.838	0

M.W. (Molecular Weight):130.0 to 725.0; #stars (few stars-more drug-like): 0 to 5; #rotor (Number of non-trivial and non-hindered rotatable bonds):0 to 15; CNS (Central Nervous System activity): -2 to +2; HBA (Hydrogen bond acceptor): 2.0 to 20.0; HBD (hydrogen bond donor): 0.0 to 6.0; QPlogKhsa (binding to human serum albumin): -1.5 to 1.5; HOA (Human Oral Absorption): 1, 2, or 3 for low medium, and high; QPlogPo/w (octanol/water partition coefficient): -2.0 to 6.5; QPlogS (Aqueous solubility): -6.5 to 0.5; Ro5 (Number of violations of Lipinski's rule of five): maximum is 4

In order to envisage the probable binding modes of morin and LY294002 on the selected enzymes, we carried out flexible docking simulations. The crystal structures of human phosphoinositide 3-kinase (PI3K) (PDB ID: 1E8Z) and the human glucose transporter GLUT 1 (PDB ID: 4PYP) were retrieved from RCSB PDB (Protein Data Bank). At first, we carried out the docking simulations of both compounds against 1E8Z and it was found that morin shows better binding affinity with G-Score and D-score of -9.19 and -8.49 kcal/mol. The G-score and D-score of LY294004 was -6.82 and -6.73 kcal/mol (**Table 3.2**). The morin exhibited strong hydrogen bond interaction with the protein 1E8Z due to the presence of hydroxyl groups and these hydroxyl groups are act as H-bond donors as shown in interaction diagrams. The terminal hydroxyl groups of morin interact with Met804 (1.86Å), Asp964 (2.00Å), Tyr867 (1.87Å) and Val 882 (206Å) while the carbonyl oxygen link with Ser806 (2.74Å). In the case of LY294002, due to the lack of hydroxyl groups the binding interactions are somewhat less than morin even though the carbonyl oxygen with Val882 (1.88Å) and π-stacking interaction of benzene ring with Tyr867. Then we tried out the interaction of morin with 4PYP. The hydroxyl groups interact with Asn317 (2.09Å) and Gln283 (2.19Å), the carbonyl oxygen H-bonded with Asn415 (2.20Å) and the benzene is in π-stacking interaction with Phe26. All these forces of interactions together yield

a better binding affinity of G-score -8.25 and D-score -7.99 kcal/mol respectively (**Figure 3.6a, 3.6b, 3.6c, 3.6d, 3.6e, 3.6f**).

Table 3.2. G-score and D-score of morin and PI3k, morin and GLUT 1, LY294002 and PI3K

Compounds	PI3K (1E8Z)			GLUT 1 (4PYP)		
	G-Score	**D-Score**	**Interacting Residues**	**G-Score**	**D-Score**	**Interacting Residues**
Morin	-9.19	-8.49	Met804,Asp964,Tyr 867, Val882, Ser806	-8.25	-7.99	Asn317, Gln283, Asn415, Phe26
LY294002 (PI3K inhibitor)	-6.82	-6.73	Val882, Lys890, Tyr867			

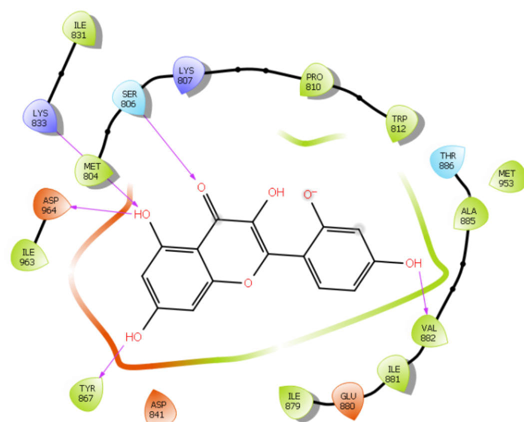


Figure 3.6a. 2D interaction diagram of morin with the protein 1E8Z (PI3K)

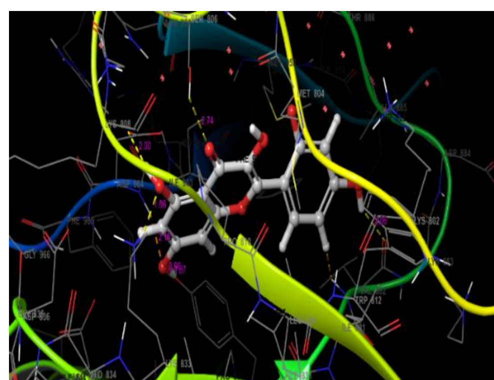


Figure 3.6b. High resolution model of interaction of morin with PI3K

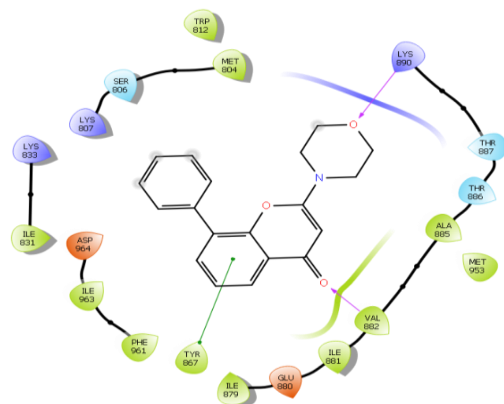


Figure 3.6c. 2D interaction diagram of LY294002 with the protein 1E8Z (PI3K)

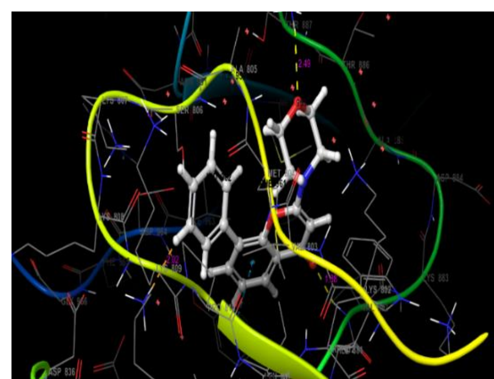


Figure 3.6d. High resolution model of interaction of LY294002 with PI3K

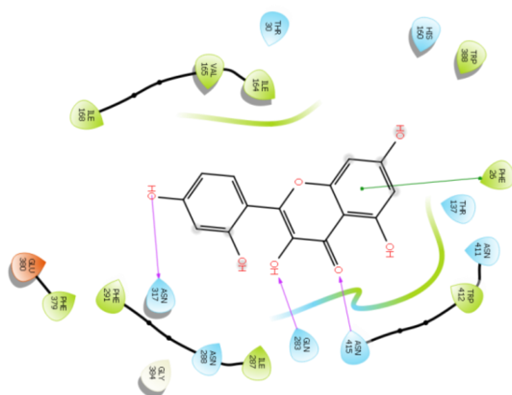


Figure 3.6e. 2D interaction diagram of morin with the protein 4PYP (GLUT 1)

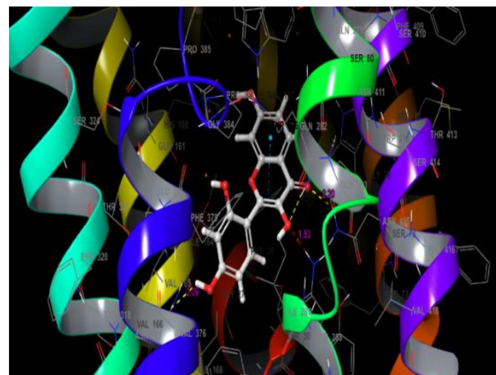


Figure 3.6f. High resolution model of interaction of morin with GLUT 1

3.4. DISCUSSION

Since cancer cells exhibit different metabolism from that of the normal cells, anticancer drugs focussing on this altered metabolism is a current area of anticancer research. Among various altered metabolism, alteration in the glucose metabolism is a major one. Dr. Warburg identified this effect in early 1980s and stated that in comparison with normal cells, which primarily rely on mitochondrial oxidative phosphorylation to generate ATP, aerobic glycolytic activity is greater in cancer cells by increasing the glucose transportation into the cytoplasm and limiting downstream mitochondrial respiration. This is known as Warburg effect and is regarded as a hallmark in cancer therapy (Warburg, 1956). This was further supported by the data obtained from epidemiological studies that the patients having diabetes mellitus are at a considerably higher risk of developing many types of cancers including colorectal cancer (Stein et al., 2010). Clinical study conducted by Meyerhardt et al (Macheda et al., 2005) pointed out that foods with high glycemic load are most strongly associated with higher reappearance of colon cancer. It is also noticed that, hyperglycemia uplifts the levels of blood insulin and levels of free Insulin-like growth factor 1 (IGF1), which can drive proliferative and antiapoptotic signaling pathways in most of the cancer cells (Meyerhardt et al., 2012, Pollak, 2008). In support of this, it was also noticed that the antiproliferative potential of 5-flurouracil (5-FU), a commonly used drug for treating colorectal cancer, was diminished by high glucose treatment indicating that to inhibit the growth of tumour in CRC patients having hyperglycemia, a higher dosage and duration of 5-FU treatment is needed (Pollak, 2008).

We investigated the potential of morin in interfering the Warburg effect using SW480 colon cancer cells in the present study. SW480 colorectal cancer cells were treated with increasing concentrations of morin, followed by NBDG uptake by flow cytometry. We could observe a significant decline in glucose uptake in a dose dependent manner on treatment with morin when compared with untreated control cells, as the concentration of morin was increased. Further to this, the relationship between the decline in glucose uptake with GLUT 1 expression was investigated by western blot analysis. The results clearly indicated a reduction in GLUT 1 expression, as the concentration of morin was increased. In order to understand the impact of both reduced glucose uptake and GLUT 1 expression on energy status of cells, we analysed the cellular ATP level by HPLC analysis. The results clearly tell that the treatment with morin resulted in decline in cellular ATP and inducing an energetic stress. Recent *in vitro* studies reports that the overexpression of transmembrane glucose transporters is the main mechanism that supports the higher glucose entry and further metabolism in carcinoma cells (HAUPTMANN et al., 2005, Rudlowski et al., 2004). Among these, the GLUT 1 is the major transmembrane protein the level of which is found to be increased with many cancers, especially with CRC (Saigusa et al., 2012). This elevated expression of Glut1 (Chung et al., 2009) have been found in CRC stage of poor prognosis and hence GLUT 1 has been regarded as a potential therapeutic target to regulate the glucose uptake by the cells to restrict the proliferative capacity of the CRC cells. The main polyphenol found in green tea, epigallocatechin gallate and quercetin decreased human breast cancer cell line viability and proliferation and found to markedly inhibiting glucose uptake in estrogen receptor (ER) negative MDA-MB-231 and ER positive MCF7 human breast cancer cell lines (Moreira et al., 2013). The glucose uptake as well as GLUT 1 protein levels in MCF-7 cells were found to be

inhibited by kaempferol also (Azevedo et al., 2015). Silybin is found to be blocking glucose uptake and impairing cell viability in cancer cell lines by inhibiting GLUT proteins (Zhan et al., 2011). Together with our early results from Chapter 2, we suggest that morin treatment resulted in impairment in mitochondrial functioning as well as reduced glucose availability/metabolism resulting an energetic stress leading to cancer cells death.

To evaluate the signaling pathway behind this we have checked the expression levels of key signaling molecules in PI3K/Akt signalling by western blotting since most of the cancer cells express elevated PI3K/Akt signalling pathway and inhibition of this pathway can have better effect on anticancer potential. When SW480 cells were treated with morin, it was observed that, the levels of all the key signaling molecules involved in the PI3K/Akt signalling pathway (PI3K, PDK, p-Akt, and PIP5K) were significantly decreased. To confirm the inhibitory potential, we have carried out the activator study. When SW480 cells were treated with morin as mentioned above, the level of p-Akt was significantly reduced, but when treated with p-Akt activator SC79 the levels of p-Akt was increased and it was further supressed when co-treated SC79 with morin. This was further confirmed by docking studies. A study by Aguilera et al., (Aguilera et al., 2016) in KRAS mutant colon cancer cells has shown that by downregulating the glucose transporter GLUT 1, vitamin C could uncouple the Warburg effect. Activation of PI3K/Akt, Ras and c-Myc will increase gene expression of GLUT 1 and which in turn facilitate glucose importation into the cancer cells (Fritz and Fajas, 2010).

Previous studies report that plant derived phytochemicals can modulate Warburg effect and hence the cancer potential. Among several phytochemicals fasentin and genistein target glucose transporter GLUT 1 and exert antitumor effects by inhibiting glucose uptake in tumor cells, thus leading to glucose deprivation facilitated cell death

(Boros et al., 2001). Resveratrol (Gwak et al., 2015), curcumin (Liao et al., 2015) and plumbagin (Sinha et al., 2013) are also reported to down-regulate GLUT 1. Honokiol (Wu et al., 2010) and epigallocatechin gallate (Moreira et al., 2013) have also been reported to alter glucose metabolism leading to anticancer effects. A number of critical enzymes that are involved in aerobic glycolysis, have also been exploited as targets for cancer therapies. Methyl jasmonate acts as a hexokinase inhibitor (Rotem et al., 2005), gossypol inhibits lactate dehydrogenase (Gomez et al., 1997) and cinnamic acid derivatives act as class of monocarboxylate transporter inhibitors (Halestrap and Denton, 1974), are the most studied enzymes involved in aerobic glycolysis. These studies propose that there is immense potential to develop anticancer drugs based on the relationship between aerobic glycolysis and cancer progression.

Since very high glucose uptake and PI3K/Akt activity are the feature associated with cancer cells and the concentrations of morin used in the present study is found to be non-toxic to liver cells (normal cells), the inhibitory potential of morin on glucose uptake and metabolism in colorectal cancer cells can be regarded as an effective strategy towards colorectal cancer prevention and management.

3.5. CONCLUSION

In the present study, the treatment of SW480 colon cancer cells with morin at different concentrations could limit entry of glucose into the cells by decreasing GLUT 1 expression with a decline in cellular ATP level. The inhibition of GLUT 1 expression represents an effective way of preventing cancer progression by blocking its major nutrient supply resulting in reduction of the glycolytic flux that further sensitizes cells to undergo mitochondria dependent apoptosis. Further studies by western blotting as well as docking suggested that alteration of Warburg effect in cancer cells are attributed to the blocking of PI3K/Akt signaling pathway. The main mechanism of

action by which morin exhibit anticancer activity in SW480 colon cancer cells as understood by the present study are related to the pro-oxidant action as well as uncoupling of Warburg effect. These evidences suggest that morin may be a promising therapeutic agent against colorectal cancer.

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CHAPTER 4

Impact of Morin on Inflammatory Status of SW480 Colorectal Cancer Cells

4.1. INTRODUCTION

Obesity, alcohol, chronic infections, radiation, environmental pollutants tobacco and consumption of high-calorie diet have been identified as major risk factors for the most common types of cancer. It is noted that there exists a link between these risk factors and cancer through inflammation. The inflammatory process as one of the predisposing conditions for tumour development was first recognized by Virchow and explained as an "Out of the 'normal' inflammatory hyperplasia" (Balkwill and Mantovani, 2001, Schmidt and Weber, 2006). Compared to acute inflammation that lasts for only short-term and mediates host defense against infections, chronic inflammation that persists for long term and can make host susceptible to several chronic illnesses, including cancer. Tumour extrinsic inflammation increase cancer risk and enhance malignant progression. In contrast, cancer-induced or cancer-intrinsic inflammation contribute to malignant progression through the recruitment and activation of inflammatory cells (Todoric et al., 2016). There exist many example that point towards the link between chronic inflammation and cancer. Approximately 25% increase in colorectal cancer risk due to ulcerative colitis (Loftus, 2006), 14% increase in prostate cancer risk due to prostatitis (Nelson et al., 2004) and 10-20-fold increase in the risk of pancreatic cancer for patients who have experienced pancreatitis has been reported (Farrow et al., 2004). Thus, the presence of inflammation appears to induce or facilitate carcinogenesis.

Phenolic compounds exhibit anticancer activities through modulating multiple carcinogenesis pathways including modulation of oxidative stress, inhibition of cell proliferation, induction of apoptosis, blockade of pro-inflammatory cascades and inhibition of tumour induced angiogenesis, which finally results in the inhibition of

progression and metastasis of cancer (Abdal Dayem et al., 2016, Abaza et al., 2015). Inhibition of inflammation plays a major role among them.

NF-κB is a regarded as a crucial regulator of inflammation and its constitutive activation is seen in various types of cancers including ovarian, breast, colon and prostate cancer. The link between inflammation and cancer is becoming mostly accepted, and the transcription factor NF-κB has appears as a key mediator of this link. Since it plays a comprehensive role in endorsing inflammation-associated cancer, the NF-κB pathway regarded as a promising target for cancer therapy (Li et al., 2005). COX-2 is an intermediate response gene that codes for a cytoplasmic protein, which catalyses the formation of prostaglandins from arachidonic acid. COX-2 expression is triggered by a number of proinflammatory cytokines, including TNF-α, IL-1β, IL-1α etc. and it is increased at sites of inflammation. Similarly, the level of inducible nitric oxide synthase (iNOS), which catalyzes the production of nitric oxide (NO) and NO act as an important signaling molecule mediating inflammation. Inhibition of the level of both COX-2 and iNOS can suppress inflammation and thereby cancer (Herschman et al., 1995).

Thus, inflammation and cancer are reported to have very strong relationship and among various types of cancers colorectal cancer is showing a very close relationship with inflammation. This may be confirmed by the higher risk of colorectal cancer in patients with inflammatory bowel disease (IBD) (McConnell and Yang, 2009). The occurrence of CRC in IBD cases is reported to be up to 60% higher than the general population (Dulai et al., 2016). Hence, it is widely accepted that suppression of inflammation can reduce development of colorectal cancer and once initiated, further worsening of cancer can be prevented by inhibiting inflammation. Thus, suppression of inflammation has a major role in both prevention and management of colorectal

cancer. With this background, the present study focusses on anti-inflammatory potential of morin on SW480 colorectal cancer cells.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Dulbecco's modified eagle's media (DMEM), antibiotic antimycotic mix, MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), morin, lipopolysaccharide (LPS) oligonucleotide primers were procured from Sigma–Aldrich Chemicals (St. Louis, MO, USA), as mentioned in previous chapters. TNF-α, IL-6, IL-1β ELISA kits were obtained from BD Biosciences (San Jose, USA). BCA protein assay kit was procured from Pierce Biotechnology, Rockford, IL, United States. Primary antibodies (p38, p-p38, JNK, p-JNK, ERK, p-ERK) and corresponding secondary antibodies for western blot analysis were purchased from Santa Cruz Biotechnology, USA, Clarity Western ECL substrate was purchased from Biorad, USA, nuclear extraction kit and NF-κB translocation assay kits were procured from Cayman Chemicals, USA, Trizol was obtained from Invitrogen Corp., (Grand Island, NY, USA). LightShift Chemiluminescent EMSA kit and Invitrogen SuperScript VILO cDNAsynthesis kit was purchased from Thermo Fisher, USA. The human colon cancer cells (SW480) were obtained from ATCC (American Type Culture Collection, Manassas, United States) were maintained in DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic mix at 37°C under a humidified 5% CO_2 and 95% air atmosphere.

4.2.2. Cell culture and treatment

The human colon cancer cells (SW480) obtained from ATCC (American Type Culture Collection, Manassas, United States) were maintained in DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic mix at 37°C under a humidified 5% CO_2 and 95% air atmosphere. Cells were exposed to 0.25% trypsin-EDTA and harvested cells

were seeded at a density of 1×10^4 cells/well on 96 well-plates, 24 well-plates and 6-well plates (Costar, United States) for different assays.

4.2.3. Experimental design

The work flow of chapter 4 is schematically represented in the flowchart (**Figure 4.1**)

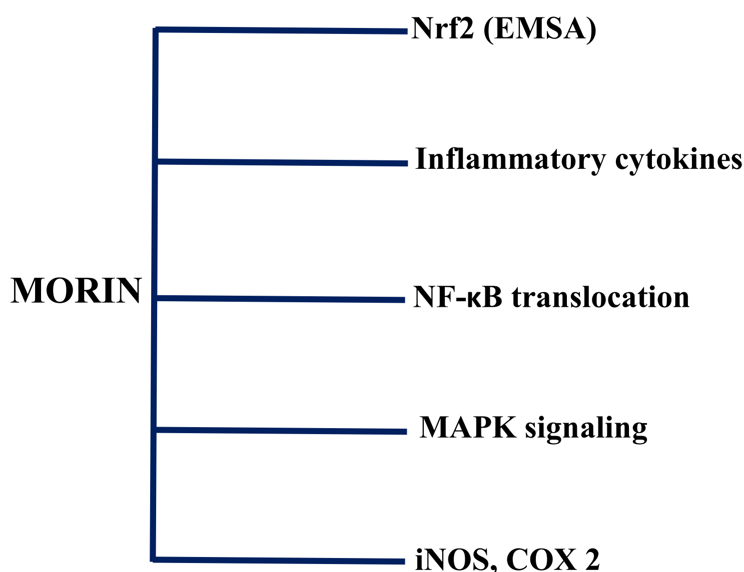

Figure 4.1. Outline of chapter 4

4.2.4.2. Cytotoxicity assay

It was clear from our previous experimental results from previous chapters that morin is inducing cytotoxicity in a time and concentration dependent manner. When SW480 cells were treated with morin for 24 h the cytotoxicity was very less compared to that of 48 h. So for evaluating the anti-inflammatory potential of morin, the cells were exposed to morin for 24 h followed by LPS (100 ng/mL) for another 18 h to induce inflammation. This was followed by MTT assay, as previously described (Mosmann, 1983).

4.2.4.1. Electrophoretic Mobility Shift Assay (EMSA)

DNA-binding activity of Nrf2 in SW480 cells were assessed by EMSA using LightShift Chemiluminescent EMSA Kit. SW480 cell lines were treated with morin for 24 h and additionally with LPS (100 ng/mL) for another 18 h. After incubation, nuclear extracts were prepared using the NEPER nuclear extraction kit. Protein concentration of the nuclear extracts was determined using the BCA kit. ThermoScientific 3′-end biotin labeling kit was used for biotinylating primers for Nrf-2. The nuclear extracts were incubated with the biotinylated probes and components of the LightShift Chemiluminescent kit at 37 °C for 60 minutes, electrophoresed on a 6% non-denaturing gel, transferred to nylon membrane and immobilized by UV cross-linking at 254 nm for 10 minutes. Streptavidin-conjugated HRP was used for visualizing by enhanced chemiluminescence. Specificity of binding was confirmed by competition with excess unlabelled Nrf-2 oligonucleotides (200-fold).

4.2.4.3. Quantification of inflammatory cytokines

SW480 cells were seeded in six well plates (1×10^6 cells/well) and treated with morin (150, 200 & 250 μM) for 24 h followed by LPS (100 ng/mL) for another 18 h. After treatment, the culture medium was harvested, and the chemokine levels (TNF-α, IL-6, IL-1β) in the supernatant was measured using ELISA kits according to the manufacturer's instructions.

4.2.4.4. NF-κ (p65) translocation assay

SW480 cells, after exposing to morin (150, 200 & 250 μM) for 24 h followed by LPS (100 ng/mL), were collected by centrifugation. Nuclear protein was isolated with the help of Cayman's nuclear extraction kit. Each samples at a concentration of 100 μg were added to the wells and incubated for 24 h. All the wells were washed with 200 μL of 1x wash buffer for five times.100 μL NF-κB (p65) primary antibodies were

added to all the wells except blank and incubated at room temperature for 1 h. The wells were washed once again (except blank) with 200 µL of 1x wash buffer for five times. Secondary antibody (100 µL, diluted goat anti-rabbit antibody) was added after the wash and incubated at room temperature for 1 h, washed again with 1x wash buffer. 100 µL developing solution was added to all the wells, and incubated for 30 min with gentle agitation. At the end of 30 min, 100 µL of stop solution was added to all the wells and absorbance was measured at 450 nm.

4.2.4.5. Western blotting

Western blotting was done to check the expression level of proteins namely, p38, p-p38, JNK, p-JNK, ERK, p-ERK. SW480 cells lines treated with morin (24 h) followed by LPS (100 ng/mL) (18 h) were washed with cold PBS, lysed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 0.5 mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100 and protease inhibitor cocktail, pH 8.0) for 30 min on ice and were centrifuged at 12000 x g for 10 min. The protein content of the lysate was measured using BCA protein assay kit. Lysates were diluted to an equal concentration of total protein and the supernatants were then stored at -80ºC until analysis. These samples were boiled for 10 min at 75°C in reducing sample buffer (62.5 mM Tris-HCl pH6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). The lysate containing 50 µg of protein was subjected to SDS-PAGE on 12% gel and transferred onto a polyvinylidene difluoride membrane (Immobilon P^{TM}, Millipore®, USA) by using Trans-Blot Turbo^{TM} transfer system (Bio-Rad Laboratories, Germany). The membranes were blocked by incubating in blocking buffer (5% skim milk in PBST, PBST-PBS buffer containing 0.1% Tween 20), for 1 h at room temperature, washed three times with PBST and probed over night at 4°C with appropriate phospho-specific or pan-specific antibodies against p38, p-p38, JNK, p-JNK, ERK, p-ERK

(each at 1: 500). Membranes were washed 3 times with PBST and incubated for 1 h at room temperature with horse radish peroxidase (HRP) conjugated secondary antibody at 1:1000 dilution and again washed three times in PBST. The bound antibodies were detected using an enhanced chemiluminescence substrate (Biorad, USA) and measured by densitometry using a Chemi Doc XRS digital imaging system and the Multi Analyst software from Bio-Rad Laboratories (USA).

4.2.4.6. Total RNA isolation and quantitative real time PCR analysis

SW480 cells lines were treated with morin for 24 h and additionally with LPS (100 ng/mL) for another 18 h. After incubation, using Trizol, total RNA was isolated according to the manufacturer's protocol (Invitrogen Corp., Grand Island, NY, USA). Reverse transcription was carried out with 1 µg RNA using SuperScript VILO cDNA synthesis kit. The primer sequences used were as given below

Genes	**Oligonucleotide primers**
COX-2	forward 5′-ACTCACTCAGTTTGTTGAATCATTC-3′,
	reverse 5′-TTTGATTAGTACTGTAGGGTTAATG-3′,
iNOS	forward 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′
	reverse 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′

4.2.4.7. Statistical analysis

Results were expressed as mean ± SD (standard deviation) from three independent experiments. The differences between treatments in comparison with control were assessed using one-way ANOVA and the significance of differences between means was calculated by Duncan's multiple range test, using SPSS for Windows, standard version 16 (SPSS, Inc.), and significance was accepted at $p \leq 0.05$.

4.3. RESULTS

4.3.1. Combined effect of morin and LPS on cell viability

From our previous data, it was clear that when SW480 cells were exposed to morin for 48 h, the viability declined significantly as compared to 24 h of incubation. Therefore, in order to study the protective effect of morin against inflammation, the cells were exposed to morin (10 µM to 400 µM) for 24 h before inducing inflammation with LPS (100 ng/mL, 18 h). The results of MTT assay clearly demonstrated that there was not much difference in viability of cells when cells were treated with morin prior to LPS treatment **(Figure 4.2).** Since the pre-treatment of the cells with morin before exposure to LPS did not affect cell proliferation to a larger extent, 150, 200 and 250 µM morin for 24 h followed by 100 ng/mL LPS for 18 h was employed for further studies to understand the effects of morin in suppressing inflammation.

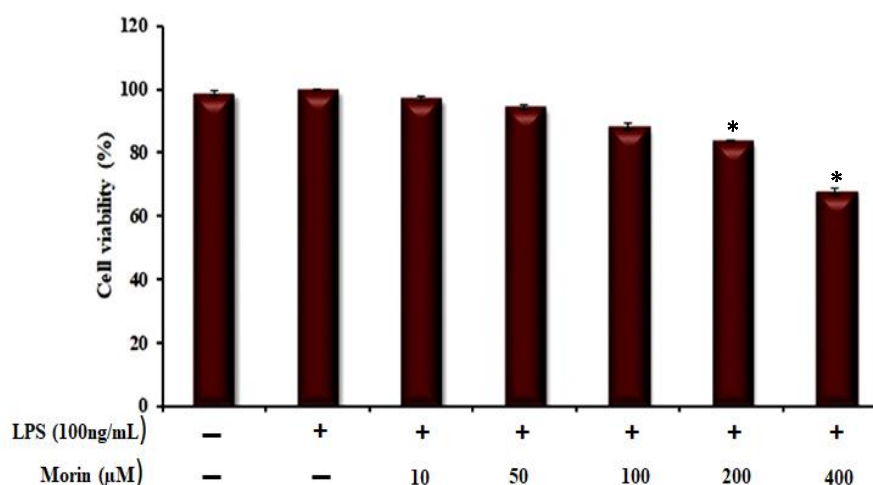

Figure 4.2. Effect of LPS and morin on Viability of SW480 cells by MTT assay. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. Cell viability was measured using MTT assay. Results are shown as percentage reduction in cell viability of treated cells compared to untreated control cells. Values shown are the means ± SD obtained from three independent experiments. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

4.3.2. Effect of morin on nuclear levels of Nrf2

Nrf2 is a transcription factor regulating the expression of antioxidant proteins and it protects cancer cells from the damages induced by ROS and anticancer drugs (Sporn and Liby, 2012) and a pivotal relationship exists between chemo-resistance and Nrf2. So, concurrent down-regulation of Nrf2 and induction of ROS in cancer cells can result in induction of apoptosis. In order to determine whether morin is having any impact on Nrf2 activity under inflammatory conditions, SW480 cells were exposed to morin (150, 200 and 250 µM) for 24 h followed by LPS (100 ng/mL) for another 18 h to induce inflammation. At the end of the incubation time, activation status of Nrf2 was assessed by EMSA using nuclear extracts from SW480 cells (**Figure 4.3**). As can be seen, the nuclear level of Nrf2 was increased on treatment with LPS alone compared to the control. When cells were exposed to morin before exposing to LPS, the expression was significantly less compared with LPS alone treated group.

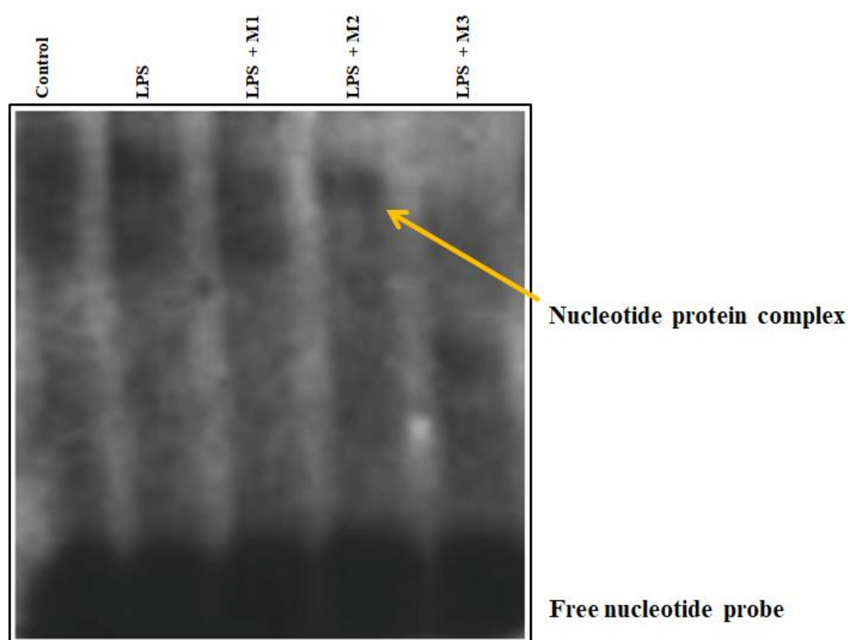

Figure 4.3. Effect of morin on Nrf2 in SW480 cells using EMSA. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. EMSA was performed for evaluating the effect of morin on nuclear translocation of Nrf2.

4.3.3. Inhibitory effects of morin on generation of inflammatory cytokines in SW480 cells

The level of pro-inflammatory cytokines such as TNF-α, IL-1 β and IL-6 are increased during the progress of inflammatory diseases that play central roles in response towards inflammatory insult in cells. In the case of colon cancer, these mediators produced during chronic inflammation are coordinated through different molecular signaling pathways and results in the formation of a microenvironment that further augment of tumorigenesis. In the present study, SW480 cells were exposed to morin (150, 200 and 250 µM) for 24 h followed by LPS (100 ng/mL) for another 18 h to induce inflammation and the level of cytokines in culture supernatants was analysed by ELISA technique. The level of all the three pro-inflammatory cytokines (TNF-α, IL-1 β and IL-6) were upregulated following the treatment with LPS compared to the control. The expression of pro-inflammatory cytokines reduced significantly when cells were pre-treated with morin ie, before treating with LPS, compared to cells without morin pre-treatment (LPS alone treated group) **(Figure 4.4a, 4.4b, 4.4c)**.

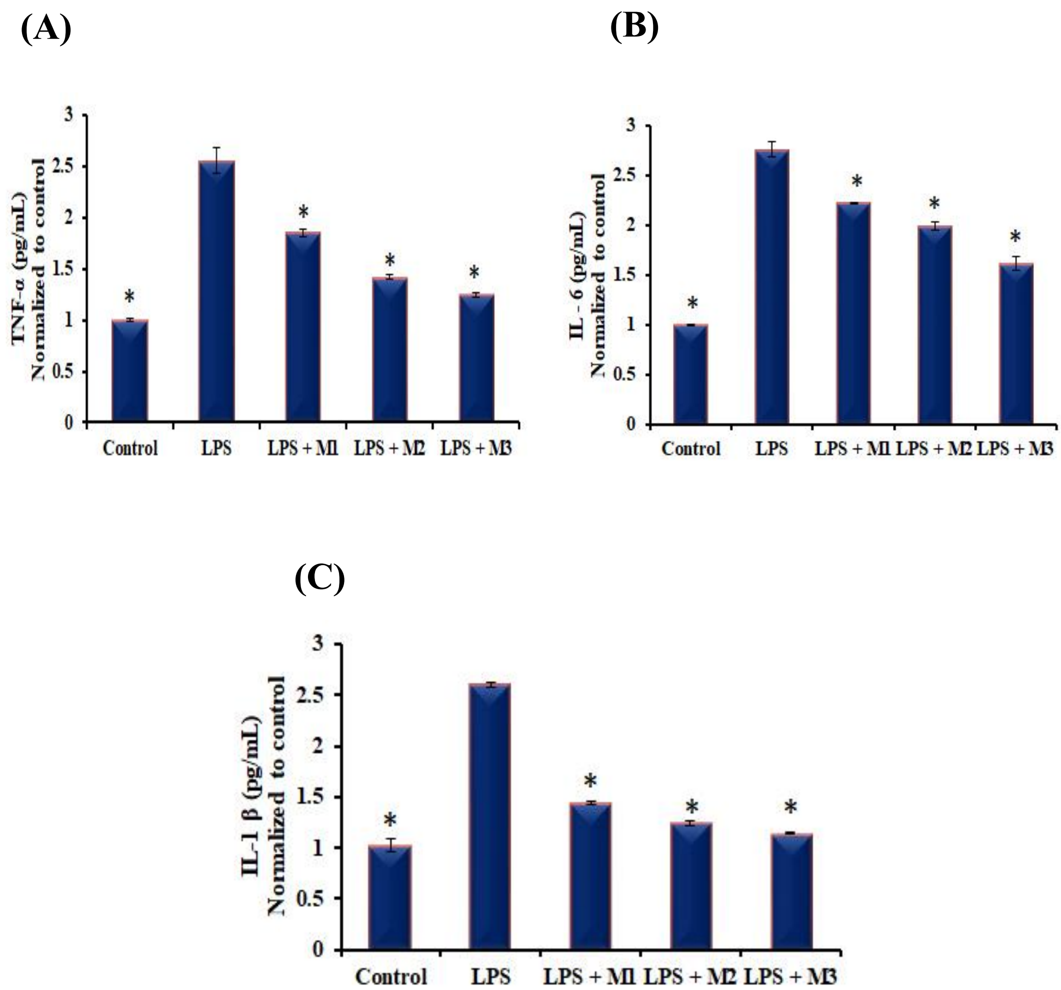

Figure 4.4. Effect of LPS and morin on generation of pro-inflammatory cytokines in SW480 cells. (A) TNF-α level, (B) IL-6 level and (C) IL-1 β level. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. Cytokine levels were determined by ELISA. Values shown are the means ± SD obtained from three independent experiments. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus LPS alone treated group.

4.3.4. Effect of morin on LPS induced nuclear translocation of NF-κB (p65)

NF-κB (p65) is regarded as one of the major transcription factors in inflammatory pathways. LPS binds to toll-like receptor 4 (TLR4) and activates NF-κB pathway which in turn activates a series of factors. NF-κB (p65) transcription factor assay, is a non-radioactive sensitive technique for detecting specific transcription factor DNA binding activity in nuclear extracts. NF-κB present in the nuclear extract, binds specifically to the NF-κB response element. The effect of morin in NF-κB translocation was studied by exposing SW480 cells for morin (150, 200 and 250 μM) for 24 h and LPS (100 ng/mL) for a further 18 h. Pre-treatment with LPS increased the level of NF-κB (p65) nuclear fraction compared with control group. Morin treatment inhibited the LPS induced nuclear translocation of NF-κB (p65) and increased the level of p65 in the cytoplasmic fraction. **(Figure 4.5)**.

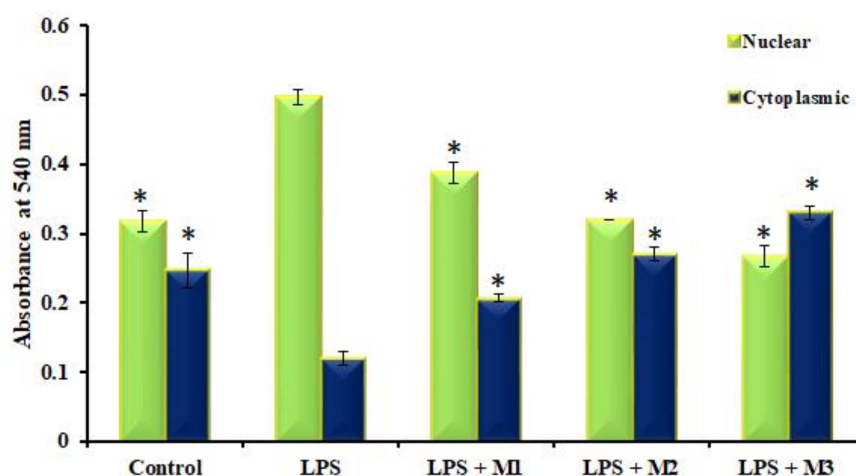

Figure 4.5. Effect of LPS and morin on NF-kB translocation in SW480 cells. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. Nuclear translocation of NF-κB was determined by NF-κB translocation assay. Values shown are the means ± SD obtained from three independent experiments. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus LPS alone treated group.

4.3.5. Effect of morin on MAPK signalling pathway

It is reported that chronic inflammation upsurges the risk of progress of several types of malignancies including colon cancer. Many cancers show resistance to anticancer drugs and this resistance has been associated with increased activity of mitogen-activated protein kinase (MAPK) signalling, in particular p38 MAPK. They are mainly involved in the regulation of the synthesis of mediators of inflammation and is the one of potential targets for anti-inflammatory therapeutics that can fight against cancer (Kaminska, 2005, Grossi et al., 2014). To confirm the anti-inflammatory pathway of morin, SW480 cells were pre-treated with morin (150, 200 and 250 μM) for 24 h followed by LPS (100 ng/mL) for a further 18 h and immunoblotting study was done to evaluate the expressions of p38, p-p38, JNK, p-JNK, ERK and p-ERK **(Figure 4.6a, 4.6b, 4.6c)**. Protein expression studies confirmed that the level of p- p38, p- JNK and p-ERK were upregulated on treatment with LPS. Morin pre- treatment prior to LPS exposure could significantly inhibit LPS induced phosphorylation of p38, JNK and ERK

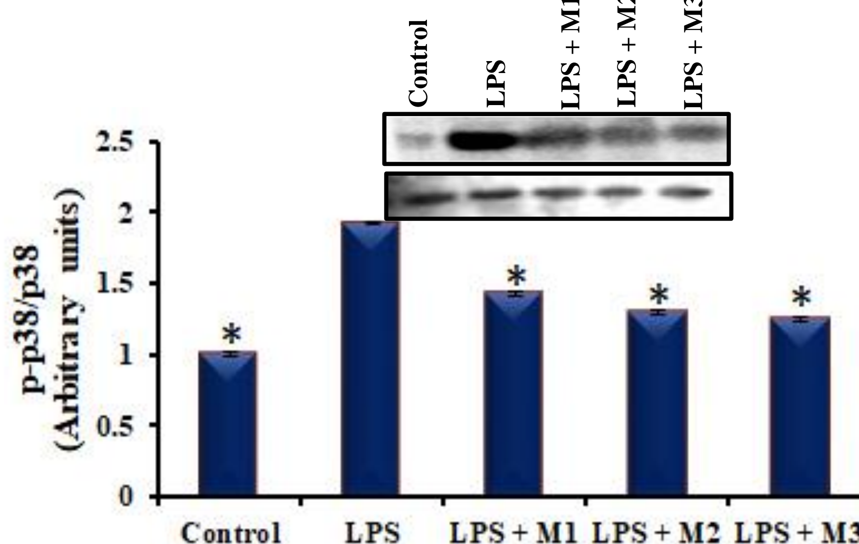

Figure 4.6a. Effect of LPS and morin on p-p38 levels in SW480 cells. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. Western blot analysis for expression of p-p38 was carried out. Values shown are the means ± SD obtained from three independent experiments. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *p ≤ 0.05 versus LPS alone treated group.

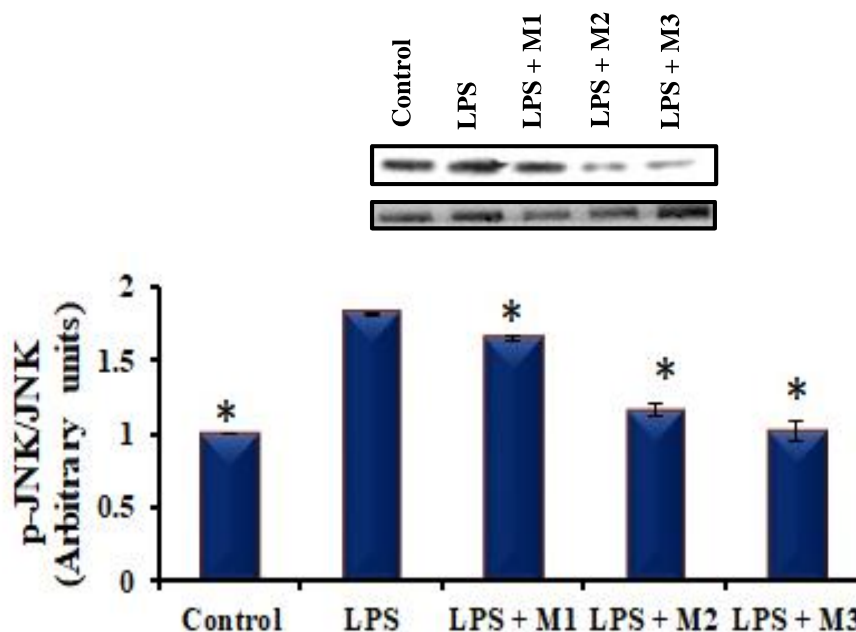

Figure 4.6b. Effect of LPS and morin on p-JNK levels in SW480 cells. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. Western blot analysis for expression of p-JNK was carried out. Values shown are the means ± SD obtained from three independent experiments. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus LPS alone treated group.

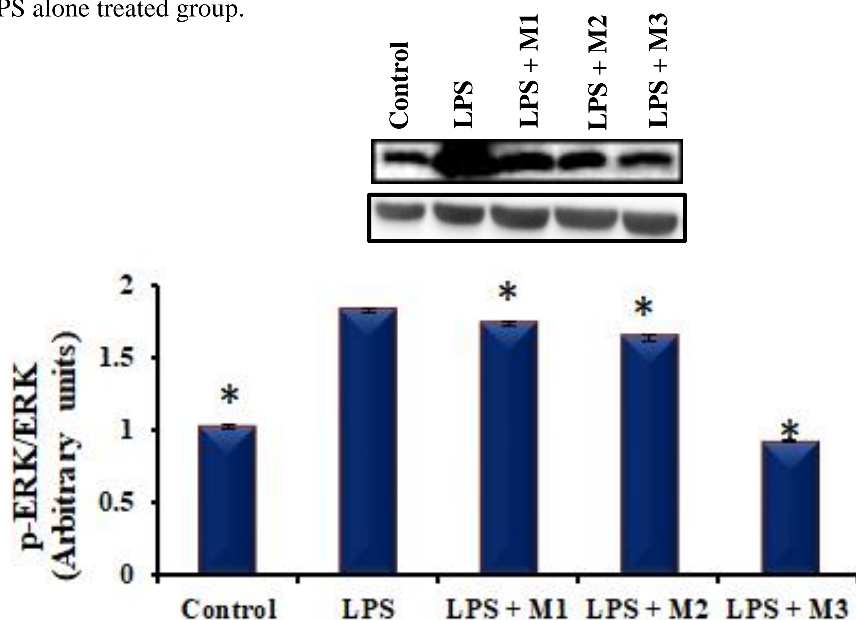

Figure 4.6c. Effect of LPS and morin on p-ERK levels in SW480 cells. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. Western blot analysis for expression of p-ERK was carried out. Values shown are the means ± SD obtained from three independent experiments. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus LPS alone treated group.

4.3.6. Impact of morin on iNOS and COX-2 gene expression

The inducible isoform of nitric oxide synthase (NOS), iNOS produces nitric oxide (NO) and is a major downstream mediator of inflammation. It has been generally reported that iNOS is involved in chronic inflammation, generates a microenvironment conducive for colon carcinogenesis (Watanabe et al., 2000). Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase (PTGS), is an enzyme accountable for the formation of prostanoids. Several studies indicate that nonsteroidal anti-inflammatory drugs that inhibit the cyclooxygenase (COX) enzyme were shown to inhibit the development of colon cancer in animal models of carcinogenesis (Sano et al., 1995). In the present study, in order to evaluate the effect of morin on iNOS and COX-2 in inflammatory pathway, SW480 cells were treated with morin (150, 200 and 250 μM) for 24 h and LPS (100 ng/mL) for a further 18 h and iNOS and COX-2 mRNA levels were examined. The mRNA level of iNOS and COX-2 were significantly increased in LPS treated cells compared to control. The pretreatment with morin could significantly downregulate the expression of iNOS and COX-2 induced by LPS challenge. (**Figure 4.7a, 4.7b**).

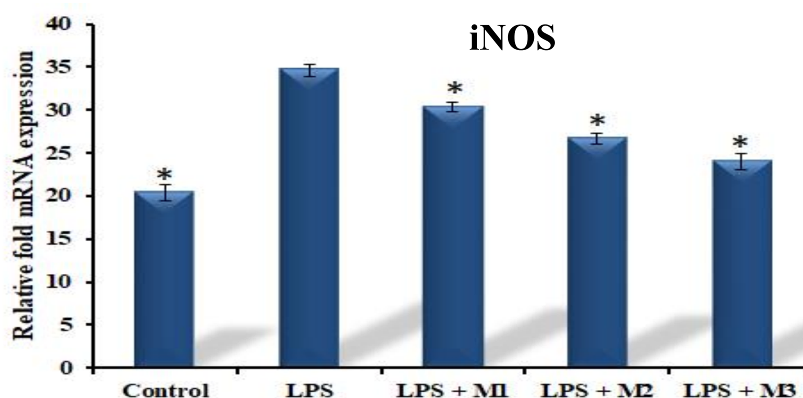

Figure 4.7a. Effect of LPS and morin on iNOS expression in SW480 cells. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. PCR analysis for expression of iNOS was carried out. Values shown are the means ± SD obtained from three independent experiments. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *p ≤ 0.05 versus LPS alone treated group.

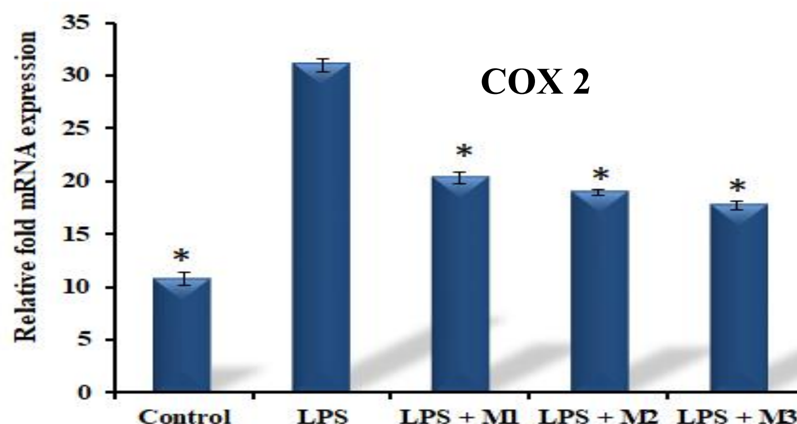

Figure 4.7b. Effect of LPS and morin on COX-2 expression in SW480 cells. SW480 cells were treated with morin for 24 h and then cells were further treated with LPS for 18 h to induce inflammation. PCR analysis for expression of COX-2 was carried out. Values shown are the means ± SD obtained from three independent experiments. Statistical significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *p ≤ 0.05 versus LPS alone treated group.

4.4. DISCUSSION

Inflammation and cancer are reported to have very strong relationship and among various types of cancers colorectal cancer is showing a very close relationship with inflammation. This may be confirmed by the higher risk of colorectal cancer in patients with inflammatory bowel disease (IBD) (McConnell and Yang, 2009). Compared to normal cells, cancer cells usually have higher reactive oxygen species (ROS) levels and can tolerate higher ROS activity (Sporn and Liby, 2012). It has been established that further increasing ROS exposure induced by ROS-generating agents can deplete the cellular antioxidant capacity and thus induce apoptosis in tumour cells. In line with this, as discussed in chapter two, it was observed that when SW480 colon cancer cells were treated with different concentrations of morin, it induced formation of reactive oxygen species (ROS) in the cells. The formation of ROS led to the disruption in the mitochondrial functioning resulting in the extrinsic as well as intrinsic pathway of apoptosis (Sithara et al., 2017, Sporn and Liby, 2012, Du et al., 2008).

Nrf2 is a chemo-resistant anti-apoptotic transcription factor induced by ROS with robust antioxidant efficacy that plays a vital role in activating antioxidant response that protects cancer cells from the ROS induced damages as well as anticancer drugs (Sporn and Liby, 2012). NF-κB is an another major chemo-resistance related anti-apoptotic factor and malignant cells with high levels of constitutive NF-κB activity show resistance to apoptosis (Jung et al., 2012). Nrf2 and NF-κB are usually activated during inflammation that can increase the chemo resistance of cancer cells against various anticancer approaches. Therefore, in cancer cells, agents that can simultaneously induce ROS formation and down-regulate of Nrf2 and NF-κB can result in induction of higher rate of apoptosis and are regarded as attractive molecular targets in therapeutic intervention.

Hence in the present study we have evaluated the effect of pre-treatment with morin on SW480 cells against LPS induced inflammation via analysing Nrf2 expression by EMSA. It was found that, when SW480 cells were exposed to LPS alone the nuclear level of Nrf2 was increased significantly compared with untreated control cells. On pre-treatment with morin prior to inflammation induction by LPS, the LPS induced nuclear translocation of Nrf2 was found to be significantly suppressed. The nuclear level of NF-κB when evaluated by nuclear translocation assay also showed reduction in LPS induced nuclear translocation of NF-κB on pre-treatment with morin, in significant amount.

There are three major MAPK cascades in humans are c-Jun N-terminal kinase (JNK) extracellular signal-regulated kinase (ERK1/2) and p38 MAPK. The chief inflammatory signaling highways from the cell surface to the nucleus is mediated by MAP kinases (Dong et al., 2002). Mounting evidence suggests that MAPK pathway plays a key role in the development and progression of cancer (Khavari and Rinn,

2007) by enhancing cell proliferation, migration, senescence of cancer cells (Yang et al., 2013). It is reported that the JNK pathway can regulate apoptosis-related proteins, including Bax and Bcl-2 (Lei and Davis, 2003). The effect of p38 MAPK signaling is various and some studies report that p38 MAPK promotes cell growth and survival (Yosimichi et al., 2001) and ERK activation is associated with the oncogenic nature, pathogenesis and progression of colorectal cancer and breast cancer (Adjei, 2005, Santen et al., 2002). Therefore, in the present study we have evaluated the expression levels of key signaling molecules in MAPK pathway by western blotting and the achieved results show that when SW480 cells when treated with LPS, the levels of p-ERK, p-JNK and p-p38 were increased and pre-treatment with morin could significantly suppress the LPS induced phosphorylation of ERK, JNK and p-38.

The MAP kinases has been reported to activate NF-κB signalling and the consequent expression of pro-inflammatory cytokines (Sun et al., 2016). Inflammatory cytokines support growth of tumour cells, disturb their differentiation and support of cancer cell survival. Hence pro-inflammatory cytokines, or transcription factors that are essential for signaling by these cytokines, including NF-κB, are indeed evolving as impending targets for anticancer therapy (Lin and Karin, 2007). Out of the pro-inflammatory cytokines, interleukin-6 is involved in the development of sporadic CRC and colitis associated cancer (CAC) (Waldner et al., 2012) and higher levels of serum IL-6 is associated with larger tumour size, liver metastases and decreased survival (Chung and Chang, 2003). TNF expression found to be higher in CRC tissues and elevated serum level of TNF directly correlate with CRC progression and diminished patient survival (Stanilov et al., 2014). IL-1β also reported as supporting cancer progression (Bunt et al., 2006). In the present study, the levels of chief pro-inflammatory cytokines like TNF-α, IL-6 and IL-1β were checked by ELISA method and found that, on exposure

of SW480 cells with LPS, the levels of all the three pro inflammatory cytokines were amplified. The levels of these were down regulated on pre-treatment of cells with morin before inducing inflammation with LPS.

iNOS and COX-2 are regarded as the major pro-inflammatory genes and studies have reported that IL-1β and TNF-α induce the production of iNOS and COX-2, thereby increasing the amounts of their products (El Mansouri et al., 2011). During the course of chronic inflammation, their long term dysfunction is observed leading to various pathological conditions including cancer (Chung et al., 2009). Thus inhibiting these pro-inflammatory mediators is regarded an effective approach for developing anti-inflammatory therapeutic compounds. In our study, we observed the expression of iNOS and COX-2 were significantly elevated when SW480 cells were treated with LPS and which was very effectively inhibited when SW480 cells were pre-treated with morin prior to LPS exposure.

It was demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) have anti-colon cancer effects (Kune et al., 1988). Many clinical trials have indicated that long-term use of aspirin or other NSAIDs drop the incidence of colorectal cancer (Wang and DuBois, 2006). Specific COX-2 inhibitors and corticosteroids are also known to exhibit anti-inflammatory and anti-cancer activity when used alone or in combination with chemotherapeutic agents. The most abundant polyphenol in green tea, (-)-Epigallocatechin-3-gallate (EGCG) and the cysteine metabolites of EGCG were examined by Lambert et al (Lambert et al., 2010) for their growth inhibitory, pro-oxidant and anti-inflammatory activities. Both compounds dose-dependently inhibited the growth of colon cancer cells, prevented aberrant arachidonic acid release and nitric oxide production by lipopolysaccharide-stimulated RAW264.7 cells. Lycopene inhibited the protein expression of NF-κB and JNK levels, that lead to reduced

production of pro-inflammatory cytokines and also inhibited expression of COX-2 and iNOS mRNA with subsequent inhibition of PGE2 and NO production in LPS exposed SW480 colorectal cancer cells (Cha et al., 2017).

4.5. CONCLUSION

As there exists a close association between colorectal cancer and inflammation, in this chapter we have evaluated anti-inflammatory potential of morin in LPS stimulated SW480 colorectal cancer cells. Treatment with morin could reduce the nuclear levels of Nrf2 a, major chemo-resistance factor against ROS induced apoptosis in cancer cells. NF-κB is usually activated during inflammation and which also will increase the chemo resistance of cancer cells against various anticancer treatments. In the present study, the NF-κB nuclear translocation assay showed that LPS induced nuclear translocation of NF-κB was inhibited when SW480 cells were treated with morin.

Because of cross-talk between different pathway, cancers are caused by dysregulation of multiple pathways and hence, agents that can suppress more than one pathway are likely to be more effective against cancer. As inflammatory pathways play critical role in both prevention and therapy of cancer and MAP kinases has been reported to activate NF-κB signaling, next we have evaluated impact of morin on MAPK signaling pathway and found that morin could significantly suppress MAPK signaling pathway. As cytokines secreted by the activated tumour stroma modulate tumour growth and enhance invasiveness of tumour cells by activation of oncogenic signaling pathways in tumour cells, including activation of NF-κB by TNFα and IL-1β, and activation of STAT3 by IL-6. Also, iNOS and COX-2 are the major pro-inflammatory genes and studies have reported that IL-1β and TNF-α induce the production of iNOS and COX-2, we have also checked the levels of major inflammatory cytokine levels and iNOS and COX-2 expression and found that morin could significantly reduce secretion these

inflammatory cytokines as well as iNOS and COX-2 expression. In short, treatment with morin can bring about anti-inflammatory activity and which can inhibit development as well as further worsening of colorectal cancer conditions, if once it has been started.

4.6. REFERENCE

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CHAPTER 5

Effect of Morin on Metastasis of SW480 Colorectal Cancer Cells

5.1. INTRIDUCTION

Metastasis refers to spreading of cancer cells from their primary location to another region of the body either through the bloodstream or lymphatic vessels or locally. Even though cancers can metastasize to nearly any regions of the body, lungs, bones, liver and brain are most common sites of metastasis (Seyfried and Huysentruyt, 2013). The capacity to metastasize is one main characteristic that differentiates malignant (cancerous) tumours from benign (non-cancerous) tumours. The treatment for cancer vary with the point of origin of cancer and the region to which it has spread. Hence understanding metastasis is a critical area of cancer research, as it is responsible for roughly 90% of the cancer deaths (Chaffer and Weinberg, 2011).

It is reported that more than half of the CRC patients are prone to develop metastatic cancer (Ferlay et al., 2010) and metastasis is the major cause of death among CRC patients (Siegel et al., 2012). Thus, research focussing on anti-metastatic agents is important for CRC prevention and therapy. In the early stage of metastasis, intravasation and migration occur when the extracellular matrix (ECM), is degraded by matrix metalloproteinases (MMPs) (Valastyan and Weinberg, 2011). After intravasation and migration, cancer cells will be released from the original tumour organ and enter the circulation. MMP-2/−9 play important roles in degrading ECM and its involvement is complex in the process of cancer metastasis (Chen et al., 2013, Gialeli et al., 2011). Studies have been reported that several approaches that block and modify the actin cytoskeleton, the major structural protein network, can bring about great therapeutic potential against cancer cell invasion, migration and metastasis suppression (Fife et al., 2014). There exists a number of cellular signalling pathways related with CRC metastasis, including Wnt/β-catenin, TGF-β/Smad, p53, Notch, VEGF, JAKs/STAT3 signaling pathways. Modulation of these signaling pathways

also have regarded as a novel approach toward colorectal cancer therapy. With this background, the present study examined the anti-metastatic potential of morin on SW480 colorectal cancer cells.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Morin, antibiotic antimycotic mix, DMEM, fluorescein isothiocyanate labeled phalloidin, Coomassie Blue, Triton X-100, gelatin and crystal violet were purchased from Sigma-Aldrich Chemicals (St Louis, MO, U.S.A.). Foetal bovine serum (FBS) was obtained from Gibco-BRL (Auckland, New Zealand). Primary antibodies (β actin, MMP-2, MMP-7, MMP-9, β-catenin, cyclin D1 and c-myc) and corresponding secondary antibodies were purchased from Santa Cruz Biotechnology, United States. Clarity Western ECL substrate was procured from Bio-rad, United States. All other chemicals used were of the standard analytical grade.

5.2.2. Cell culture and treatment

The human colon cancer cells (SW480), obtained from ATCC (American Type Culture Collection, Manassas, United States) were maintained in DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic mix at 37°C under a humidified 5% CO_2 and 95% air atmosphere. Cells were exposed to 0.25% trypsin-EDTA and harvested cells were seeded at a density of 1×10^4 cells/well on 24 well-plates and 6-well plates (Costar, United States) for different assays.

5.2.3. Experimental design

The work flow in this chapter is schematically represented as in **Figure. 5.1**

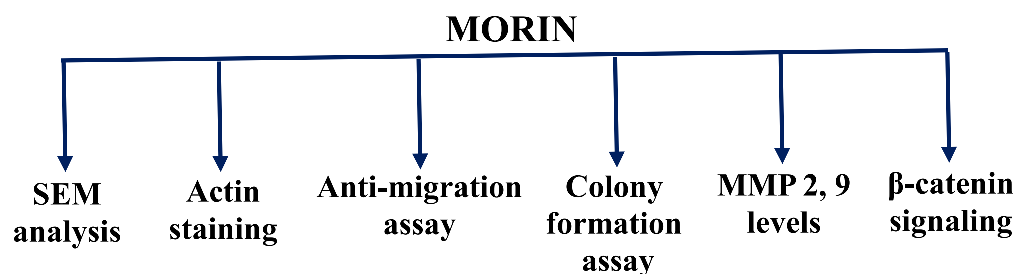

Figure 5.1. Outline of chapter 5

5.2.4.1. Scanning electron microscope (SEM) observation

The morphological variations on treatment with morin were observed using SEM. SW480 cells were treated with morin (150, 200 & 250 µM) and camptothecin (50 µM) for 48 h and cells in each group were fixed in phosphate-buffered 2.5% glutaraldehyde solution. After that cells were rinsed in PBS three times and dehydrated by ascending ethanol series (50,70, 80, 90, 95 and 100%) and samples were stuck on to Aluminum stub with the help of a carbon tape, sputter coated with gold–palladium to render them electrically conductive by using HUMMLE VII Sputter Coating Device (Anatech Electronics, Garfield, N.J., USA). The micrographs were taken using JEOL JSM-5600LV (Italy) at magnification of 1500× and 5000×.

5.2.4.2. Microfilament analysis

SW480 cells were pretreated with different concentrations of morin (150, 200 & 250 µM). After treatment, cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at 37°C, permeabilized with 0.1% Triton X-100 in PBS for 7 min and blocked with 1% BSA in PBS for 1 h at 37°C. Between each step, cells were washed three times with PBS. After blocking, cells were stained with 5 µg/mL FITC-phalloidine for 1 h at 37°C in the dark (Zhang et al., 2012). Images were obtained by fluorescent

microscope (Pathway 855, BD Bioscience, USA) equipped with filters in the FITC range (Excitation, 490 nm; and Emission, 525 nm). Camptothecin (50 µM, 48 h incubation) was the positive control.

5.2.4.3. Anti-migration assay

Cell migration was measured using a monolayer scratch injury assay as described by Tang et al., (Tang et al., 2012) with slight modification. SW480 cells were seeded into a 6 well assay plate and allowed to attach, spread and form a confluent monolayer. Once the monolayer was formed, a fine pipette tip was used to scratch and remove cells from a discrete area of the confluent monolayer to form a cell-free zone into which cells at the edges of the wound can migrate. Morin (150, 200 & 250 µM) and positive control, camptothecin (50 µM) were added to the wells and images of cell migration were captured after 48 h of incubation using phase contrast microscope attached with camera (Nikon Eclipse TS-100, Nikon instruments Inc. Melville, USA).

5.2.4.4. Colony formation assay

Colony formation assay was done as explained by Wang et al., with slight modification (Wang et al., 2014). SW480 cells were suspended in DMEM medium containing 10% FBS and plated in 6-well plates at a density of 200 cells/well. After the attachment of cells for 24 h, they were treated with morin (150, 200, and 250 µM). After 24 h of treatment, fresh complete culture medium was changed and cell colonies were allowed to grow for 14 days. Colonies were then fixed with 3% paraformaldehyde and stained with 0.1% crystal violet for 30 min. Stained cell colonies were washed with phosphate buffered saline (PBS) for three times, dried and images were obtained by phase contrast microscope attached with camera (Nikon Eclipse TS-100, Nikon instruments Inc. Melville, USA). Camptothecin (50 µM) was used as positive control.

5.2.4.5. Gelatin zymography

MMP-2 and MMP-9 levels in the cell culture medium was assessed by assessing the activity of gelatinases by gelatin zymography (Toth et al., 2012). Briefly, after treatment, culture medium was collected and samples were normalized to an equal amount of protein (60 μg), electrophoresed in 7.5% SDS-PAGE containing 0.1% gelatin, at 120 V for 2.5 h. After electrophoretic run, the gels were incubated with 2.5% triton X-100 for 30 min and afterward treated with incubation bufferr (pH 7.5), at 37°C overnight. The gels were then stained with 0.25% Coomassie Blue R-250 for 15 min. Excess stains were removed by washing with distilled water. The proteolysis by gelatinases, MMP-2 (72 kDa) and MMP-9 (92 kDa) were detected as white bands against a blue background. The image was taken using a Chemi Doc XRS digital imaging system and the Multi-Analyst software from Bio-Rad Laboratories (USA).

5.2.4.6. Western blot analysis

Cells were treated with 150, 200 and 250 μM concentrations of morin and 50 μM concentration of camptothecin for 48 h and after incubation, cells were washed twice with ice cold PBS, lysed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 0.5 mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100 and protease inhibitor cocktail, pH 8.0) for 30 min on ice and were centrifuged at 12000 x g for 10 min. BCA protein assay kit was used for determining the protein concentration of the lysate. Lysates were diluted to an equal concentration of total protein and supernatants were then stored at -80ºC until analysis. These samples in reducing sample buffer (62.5 mM Tris-HCl pH6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue) were boiled for 10 min at 75°C. The lysate containing 50 μg of protein was subjected to SDS-PAGE on 12% gel and transferred onto a polyvinylidene difluoride membrane (Immobilon PTM, Millipore®, USA) by using Trans-Blot

TurboTM transfer system (Bio-Rad Laboratories, Germany). The blocking buffer (5% skim milk in PBST, PBST-PBS buffer containing 0.1% Tween 20), for 1 hr at room temperature was used for blocking. The membranes were washed three times with PBST and probed over night at 4°C with primary antibodies (β actin, MMP-2, MMP-9, MMP-7, β-catenin, c-myc and cyclin D1 at 1:500 dilution). After washing three times with PBST for 5 min each, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody at 1:1000 dilution and again washed three times in PBST. The bound antibodies were sensed using an enhanced chemiluminescence substrate (Biorad, USA) and measured by densitometry using a Chemi Doc XRS digital imaging system and the Multi-Analyst software from Bio-Rad Laboratories (USA).

5.2.4.7. Statistical analysis

All the experiments were done at least in triplicate and results were expressed as mean ± SD (standard deviation). The differences between treatments in comparison with control were assessed using one-way ANOVA and the significance of differences between means was calculated by Duncan's multiple range test, using SPSS for Windows, standard version 16 (SPSS, Inc.) and significance was accepted at $p \leq 0.05$.

5.3. RESULTS

5.3.1. Morphological changes in the cells when treated with morin examined using SEM

The changes in the surfaces of the cells after treatment with morin were examined using SEM (Kang et al., 2012). The untreated control cells displayed a smooth surface and cells treated with morin showed severe damage of the cells with deformation, shrinking to abnormal round type and a significant reduction in cell number. At higher

concentration of morin, on the surface of cells, we could see separated apoptotic bodies as well as papillous protuberances.

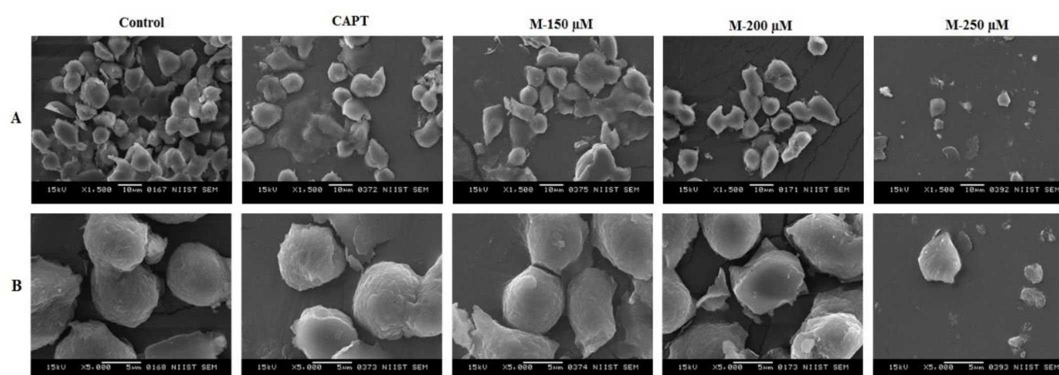

Figure 5.2. Scanning electron microscopic images of SW480 cells after morin treatment. SW480 cells were treated with various concentrations of morin (150, 200, and 250 µM) and camptothecin (50 µM) for 48 h. After incubation, morphological changes were observed under SEM at magnification of 1500× (A) and 5000× (B) respectively.

5.3.2. Microfilament analysis

It is a known fact that cell movements and cytoskeleton elements are closely linked (McInroy and Määttä, 2007). In order to evaluate the cell migration inhibition potential of morin, the alteration in the organization of F-actin microfilaments in SW480 cells after morin treatment was investigated by fluorescence microscopy using fluorescent (FITC) dye labelled phalloidin toxin. A regular and defined array of actin filaments, evenly distributed in the cytoplasm along the cells were observed in the control cells. The cells treated with a lower concentration of morin demonstrated disorganization of actin filament with the actin stress fibers exhibiting green fluorescence spots and those cells treated with morin at higher concentration showed complete disappearance of actin filaments and almost complete damage of actin network in the cells.

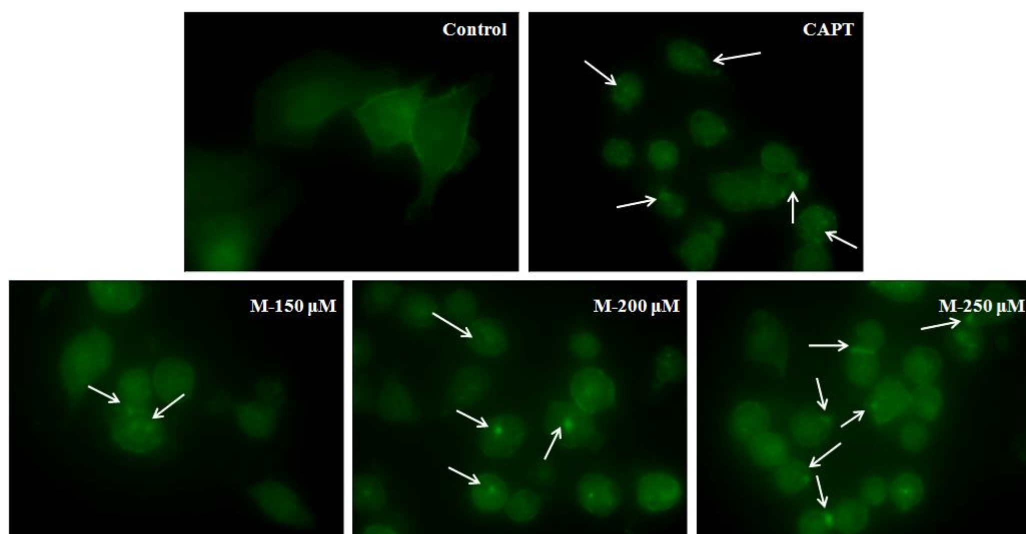

Figure 5.3. Microfilament analysis after morin treatment. Changes in the actin cytoskeleton of SW480 cells after morin (150, 200, and 250 µM) and camptothecin (50 µM) treatment were observed by staining the cells with phalloidin (green) for F-actin and disorganization of the microfilaments was observed (magnification 40×).

5.3.3. Migration of SW480 cells were inhibited by morin

Anti-migration assay was done to validate the inhibitory effect of morin on SW480 cell migration. This assay reveals the migration and movement of the cells on the surface on which they are anchored for growth. It was noted that after incubation for 48 h, cells in the control group started healing the wound and grown as compact mass of cells, while in the morin treated cells exhibited only a few cells grew to the internal space of the wound and a significant reduction in the cell density was seen as the concentration of morin increased.

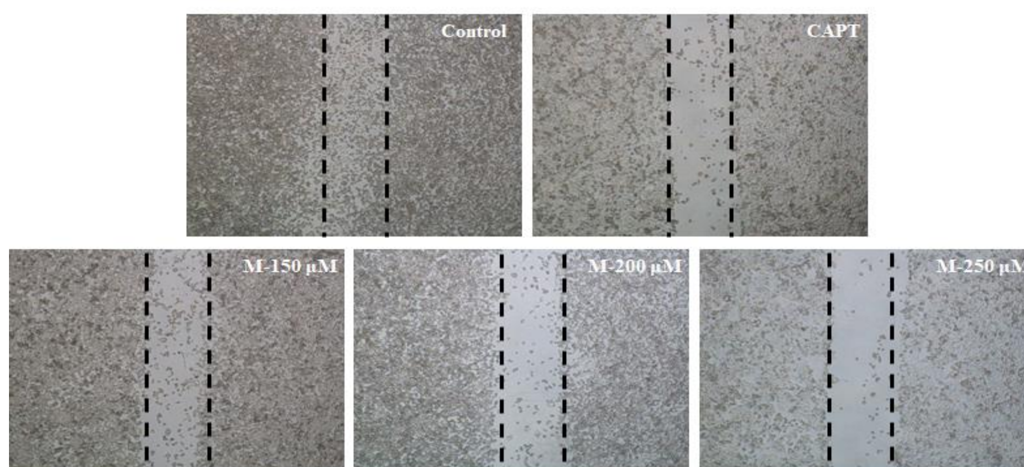

Figure 5.4. Effect of morin on the migration of SW480 cells *in vitro*. Cells in 6 well plates were wounded by scratching them with a pipet tip and the cells were incubated with morin (150, 200 and 250 μM) and camptothecin (50 μM) for 48 h. Cells were photographed under phase-contrast microscopy (4× magnification).

5.3.4. Colony formation assay

We further determined the long-term effects of morin on the growth of SW480 cells by colony formation assay. Here we treated SW480 cells with morin (150, 200, and 250 μM) for 24 h and cells were allowed to grow for further 14 days, fixed, stained and observed under microscope. The results from clonogenic assay demonstrated that morin could significantly inhibit the reproductive potentials of colorectal cancer compared with the untreated SW480 control cell group.

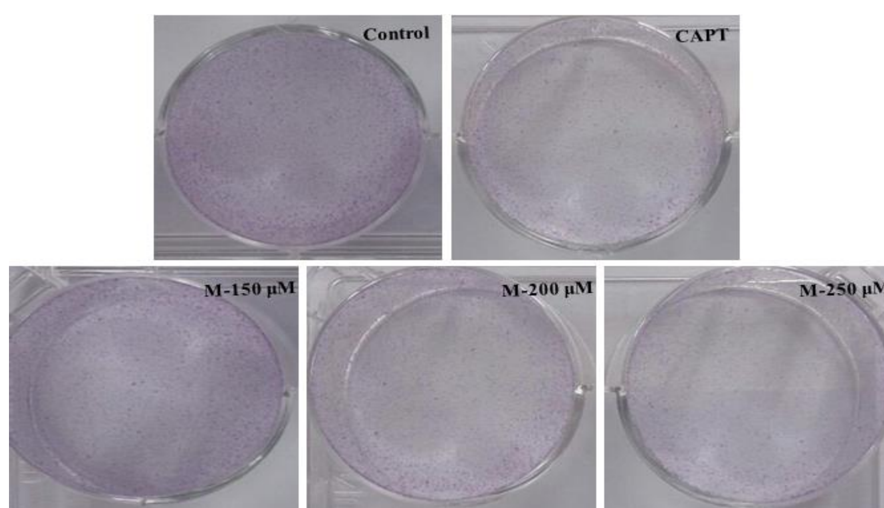

Figure 5.5. Effect of morin on the colony formation of SW480 cells *in vitro*. SW480 cells treated with morin (150, 200, and 250 μM) for 24 h, allowed to grow for further 14 days, fixed, stained and observed under phase-contrast microscopy (4× magnification).

5.3.5. MMP-2 and MMP-9 expression by gelatin zymography

MMP-2 and MMP-9 are reported to play important roles during cancer metastasis by degrading extracellular matrix (ECM). Gelatin zymography is a simple and powerful technique to detect the levels of these proteolytic enzymes from different biological sources, due to their potent gelatin-degrading activity. In this study, when SW480 cells were treated with morin, we could see a significant reduction in the levels of both MMP-2 and MMP-9 in culture media, in a concentration dependant manner.

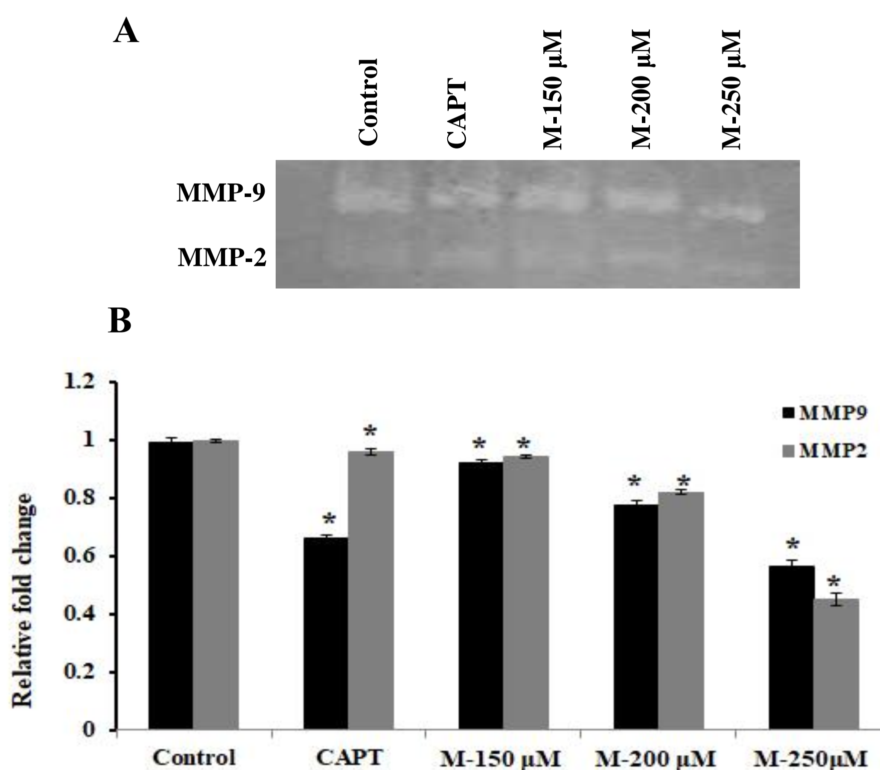

Figure 5.4. Effect of morin on MMP-2 and MMP-9 in SW480 cells by gelatin zymography. SW480 cells treated with morin (150, 200, and 250 µM) and camptothecin (50 µM) for 48 h and the levels of MMP-9 and MMP-2 were determined by gelatin zymography. (A) Representative results. (B) The protein levels were quantified and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

5.3.6. MMP-2 and MMP-9 expression by western blotting

To confirm the gelatin zymography results further, expression levels of both MMP-2 and MMP-9 were evaluated by western blotting. A significant reduction in both MMP-2 and MMP-9 protein expression were observed on morin treatment compared with untreated SW480 control cells in a concentration dependent manner, confirming the results of gelatin zymography, indicating the inhibitory potential of morin on extracellular matrix degradation and thereby CRC metastasis.

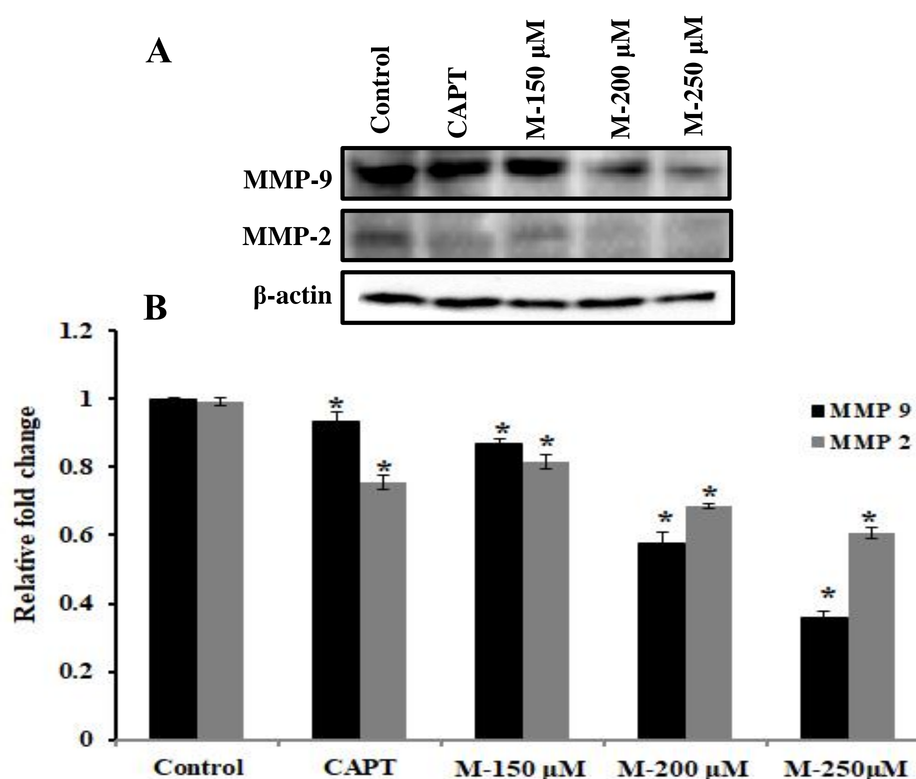

Figure 5.5. **Effect of morin on MMP-2 and MMP-9 level**. SW480 cells were treated with various concentrations of morin (150, 200 and 250 µM) and camptothecin (50 µM) for 48 h. Western blot analysis for expression of MMP-2 and MMP-9 was carried out. (A) Representative results. (B) The protein levels were quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus control.

5.3.7. Wnt/β-catenin signaling pathway regulate the metastatic arrest of SW480 cells by morin

β-catenin is the central molecule in the Wnt signalling pathway and plays a key role in the genesis and development of tumours. The Wnt/β-catenin signalling pathway has been found to be deregulated in colorectal cancer (Kang et al., 2012, Scholer-Dahirel et al., 2011). This highly conserved signalling pathway plays key regulatory roles in migration of colorectal cancer (Polakis, 2000). Western blot analysis targeting on Wnt/ β-catenin indicated that morin treatment in SW480 cells significantly reduced the protein expression levels of Wnt/β-catenin signalling pathway components, including β-catenin, c-myc, cyclin-D1 and MMP-7 compared to untreated SW480 control cells in a dose dependent manner.

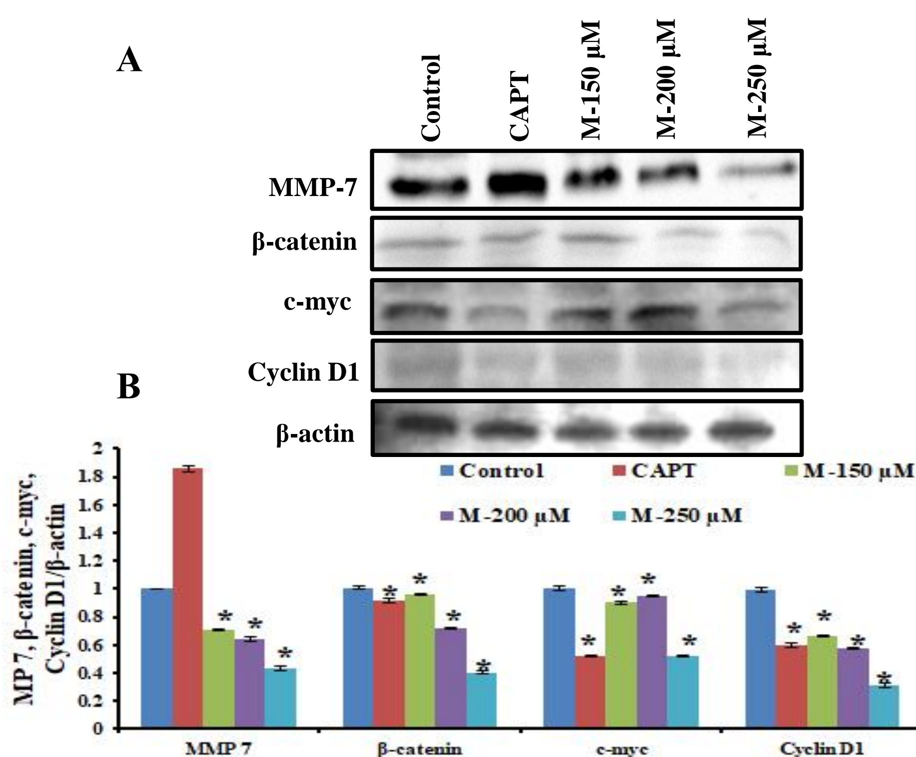

Figure 5.6. **Effect of morin on MMP-7, β-catenin, c-myc and cyclin D1 level**. SW480 cells were treated with various concentrations of morin (150, 200 and 250 µM) and camptothecin (50 µM) for 48 h. Western blot analysis for expression of PI3K was carried out. (A) Representative results. (B) The protein levels were quantified and normalized to β actin and are shown in a histogram. Significance levels between distinct groups were determined by using one way ANOVA, followed by Duncan's multiple range test. *$p \leq 0.05$ versus

5.4. DISCUSSION

Metastasis of the cancer is the major challenge against effective treatment of cancer. Therefore, recent research is focussing more on diagnosis of cancer at its early stage and inhibition of its spread to other parts of the body. None of the available treatment for cancer effectively address the metastasis. It is also reported that many of them possess a large number of side effects that further induce other complications in cancer patients. Therefore, researchers are focussing on compounds derived from nature with lesser side effects and having better anticancer activity for effective management of progression of cancer.

Various phytochemicals have been studied for their anticancer activity and anti-metastatic activity. These anticancer phytochemicals exhibit anti-metastatic potential by various mechanisms. Celastrol, a penta cyclic triterpene derived from the traditional Chinese medicinal plant *Tripterygium wilfordii Hook* F, induced antitumor effects through MMP-3 and MMP-7 by the PI3K/AKT signaling pathway (Bufu et al., 2018). β elemene, a component of *Rhizoma zedoariae* oil inhibited cell proliferation and invasion, in addition to inducing apoptosis, through attenuation of the Wnt/β-catenin signaling pathway in cervical cancer cells (Wang et al., 2018). Latrunculin A inhibited the invasiveness of human prostate cancer PC3 cells and T47D breast carcinoma cells and blebbistatin, a 1-phenyl-2-pyrrolidinone derivative capable of inhibiting non-muscle myosin II activity, inhibited the invasiveness of pancreatic adenocarcinoma (Duxbury et al., 2004). Shao et al., (Shao et al., 2002) demonstrated that curcumin has a strong anti-invasive effect by down-regulating MMP-2 and up-regulating TIMP-1, 2.

Intact morphology of cells with proper cytoskeletal features are mandatory for the tumorigenic potential of the cancer cells and during the course of metastasis, changes

in shape and cell movement impose reorganization of the cell cytoskeleton. Studies have been reported that various approaches for blocking actin cytoskeleton alteration can bring about great therapeutic potential against tumour cell metastasis (Klimaszewska-Wiśniewska et al., 2018, Leduc and Etienne-Manneville, 2015). In the present study, as a part of evaluating the anti-metastatic potential of morin against SW480 colorectal cancer cells, the alteration in cellular morphology was assessed by SEM analysis. We could observe that low concentration SW480 cells were seriously damaged with obvious deformation, contracted to abnormal round type and the cell number was considerably declined. Upon treatment with higher concentration of morin, few papillous protuberances were observed on the surface of cells with extrusion of cytoplasm through the cell membrane boundary. These findings were in line with the study conducted by Yan et al.,(Yan et al., 2014). As actin plays major role in cell movements and cellular migration, drugs targeting actin and its contractility are captivating area of pharmacological research. Geodiamolides are actin-targeting drugs that disrupt actin filaments and are derived from marine sponges (Tannert et al., 2010), latrunculins are microfilament-directed agents, also derived from marine sponges, that inhibit actin polymerization through the sequestration of G-actin monomers (Yarmola et al., 2000). Both of them are found to impart anti-metastatic activities in different cancer cells. Hence, in the present study, for checking the impact of morin on actin cytoskeleton, fluorescent (FITC) dye labelled phalloidin staining was performed and we observed a severe distortion of F-actin arrangement with collapse of the microfilament network.

As metastasis is one of major the reason for most of the cancer deaths, where tumour cells migrate, the effect of morin on the invasion and migratory activity of SW480 cells were evaluated. We performed anti-migration assay and colony formation assay

which indicated a decline in migration as well as colony formation properties of SW480 cells, in a concentration dependent manner. In a similar study, Bigelovin is a sesquiterpene lactone purified from *Inula helianthus-aquatica* exhibited potent anti-migratory potential against CRC through suppressing cell proliferation and colony formation (Li et al., 2017). Nassar et al., (Nassar et al., 2012) showed that koetjapic acid, a natural triterpene inhibited cell migration, invasion and colony formation properties of breast cancer cells.

Earlier studies have reported that the downregulation of matrix metalloproteinase can repress the expression of malignant phenotypes that intern suggests the importance of MMPs in tumour invasion and metastasis (Fang et al., 2003). Many phytochemicals have been reported to inhibit matrix metalloproteinase and exhibit anti-metastatic potential in various cancers. Epigallocatechin-3-gallate (EGCG) treatment resulted in the downregulation of MMP-2 and MMP-9 in renal cell carcinoma (RCC) cells and reduced metastatic behaviour of human RCC cells (Chen et al., 2016). Inhibition of MMP-9 and COX-2 expression by sanguinarine is reported to suppress the invasiveness of breast cancer cells (Park et al., 2014). Sun et al., (2015) reported that emodin, an anthraquinone present in the aloe plant exudate, decreased the secretion of MMP-2 and MMP-9 in MDA-MB-231 breast cancer cells that resulted in inhibition of migration, invasion and metastatic potential. Hence, to understand the underlying mechanism of anti-metastatic potential of morin, we have checked the levels of two major MMPs, MMP-2 and MMP-9 by both gelatin zymography as well as western blotting. The obtained results indicated that there was a clear down regulation of MMP-2 and MMP-9 levels when the cells were exposed to morin. The results from the above studies strongly suggest that morin can significantly inhibit metastatic potential of colorectal cancer cells.

The Wnt/β-catenin signaling pathway is evolutionarily conserved in animals, that means they are similar across animal species from fruit flies to humans (Nusse, 2005) and mediates numerous biological functions like axis patterning, cell proliferation, cell fate specification and cell migration (Kučerová, 2012). Unusual activation of the Wnt/β-catenin signaling pathway shows close relationship with tumorigenesis, invasion and metastasis of several types of cancers including CRC (Hoffmeyer et al., 2012, Polakis, 2000, Shan et al., 2009). The canonical Wnt/β-catenin signaling pathway has been found to play an important role in the promotion of cancer metastasis (Fu et al., 2011, Valenta et al., 2012). APC mutations, the most predominant genetic variations in colorectal cancers, results in the accumulation of β-catenin and these mutations ultimately activate the Wnt pathway in colorectal cancer cells. This will lead to the binding of the Wnt ligand to its receptor and the inhibition of the cytoplasmic degradation complex and stabilization of β-catenin. β-catenin then accumulates in the cytoplasm and translocate into the nucleus, resulting in the induction of downstream Wnt genes, which are factors for cancer metastasis (Liu et al., 2002). Hence, the modulation of Wnt/β-catenin signalling is regarded as a promising target in the field of colorectal cancer drug research.

Cyclin-D1, c-myc and MMP-7 are the targets of Wnt/β-catenin pathway and their expression have been shown to be a negative predictor of prognosis in CRC, by regulating various aspects connected with regulation of cell proliferation and cellular metabolism (Miller et al., 2012, Xing et al., 2014). Phytochemicals have been explored for inhibiting metastasis in various cancer cells and their mechanism of action has been explored. Fangchinoline, an active component purified from medicinal plant *Stephania tetrandra* is found to be exerting its anti-metastatic potential against human gastric cancer cells by suppressing MMP-2 and MMP-9 levels, increasing

levels of tissue inhibitor of metalloproteinase (TIMP1 and TIMP2) genes and inhibiting phosphorylation of Akt (Chen et al., 2017). Ergosterol peroxide isolated from Chaga mushroom displayed anti-migratory activity in CRC by downregulating of β-catenin signaling pathway (Kang et al., 2015). Rosmarinic acid inhibited adhesion, migration and invasion of CRC cells and reduced expressions of MMP-2, MMP-9, intercellular adhesion molecule (ICAM-1) and integrin β1 levels in CRC cells (Han et al., 2018). In our current study, western blotting results showed that morin treatment could significantly downregulate the expression of β-catenin, cyclin-D1, c-myc and MMP-7 expression in a dose dependant manner. The results suggest that, morin inhibited Wnt/β-catenin signalling effectively and thereby it can play a promising role in inhibition of metastasis in CRC.

5.5. CONCLUSION

Migration of colorectal cancer cells is one of the major challenge against successful recovery from colorectal cancer and diagnosis of most of the colorectal cancer cases occurs after the initiation of metastasis. Therefore, anticancer agents having anti-migratory potential has great significance towards treatment of colorectal cancer. The present study investigated the anti-migratory potential of morin on SW480 colorectal cancer cells using various assays. The results suggest that treatment with morin could significantly altered cellular morphology and intactness, with severe distortion of cytoskeletal actin filament organisation which intern found to inhibit migration as well as colony formation property of SW480 cells as evidenced from colony formation assay and anti-migration assay. Since the canonical Wnt/β-catenin signalling pathway has been found to play an important role in the promotion and metastasis of CRC, further investigation was carried out on the impact of morin on Wnt/β-catenin signaling pathway. The results confirmed that morin induces anti-metastatic effect by

the downregulation of Wnt/β-catenin signaling pathway target genes, including β-catenin, c-myc and cyclin-D1and major MMPs (MMP-2, MMP-7 and MMP-9) which intern contribute to the motility suppression of colon cancer cells.

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CHAPTER 6

Encapsulation of Morin with Dietary Fiber (Inulin) & Anticancer Efficacy of the Encapsulated Product

6.1. INTRODUCTION

Colorectal cancer occurs due to multiple factors and the most important type is inherited ones (Jasperson et al., 2010). Numerous studies suggest the close association between CRC and dietary factors. Increased fat intake along with decreased carbohydrate intake, smoking, increased alcohol consumption, intake of refined grains and red meat are recognized as key factors contributing for colon cancer development (Levi et al., 1999). Lot of researchers have documented the promising role of a diet rich in fruits and vegetables in combating the risk of developing colon cancer (Terry et al., 2001). Phytochemicals isolated from vegetables, fruits, grains and spices have shown chemopreventive potential (Vainio and Weiderpass, 2006). Protective elements present in them include vitamins, selenium, polyphenols, such as phenolic acids, indoles, flavonoids, phytoalexins, carotenoids, etc (Surh, 2003, Russo, 2007). Quercetin, curcumin, kaempferol, epigallocatechin gallate, lycopene, catechin, naringin and resveratrol are few compounds coming under this group and have studied extensively (Aggarwal et al., 2004, Wong and Fiscus, 2015, Hwang and Lee, 2006, ACKLAND et al., 2005, Bishayee, 2009).

Many studies have assessed the association between fibre and colorectal cancer and have clearly shown the reduced risk of colorectal cancer with dietary fiber intake (Aune et al., 2011, Schatzkin et al., 2007, Zhu et al., 2013, Park et al., 2005, Larsson et al., 2005, Levi et al., 2001). Many biologically plausible mechanisms have been postulated to elucidate the link between fibre and prevention of colorectal cancer like, increased fibre intake may lead to dilution of faecal carcinogens, reduced gut transit time and bacterial fermentation of fibre resulting in the production of short-chain fatty acids with anti-carcinogenic properties (Sengupta et al., 2006, Lipkin et al., 1999, Goodlad, 2001, Song et al., 2015) etc. It is reported that around 50% of the total dietary

polyphenolics, traverse the small intestine associated with dietary fiber. They release the fiber matrix in the colon by the action of the bacterial microbiota, producing metabolites and an antioxidant environment. It is established that the transportation of dietary antioxidants through the gastrointestinal tract may be an essential function of dietary fibre (Saura-Calixto, 2010).

Many *in vitro* studies have been reported the anticancer properties of various polyphenols using various colorectal cancer cell lines like SW480, HCT116, HT29, CaCo-2 (Wang et al., 2004, Kim et al., 2014, Lim and Park, 2009, Lee et al., 2014) etc. In the previous chapters we have evaluated and established the anticancer potential of morin on CRC using SW480 cells. Even though *in vitro* studies are used to mimic *in vivo* condition, validation of these finding in *in vivo* is very important to mimic real biological condition. Hence for scientifically validating the results from *in vitro* studies, it's *in vivo* evaluation is mandatory.

In vivo assessments in a suitable animal model can be well appreciated, when used in combination with *in vitro* systems. This will help to verify *in vitro* results using *in vivo* animal-based studies and for further extrapolation of the results to human clinical conditions. The logic behind the selection of an animal model is based on broad similarities to humans, in important physiological and biochemical parameters governing drug absorption, distribution, metabolism and excretion processes (Tang and Prueksaritanont, 2010). Different animal models are used for different disease conditions and different disease conditions are established through various ways.

In the case of CRC, there are mainly two different ways to create *in vivo* CRC models, either by injecting live CRC cells or by using chemical carcinogens (Golovko et al., 2015). The xenograft model exploits use of cultured or primary CRC cells that are implanted under the skin of immune deficient rats and mice. As these animals have

defects in the immune system, that inhibits tumor growth and the subcutaneous microenvironment around the transplanted tumors is different from that of original CRC. Hence this approach does not exactly mimic tumor development in man (Ding et al., 2010, Voskoglou-Nomikos et al., 2003). As the characteristics of the chemically-induced CRC are very similar to those of human CRC, it is regarded as the superior models. Often used carcinogens for chemically induced colorectal cancer include dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU) and dextran sodium sulphate (Kobaek-Larsen et al., 2000). Among these, Azoxymethane (AOM) and its precursor compound, di methyl hydrazine are the commonest carcinogens used for chemical induction of CRC. In the current study, we have used azoxymethane and are reported to cause DNA mutation by binding methyl or alkyl groups to guanine residues, leading to G to A transition mutation (Dipple, 1995). Aberrant crypt foci (ACF) formed are been regarded as intermediate biomarkers to quickly evaluate the chemo preventive potential of several agents, including naturally occurring agents against colon cancer (Corpet and Taché, 2002).

It was very clear from our previous studies that morin demonstrate good anticolorectal cancer potential in *in vitro* conditions, as the next step, in this chapter we aimed to assess the anticolorectal cancer efficacy of morin in *in vivo* condition using azoxymethane induced colon carcinogenesis model. Here, our hypothesis is that, as dietary fibers are not digested in the gastro intestinal tract other than undergoing fermentation in the large intestine, morin encapsulated with dietary fibre can be delivered to the colon area without exposing to upper gastro intestinal condition and can reach in to the colon area safely. Second hypothesis is that, as there is lot of evidences on dietary fibre and colon cancer prevention, encapsulates of morin with

dietary fibre can be fermented by the colon microbiota generating the beneficial metabolites, thus helps in maintaining the colon homeostasis. Based on this, the present chapter was aimed to (I) optimize the conditions for encapsulating morin with dietary known fibre inulin and (2) to assess the anticancer activity of encapsulated morin with that of morin alone, against CRC, using *in vivo* CRC models.

6.2. MATERIALS AND METHODS

6.2.1. Materials

Morin, 5-fluorouracil, camptothecin and azoxymethane were purchased from Sigma-Aldrich Chemicals (St Louis, MO, U.S.A.) Inulin (Orfati®Inulin) was supplied by Beneo Asia Pacific Pvt.Ltd., Singapore. Primary antibodies (β actin, cleaved caspase 3, cleaved PARP, Bcl 2 and Bax) and corresponding secondary antibodies were purchased from Santa Cruz Biotechnology, United States. Clarity Western ECL substrate was procured from Bio-rad, United States. All other chemicals used were of the standard analytical grade.

6.2.2. Experimental design

The work flow in this chapter is schematically represented as in **Figure 6.1**

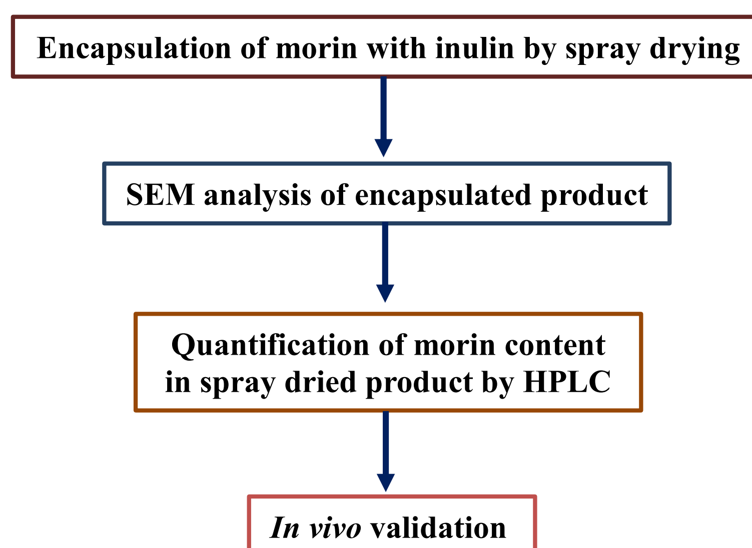


Figure 6.1. Outline of chapter 6

6.2.3. Encapsulation by spray drying

Solution of 10% maltodextrin, 1% inulin, 1 % morin was prepared in 100 mL of sterile distilled water and stirred for 1 h to hydrate. These feed solutions were directly used for spray drying. A bench top scale dryer (LU – 228 ADVANCED, LabUltima, Mumbai, India) was used for spray drying the feed solution. The inlet and outlet temperatures were 140 ± 2°C and 60 ± 2°C respectively with a flow rate of 1.5 mL/min. The feed solution was pumped in to the drying chamber by a peristaltic pump. The feeding solutions were constantly stirred during the spray drying process. The aspiration rate was about 55% while the feeding rate of the solutions was 2 mL/min. The microcapsules were separated by cyclone and gathered in the collection vessel.

6.2.3.1. Assessment of Product yield (PY)

The product yield was calculated as the percentage of the ratio of weight of collected samples (g dry matter) to weight of feed solution (g dry matter).

6.2.3.2. Assessment of moisture content

The moisture content of the sample was analyzed using HC 103 moisture analyser (Mettler-Toledo, Switzerland).

6.2.3.3. Morphological analysis of spray dried product

The topographical properties of encapsulated morin were investigated by scanning electron microscopy (SEM). For this, samples were stuck on to Aluminium stub with the help of a carbon tape. Then the sample whose SEM images have to be captured is shadow casted with thin layer of gold (30-40 nm). SEM analysis was carried out using JEOL JSM-5600LV (Italy). Prior to examination, samples were sputter coated with gold–palladium to render them electrically conductive by using HUMMLE VII Sputter coating device (Anatech Electronics, Garfield, N.J., USA). The micrographs were taken at magnification of 1500×, 3000× and 5000×.

6.2.3.4. Standardization and quantification of morin content in spray dried product by HPLC

Morin was encapsulated with inulin and the content of morin in the spray dried powder was quantified using Prominence HPLC system (Shimadzu, Japan) containing LC-20 AD system controller, Phenomenex Gemini C18 column (250mm×4.6mm,5 μm), a column oven (CTO-20A), a Rheodyne injector (United States) with a loop of 20 μL volume and a diode array detector (SPD-M20A). The flow rate was 1 ml/min, LC LabSolutions software was used for data acquisition and analysis.

6.2.4. *In vivo study:* Anticancer efficacy of encapsulated product

6.2.4.1. Animals and maintenance condition

Male Swiss albino mice weighing about 25–30 g were used for the study. The animals were housed in distinct cages and acclimatized for a week before the start of the experiment. They were maintained under standard temperature and humidity on a 12-h light/dark cycle with access to food and water ad libitum. The experiments were designed and conducted according to the guidelines of the animal ethics committee for the purpose of control and supervision of experiments on animals (CPCSEA). Approval for conducting animal study was obtained from animal ethics board (IAEC No. CBLRC/IAEC/02/01-2018).

6.2.4.2. Carcinogen and drug administration

For inducing of colon cancer, Swiss albino mice were injected with azoxymethane (AOM) intraperitoneally at a dose of 15 mg/Kg body weight once a week for three consecutive weeks. AOM was suspended in 0.9 % NaCl. Control mice received intraperitoneal injections of saline. The treatment regimen followed for the mice in other groups are given below.

Table 6.1. Experimental groups

Group 1	Control group	Basal diet
Group II	AOM group	AOM (azoxymethane) 15mg/Kg body wt once a week for 3 weeks to induce ACF (intra peritoneal injection)
Group III	5-Fluorouracil group	AOM (azoxymethane) 15mg/Kg body wt once week for 3 weeks to induce ACF (intra peritoneal injection) + 5-FU 10mg/Kg body wt, intra peritoneal injection for 8 weeks
Group IV	Morin group	AOM (azoxymethane) 15mg/Kg body wt once week for 3 weeks to induce ACF (intra peritoneal injection) + Morin 50 mg/kg body weight for 8 weeks orally
Group V	Encapsulated morin group	AOM (azoxymethane) 15mg/Kg body wt once week for 3 weeks to induce ACF (intra peritoneal injection) + Spray dried powder (morin content 50 mg/Kg body weight) to each mice orally

For the induction of colon carcinoma in biological research, azoxymethane, a carcinogenic and neurotoxic chemical compound is widely used. Fluorouracil (5-FU), sold under the brand name Adrucil among others, is a medication used to treat cancer. In order to study the effect of morin encapsulation with dietary fibre, studies were carried out using morin alone and compared with encapsulated morin (with inulin), against AOM-induced mice colon cancer model.

Briefly, mice were divided in to five groups with six mice in each group. Mice in the group I, control group; received intraperitoneal injections of physiological saline, mice in group II, AOM group; were administered AOM (15 mg/kg body weight) intraperitoneally once a week for 3 consecutive weeks, mice in group III, 5-Fluorouracil group; were administered AOM (azoxymethane) 15mg/Kg body weight once week for 3 weeks to induce ACF by intra peritoneal injection and 5-FU 10mg/Kg body weight by intra peritoneal injection for 8 weeks. mice in group IV, morin group; were administered AOM (azoxymethane) 15mg/Kg body weight once week for 3 weeks to induce ACF by intra peritoneal injection and morin 50 mg/Kg body weight for 8 weeks orally, mice in group V, encapsulated morin group were administered AOM (azoxymethane) 15mg/Kg body weight once week for 3 weeks to induce ACF by intra peritoneal injection and encapsulated morin (morin at a concentration of 50 mg/Kg body weight) to each mouse orally.

At the beginning of the study, initial body weight of all mice in all groups were recorded. At the end of the study period, the final body weight of all mice were recorded. All groups of animals were sacrificed by exposing to 0.8L/min of CO_2.

6.2.4.3. Histopathological analysis

Liver tissue was collected at the time of sacrifice and fixed in 4% formaldehyde for overnight and stored in 70% ethanol. Fixed sections of tissues were embedded in paraffin, cut into 6-μm sections, stained with hematoxylin–eosin (H&E) and examined under phase contrast microscope attached with camera (Nikon Eclipse TS-100, Nikon instruments Inc. Melville, USA).

6.2.4.4. Examination for aberrant crypt foci (ACF) formation

Method of Bird (Bird, 1987) was used for examining number of ACF formed. The colon was cut in to different pieces, slit opened longitudinally and placed on a strip of

filter paper with its luminal surface open and exposed. Another filter paper was positioned on the top of the luminal surface. This setup was fixed using 10 % formalin and kept for overnight. The fixed colonic sections were stained with 0.2 % methylene blue for 5 min. The sections were placed on a slide in such a way that the mucosal surface facing upwards. The stained specimens were observed under a light microscope at 40× magnification. The number of ACF observed in each focus of colon were counted and recorded.

6.2.4.5. Colon length and weight

Colon was excised, freed of adherent adipose tissue. Subsequent to washing in ice cold 0.9% saline solution, weighed and colon length (from the colocecal junction to the anus) was measured by placing on filter papers. Then the colon was longitudinally slit opened and examined for tumours. Homogenates of colon tissues were prepared in appropriate homogenizing buffer and were used for further assays.

6.2.4.6. Protein extraction and western blotting

The colonic tissues of the mice from control and experimental groups were collected and chopped in to small pieces. Chopped tissues were homogenized in RIPA buffer. The homogenates were centrifuged at 10,000 rpm for 20 min at 4°C. After centrifugation the supernatants were collected and protein concentration in the supernatants were was estimated using BCA protein assay kit. Lysates were diluted to an equal concentration of total protein and supernatants were then stored at -80ºC until analysis. These samples were boiled for 10 min at 75°C in reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). The lysate containing 50 µg of protein was subjected to SDS-PAGE on 12% gel and transferred onto a polyvinylidene difluoride membrane (Immobilon PTM, Millipore®, USA) by using Trans-Blot TurboTM transfer system

(Bio-Rad Laboratories, Germany). The membranes were blocked by incubating in blocking buffer (5% skim milk in PBST, PBST-PBS buffer containing 0.1% Tween 20), for 1 h at room temperature, washed three times with PBST and probed over night at 4°C with appropriate primary antibodies at 1: 500 dilutions (β actin, Bax, Bcl 2, cleaved PARP, cleaved caspase 3). Membranes were washed 3 times with PBST and incubated for 1 h at room temperature with horse radish peroxidase (HRP) conjugated secondary antibody at 1:1000 dilution and again washed three times in PBST. The bound antibodies were detected using an enhanced chemiluminescence substrate (Biorad, USA) and measured by densitometry using a Chemi Doc XRS digital imaging system and the Multi Analyst software from Bio-Rad Laboratories (USA).

6.2.4.7. Statistical analysis

Statistical analysis was performed using SPSS for Windows, standard version 16 (SPSS, Inc.). Data were expressed as mean values ± standard deviation (SD). One-way analysis of variance (ANOVA) tests were performed for qualitative comparative analysis and the significance of differences between means was calculated by Duncan's multiple range test. Differences were considered to be statistically significant when $p \leq 0.05$.

6.3. RESULTS

6.3.1. Encapsulation of morin with inulin by spray drying

Dietary fiber is known to exhibit various health benefits and anticancer activity exhibited by dietary fiber is regarded as the most important one. Studies have proven that dietary fiber on fermentation by intestinal microbiota (probiotics) can produce fermentation by-products, especially short chain fatty acids which is responsible for maintaining colon homeostasis and thus demonstrate anti-anticancer efficacy against CRC. In the present study we have chosen inulin, a standard soluble dietary fiber for

encapsulating our compound of interest, morin, which has shown significant anticancer properties as discussed in the previous chapters. We have chosen spray drying for encapsulating morin with inulin and the procedure was standardized.

6.3.1.1. Product yield, encapsulation efficiency, moisture content

Yield of the product was calculated as the percentage of the ratio of weight of collected samples (g dry matter) to weight of the feed solution (g dry matter) and found to be 42.09 ± 0.32 (g/100 g feed dry matter). Moisture content in the spray dried product is one of the important variable in spray drying that influence on the physicochemical properties, commercial value and quality of spray dried product (De Knegt and Van den Brink, 1998). A low moisture content favours a low water activity, increases resistance to fungal and microbial growth and hinders enzymatic activity (Blandamer et al., 2005, Krishnaiah et al., 2014). Spray dried product with low moisture content has low powder bulk density, better powder flow, nutrient content, potential to maintain intact particle morphology (Walzel and Furuta, 2014)8 and solubility (Goula and Adamopoulos, 2010). In the present study we analyzed the moisture content of the spray dried product and it was found to be 5.7%, a low level of moisture content, indicating storage of the product is possible without damaging the property of the product.

6.3.1.2. Morphological analysis of spray dried product

The surface morphology of the spray-dried powder was visualized using scanning electron microscopy (**Figure 6.2**). Spray dried particles were soft and maintained uniform spherical shape with smooth external surface. Microphotography also revealed the absence of any crystals of the phytochemical on the surface of microsphere which intern indicates uniform distribution of the drug within the microspheres (Gowda and Shivakumar, 2007).

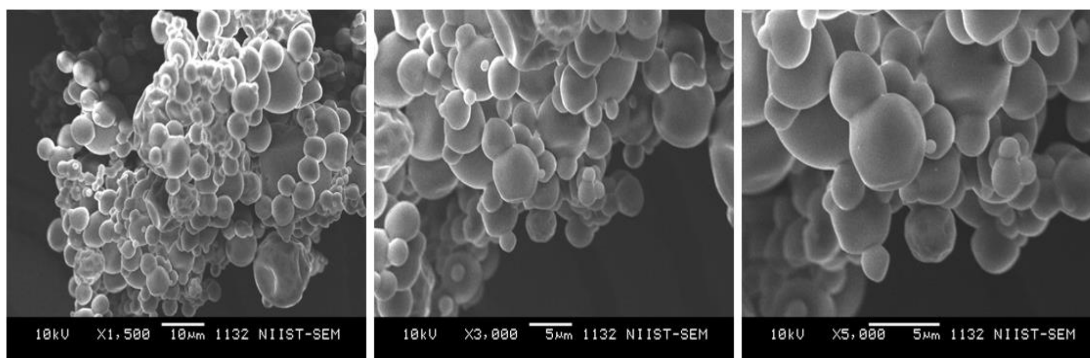


Figure 6.2. Morphological analysis of spray dried product. Morphological analysis of spray dried product was observed under under SEM at magnification of 1500×, 3000× and 5000×.

6.3.1.3. Quantification of morin in spray dried product by HPLC

The HPLC protocol was standardized by changing the polarity of the mobile phase, 0.38% sulfuric acid in 70% methanol was finalized for quantifying the morin in spray dried powder. Morin was detected in the retention time of 3.7 minutes. The fractions were monitored at 254 nm (**Figure 6.3**). Sample peaks were identified by comparing with retention times of standard peaks. It was found that when 1.0 g of morin was taken for spray drying, the spray dried powder obtained was having a morin content of 45.2 μg/ mg spray dried product.

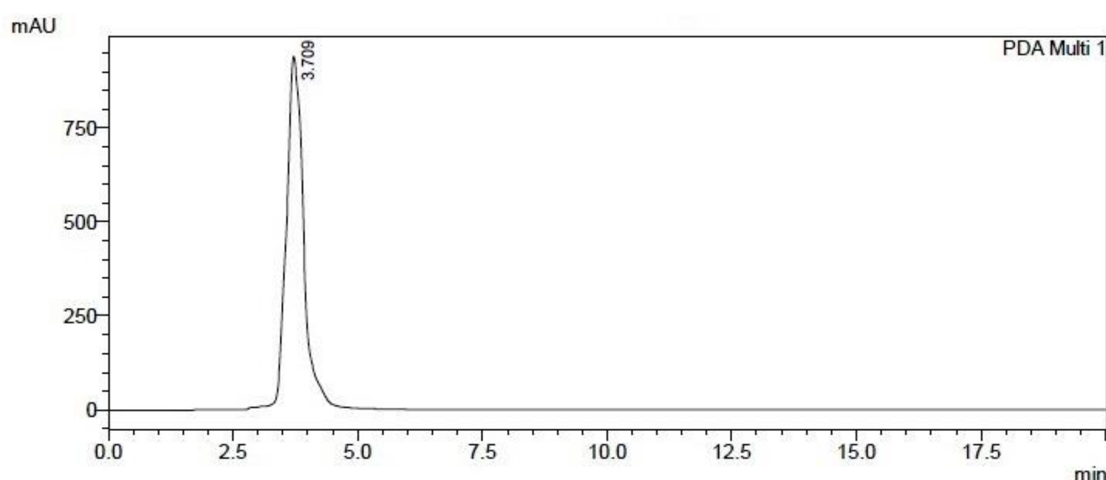


Figure 6.3. Quantification of morin in spray dried product (HPLC). Representative HPLC chromatogram showing peak for morin at a retention time of 3.7 min.

6.3.2. *In vivo* study: Anticancer efficacy of encapsulated product

It was found from our cell culture experiments that morin is able to exert good anticolorectal cancer activity and, to extrapolate the same result in *in vivo* conditions, in this chapter we have evaluated the anticancer efficiency of morin on chemically induced mice colorectal cancer model. In order to achieve the colon targeted delivery as well as to get benefit from both morin and soluble dietary fibre against CRC, we encapsulated morin with inulin, a soluble dietary fibre and it was also taken for *in vivo* evaluation.

6.3.2.1. Histopathology of liver

Liver tissues were collected from mice in all the groups under study and histopathological examination was performed to check for any abnormality. The results did not show any pathological changes in liver tissues of mice treated with 5-fluouracil, morin and encapsulated morin group indicating they are not causing any hepatic toxicity and are safer to use. At the same time, alteration in the histopathology result of mice belonging to AOM group were observed, indicating ttoxicty (**Figure 6.4**).

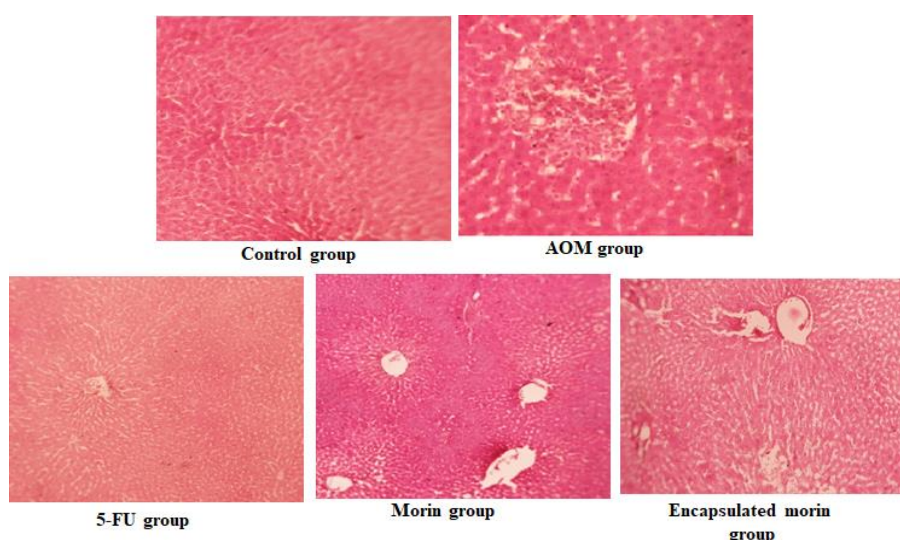


Figure 6.4. Histopathology analysis of liver of mice of various groups at the end of the study. At the end of the study animals were sacrificed and liver was collected, fixed and stained (H&E staining).

6.3.2.2. Effect of morin on the body weight of control and experimental groups of animals

The treatment with AOM resulted in a noticeable decrease in the body weight gain (%) of Group II animals compared to the control animals (Group I). Treatment with 5-flurouracil (Group III) significantly increased the percentage body weight gain as compared to Group II animals. The group IV and V animals also showed marked increase in body weight compared to Group II animals (**Table 2**). It was also noticed that the body weight gains among mice treated with encapsulated morin were significantly higher than that of mice treated with morin alone.

Table 6.2. Effect of morin on the body weight of control and experimental groups of animals

Parameters	Group I Control group	Group II AOM group	Group III 5-FU group	Group IV Morin group	Group IV Encapsulated morin group
Initial body weight (g)	26.82±0.25	26.78±0.41	26.86±0.28	26.74±0.40	26.65±0.64
Final body weight (g)	31.25±0.10	26.92±0.08	30.43±0.36	29.31±0.12	29.66±0.29
Body weight gain (%)	11.6*	10.05	11.32*	10.96*	11.09* #

At the start and at the end of the study body weight of mice in each group were weighed and weight gain % was calculated. * $p \leq 0.05$ versus AOM group, # $p \leq 0.05$ morin group versus encapsulated morin group. AOM: Azoxymethane, 5-FU: 5- Fluorouracil.

6.3.2.3. Effect of morin on the ACF formation in control and experimental groups of animals

ACF are preneoplastic lesions in colon and recognised as surrogate precursor lesions of colorectal cancer. Studies have reported that natural compounds that can inhibit carcinogen induced ACF formation are protective against colon cancer in rodents (Mori et al., 1997). Total number of ACFs in AOM treated mice (Group II) were significantly high compared to the mice in the control group (Group I). Administration of 5-flurouracil (Group III), morin (Group IV) animals and encapsulated morin (Group V) significantly inhibited the formation of AOM induced ACF formation. It was also noticed that the number of ACF formed among mice treated with encapsulated morin were significantly lower than that of mice treated with morin alone (**Figure 6.5**).

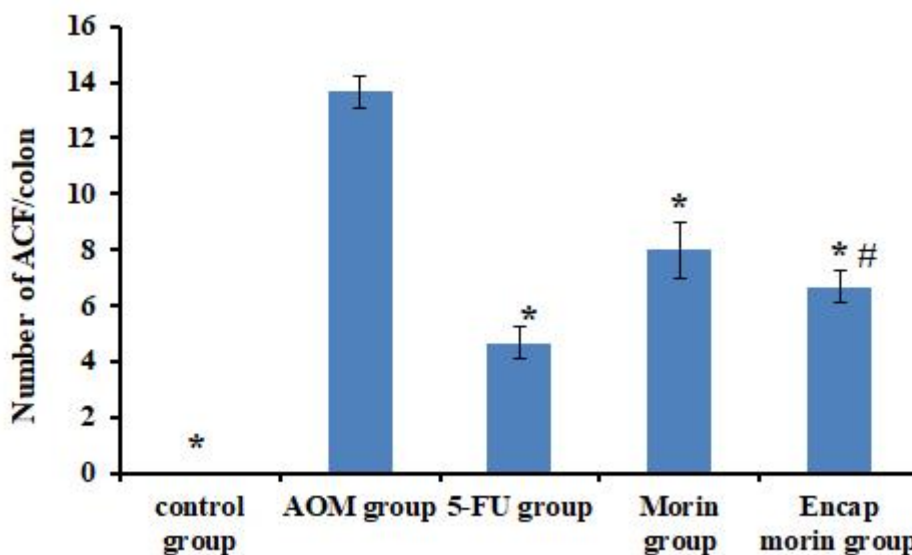
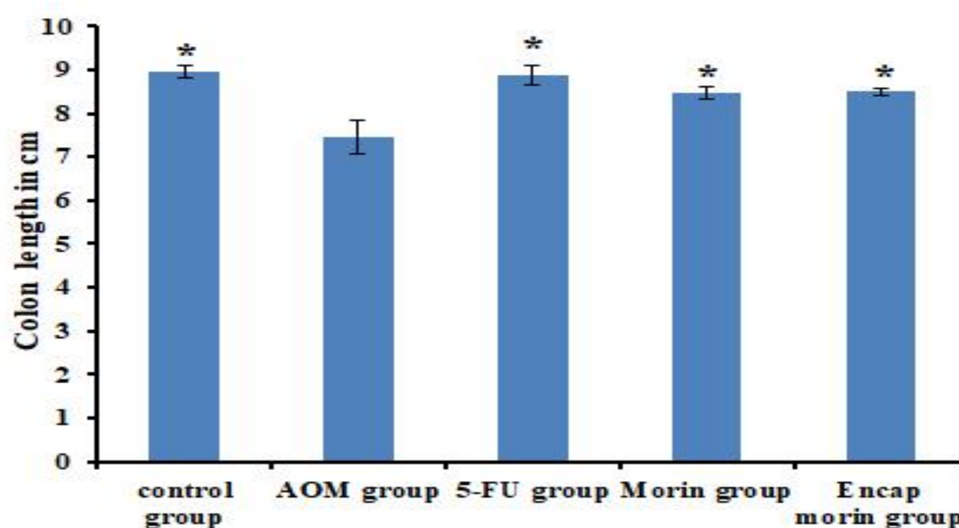


Figure 6.5. Number of ACF formed among mice of various groups. At the end of the study animals were sacrificed and colon was collected. Number of ACF formed were counted after fixing in formalin, followed by staining with methylene blue. * $p \leq 0.05$ versus AOM group, # $p \leq 0.05$ morin group versus encapsulated morin group. AOM: Azoxymethane, 5-FU: 5- Fluorouracil.

6.3.2.4. Effect of morin on the colon length of control and experimental groups of animals

Development of polyps or adenomas in the colon results in a decrease in the length of colon with an increase in its weight (Pande et al., 2017). In the present study, we measured the colon length of all mice in the entire group and the results were compared. It was found that the length of colon of mice treated with AOM (Group II) were shortened significantly compared to the mice of untreated group (Group I). Administration of 5-flurouracil (Group III), morin (Group IV) animals and encapsulated morin (Group V) significantly inhibited the shortening of colon length caused by AOM administration (**Figure 6.6**).



6.6. Colon length among mice of various groups. At the end of the study, animals were sacrificed and colon was collected. Colon length of mice in each group was noted. * $p \leq 0.05$ versus AOM group**. AOM:** Azoxymethane, 5-FU: 5- Fluorouracil.

6.3.2.5. Effect of morin on the colon weight/length ratio of control and experimental groups of animals

Since an increase in the weight of colon with a decrease in length is a feature that is associated with the development of polyps or adenomas in the colon, the weight of colon from mice belonging to all group was measured and compared. It was found that the colon weight of the mice treated with AOM (Group II) were increased significantly compared to mice of untreated group (Group I). Administration of 5-flurouracil (Group III), morin (Group IV) and encapsulated morin (Group V) significantly inhibited the abnormal increase in colon weight caused by AOM (**Figure 6.7**).

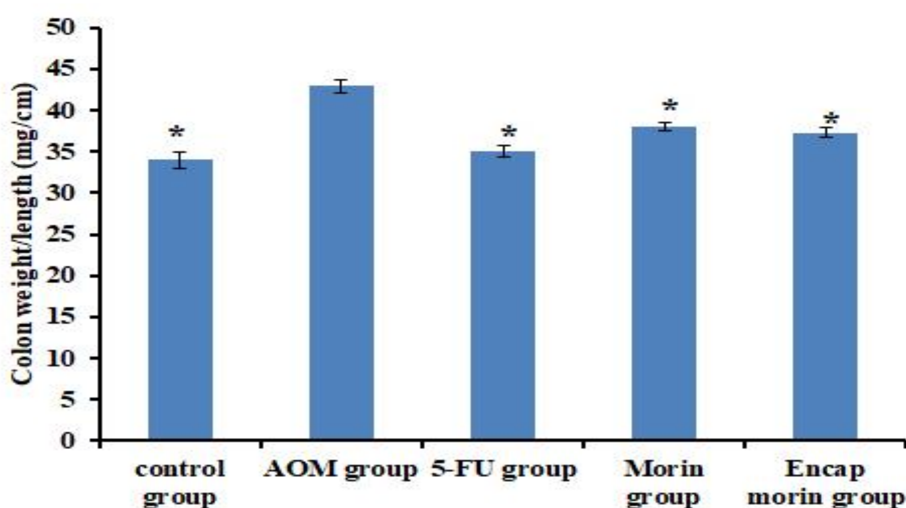


Figure 6.7. Colon weight/length ratio among mice of various groups. At the end of the study animals were sacrificed and colon was collected. Colon weight of mice in each group was noted. * $p \leq 0.05$ versus AOM group. AOM: Azoxymethane, 5-FU: 5- Fluorouracil.

6.3.2.6. Effect of morin on apoptosis associated protein expression in control and experimental groups of animals

We examined the effect of morin treatment on caspase 3 activation, proteolytic cleavage of PARP, Bcl 2 and Bax level using western blotting. Caspase 3 activation is one of the most vital events in apoptosis and PARP is a key substrate of activated

caspase 3. Activation of initiator caspase results in the cleavage caspase 3 into cleaved caspase 3 (effector caspase) and this active form will further cleave PARP into cleaved PARP. At this stage, PARP can't function as polymerase which eventually leads to DNA damage and induction of apoptosis of the cells. As can be seen in **Figure 6.8,** there were marked increment in the levels of cleaved caspase 3 and cleaved PARP in colon tissues of mice treated with 5-flurouracil (Group III), morin (Group IV) and encapsulated morin (Group V). They exhibited increased levels of both cleaved caspase 3 and cleaved PARP compared to that of mice treated with AOM alone (Group II).

Bcl 2 family members proteins are composed of two types, antiapoptotic proteins such as Bcl 2 and pro-apoptotic proteins such as Bax (Lee et al., 2002). Research have shown that Bcl 2 and Bax are identified in the in the outer-membrane of mitochondria and the ratio of Bcl 2/ Bax can be taken as a crucial factor in recognizing of the apoptotic process (Yang et al., 2006). During the process of carcinogenesis, deviations in the balance between cellular deaths and cellular recovery in the colonic mucosa is observed. In the current study, the deregulation of crypt cellular proliferation in the colon of the AOM-injected mice was associated with altered Bcl 2/ Bax ratio, with reduced level of Bax along with increased level of Bcl 2. It was noticed that treatment with 5-fluorouracil, morin and encapsulated morin resulted in a significant upregulation of proapoptotic Bax protein level and downregulation in the antiapoptotic Bcl 2 protein level leading to a reversal of the Bcl 2/Bax ratio compared to the mice in the AOM treated group. It was also noticed that the levels of proapoptotic proteins among mice treated with encapsulated morin were significantly higher than that of mice treated with morin alone and the level of antiapoptotic Bcl 2 protein level was

were significantly lower among mice treated with encapsulated morin than that of mice treated with morin alone (**Figure 6.8).**

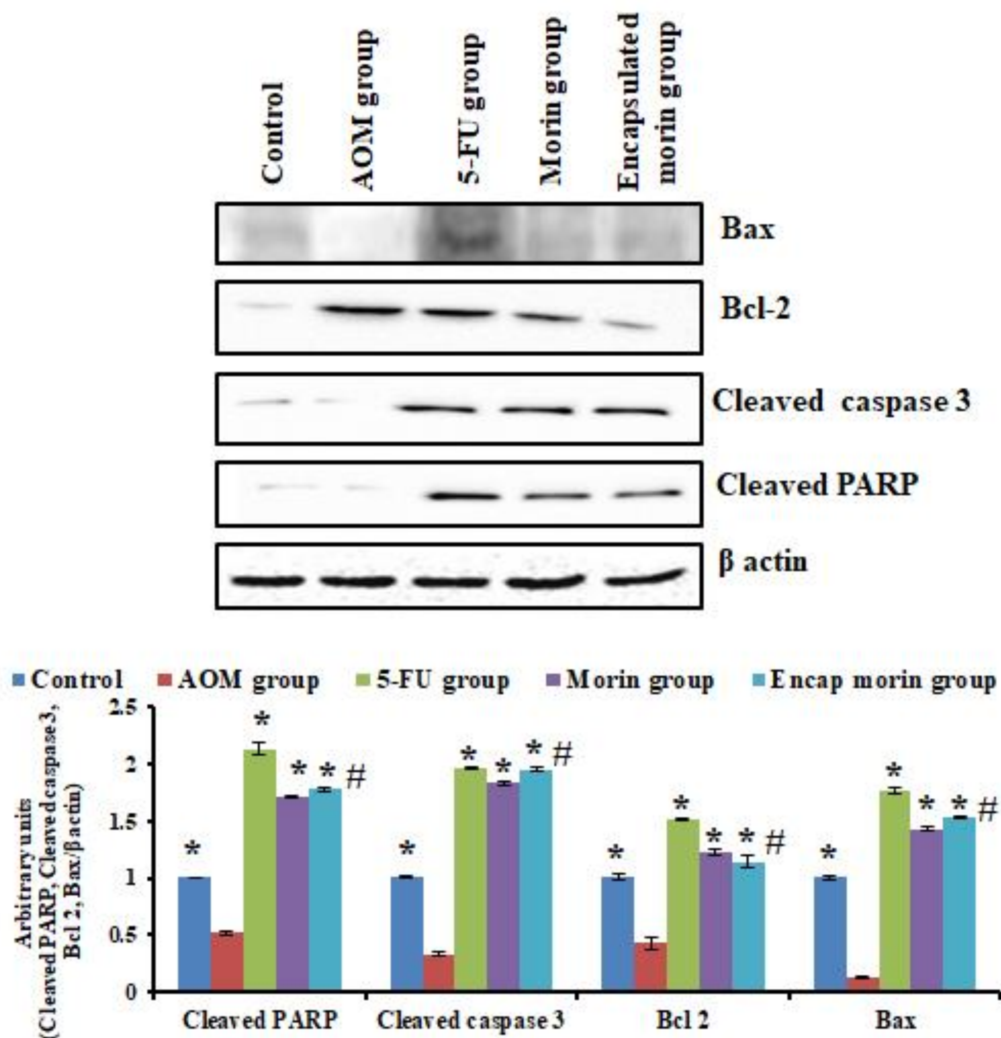


Figure 6.8. Protein expression analysis of key proteins involved in apoptotic pathways. At the end of the study animals were sacrificed and colon was collected. Western blotting for key apoptotic proteins (Bax, Bcl 2, cleaved caspase 3 and cleaved PARP) was performed. * $p \leq 0.05$ versus AOM group, # $p \leq 0.05$ morin group versus encapsulated morin group. AOM: Azoxymethane, 5-FU: 5-Fluorouracil.

6.4. DISCUSSION

A number of phytochemicals have been investigated for their anticancer potential against various types of cancers including colorectal cancer. These studies indicate promising potential of these phytochemicals, where some of them exhibited better

activity than that of the conventional anticancer drugs. Moreover, phytochemicals are known to have no/less side effects, which is commonly associated with anticancer drugs (Kim et al., 2011). From our *in vitro* experiments we found that morin exhibited potential anticancer effect against colorectal cancer and we tried to elucidate the underlying mechanism by which it exhibits anticancer potential. One of the major concern regarding *in vitro* experiments is, its reproducibility in real *in vivo* conditions. In *in vivo* circumstances, the communication between tumour cells and the host environment leads to activation of various types of cells including endothelial cells, fibroblasts, immune cells and inflammatory cells leading to the modification of genetic and phenotypic nature of the tumour (Friedl and Alexander, 2011). Hence cell culture and animal studies together are used for determining efficacy, pharmacodynamics and mechanism of action of novel anti-cancer drugs (HogenEsch and Nikitin, 2012).

There are many reports available suggesting that the results from the cell culture studies were in line with animal studies. The study by Lee et al., (LEE et al., 2012) have shown that destruxin B isolated from green muscardine fungus exhibited anticancer effect on HT-29 cells and which when administered subcutaneously in to murine xenograft model of human colon cancer, found to be safe and effective in inhibiting the CRC growth. Quercetin a plant pigment (flavonoid), exhibited anticancer activity in colon carcinoma CT-26 cells by inducing the apoptosis and in *in vivo* experiments in mice with CT-26 tumours, quercetin exhibited significant reduction in tumour volume as compared to the control group indicating quercetin exhibits anticancer properties both *in vivo* and *in vitro* (Hashemzaei et al., 2017). Wang et al., (Wang et al., 2017) found that the combination of leucovorin (a medication used to decrease the toxic effects of methotrexate) and bortezomib (an anti-cancer drug) enhanced caspase activation and increased apoptosis in colorectal cancer cells better

than either alone. Also the synergistic induction of apoptosis and inhibition of tumour growth were also observed in mouse colorectal cancer xenografts. Bahadori et al., (Bahadori et al., 2016) found that chrysin, a flavone found in honey, propolis etc., exerted a cytotoxic effect on CT26 colon cancer cells through induction of the intrinsic pathway of apoptosis (caspase 3 and caspase 9 activation) and in vivo studies revealed a remarkable reduction of the colon tumor volume in tumour induced mice with upregulation of the Bax level.

Therefore, in order to extrapolate the anticancer activity of morin that was observed in *in vitro* conditions, we further evaluated the anti-colorectal cancer potential of morin using azoxymethane induced colon cancer mice models. Designing of proper delivery system that can effectively deliver morin in the colon is very important in *in vivo* studies. Delivery of drugs specifically to the colon without being affected by the upper gastrointestinal tract allows a higher concentration of that particular drug to reach the colon with minimal systemic absorption (Kumar et al., 2010). Also, the colonic contents have a retention time up to five days and the colonic mucosa facilitate the absorption of several drugs further makes colon targeted drug delivery more practical (Philip and Philip, 2010). There exist different delivery systems that specifically targets the drugs into the colon for the effective therapeutic outcomes. These approaches comprise the usage of formulation components that interact with one or more features of gastrointestinal physiology, like presence of colonic microbiota, the pH variation along the GI tract and enzymes, for achieving colon targeting (Amidon et al., 2015). Commonly practiced colon targeted delivery systems include use of prodrugs, that are inactive form of a drug molecule which release the active ingredient on reaching colon by the hydrolysis by enzymes in the colon (Rabito et al., 2012). Matrix-based system is an another approach for the colon-targeted drug delivery

involving embedding the drug in polymer matrices and release of the drug on reaching the colon (Ahmad et al., 2012). Polysaccharide-based delivery system is a popular way for colon-specific delivery of drugs as it is having advantages like availability, stability, easy modifications, safety and biodegradability (Philip and Philip, 2010). Timed-release systems are based on the release of drug in the colon after a specified time period. Colon-specific biodegradable delivery system, bioadhesive systems and multiparticulate systems are the other approaches for colon targeted drug delivery.

Microencapsulation is a encapsulation technique in which tiny droplets or particles are surrounded by a coating to give small capsules of many useful properties and it is mainly used for reduce dosing frequency and prevent the degradation of pharmaceuticals (Singh et al., 2010). Spray drying is a microencapsulation technique and commonly used wall materials for encapsulation by spray drying includes polysaccharides (maltodextrins, starches, corn syrups and gum arabic) lipids and proteins (gelatin, casein and milk serum). Among these, maltodextrins are found to be the best thermal defenders, crucial to preserve the integrity of phytochemicals during their encapsulation (Munin and Edwards-Lévy, 2011). Cilek et al., (Cilek et al., 2012) successfully encapsulated phenolic compounds extracted from sour cherry pomace using a mixture of gum arabic and maltodextrin. A mixture of maltodextrin and gum arabic has also been used for encapsulation of procyanidins from grape seeds by Zhang et al., (Zhang et al., 2007) and soybean extract was encapsulated within a matrix composed of maltodextrin, starch or a silica by Georgetti et al.,(Georgetti et al., 2008). Dietary fibre (DF) as encapsulants for bioactives is an effective approach to increase DF consumption. Plant gums, modified celluloses and dextrins can be used as the components of an encapsulant matrix (Sun-Waterhouse et al., 2012, Sun-Waterhouse

et al., 2011). Combinations of various polymers as encapsulant can also be used for encapsulating bioactive substances.

Dietary habits are closely associated with both in prevention and management of CRC (Shike, 1999) and studies have reported that consumption of adequate amount of dietary fibre can reduce CRC up to 40% (Dahm et al., 2010). Inulin is a fructooligosaccharide, composed of fructose units with β (2–1) links with glucose units at the end of the chains. Inulin has dietary fiber actions, prebiotic effects and other health related benefits (Ranawana, 2010). By supporting the growth of beneficial gut bacteria, inulin keeps the gut bacteria balanced and may have various health benefits especially towards wellbeing of colon, through the fermentation of inulin into butyrate it reduces precancerous colon growths (Gonçalves and Martel, 2013), reduction in colonic inflammation (Hijová et al., 2013) and creates a colon environment to be less favorable for cancer development (Boutron-Ruault et al., 2005, Leenen and Dieleman, 2007). Therefore, in the current study we have encapsulated morin with inulin by spray drying, so the encapsulated product can impart benefits of morin and inulin and since inulin is resistant to digestion in the gastrointestinal tract other than in large intestine, it is assumed that morin can reach the large intestine safely. On reaching the intestine, inulin will be acted upon by colonic microbiota and morin will be released. The morin content in the spray dried product was quantified by HPLC method.

The *in vivo* study was carried out using AOM-induced mice colon carcinogenesis model. It illustrates a well appreciated model, sharing many of the molecular, pathological and clinical features with sporadic human colorectal cancer (Perše and Cerar, 2010). In the present study, results from histopathological analysis clearly revealed that morin and encapsulated morin did not cause any pathological variation in liver, further confirming their nontoxic nature.

Weight loss is a feature associated with cancer and is due to loss of both skeletal muscle mass and adipose tissue. It is considered as a prognostic factor in cancer; higher the degree of weight loss, lesser the survival time (Dhanapal et al., 2011, Petruzzelli and Wagner, 2016). In our study we observed that, AOM treatment resulted in a noticeable decrease in the body weight gain percentage (%) compared to untreated control mice and treatment with standard drug, morin and encapsulated morin significantly improved the body weight gain percentage as compared to mice in the carcinogen treated group. Also, it was noticed that mice treated with encapsulated morin showed a significant body weight gain than that of mice treated with morin alone. A similar study by Rajendran et al., (Rajendran et al., 2018) showed amelioration of 1, 2 dimethylhydrazine induced tumour promotion response by novel benzimidazole derivative nanoparticle in Wistar rats was accompanied by improvement in body weight.

Colon carcinogenesis mainly includes hyper proliferation of crypt cells and formation of aberrant crypt foci (ACF), the premalignant precursors during colon carcinogenesis (Bird, 1995) and is considered as the 'gold standard' among various colon carcinogenesis biomarkers (Takayama et al., 1998). It was found that the treatment with standard drug, morin and combination of morin and inulin for 8 weeks could significantly reduce the azoxymethane induced aberrant crypt formation. It was also noticed that the number of ACF formed in mice treated with encapsulated morin was significantly lower than that of mice treated with morin alone. As adenomatous changes in the colon is associated with alteration in colon length as well as colon weight/length ratio, in our study we have evaluated both these parameters and found that morin as well as encapsulated morin could significantly inhibit carcinogen induced alterations these parameters. This was in line with study conducted by Yun

Tian et al., (Tian et al., 2013) where they observed administration of dietary glutamine resulted in an attenuation of signs of dextran sulfate sodium (DSS)/azoxymethane (AOM)-induced colitis-associated CRC alterations in colon length, colon weight and microscopic features of colon. Another study by Chari et al., (Chari et al., 2018) showed also have shown similar results.

There exist several mechanisms for bringing about anticancer potential by drugs and induction of apoptosis is regarded as one of the major way among them. Caspases, the cysteine proteases play a critical role in the apoptosis execution. Several chemopreventive and chemotherapeutic agents have been revealed to cause apoptotic cell death through caspases activation (Chun-Guang et al., 2010). The study by Lee et al., (Lee et al., 2014) showed destruxin B from green muscardine fungus induced anticolorectal cancer activity in tumour xenografts mice models and was found to be associated with increased expression of Bax, cleaved poly (ADP-ribose) polymerase and active caspase 3. Antitumor activity of KT2 peptide, derived from leukocyte peptide of crocodile against human HCT116 colon cancer xenografts was found to be associated with increased the expression of apoptotic proteins like Bax, cleaved caspase 3 and poly (ADP-ribose) polymerase (Maraming et al., 2018). It is also reported that oxysophoridine, an alkaloid extracted from *Sophoraalopecuroides* L. inhibited the growth of the transplanted mouse CT26 tumour tissue by upregulating the expression of Bax, caspase 3 and cytochrome c and downregulating the expression of Bcl 2 (Jin et al., 2017). Deltonin (steroidal saponin) isolated from *Dioscorea zingiberensis*, on oral administration significantly inhibited the tumour growth and prolonged survival of the tumour bearing mice by inducing apoptosis in tumour tissue, which was found to be associated with increased levels of Bax, activated caspase 3,

caspase 9, and cleaved poly (ADP ribose) polymerase, decreased pro-caspase 8, pro-caspase 9, Bcl 2 expression levels (Tong et al., 2011).

Similarly, in the present study western blot analysis was performed and the immunoblot results clearly revealed that 5-fluorouracil, morin and morin along with inulin treatment could counter act with carcinogenic potential of azoxymethane, by inducing apoptosis, as evidenced by the increased levels of cleaved caspse 3, cleaved PARP and Bax protein levels along with decreased levels of Bcl 2 level. Also, we could observe that mice treated with encapsulated morin showed significantly higher apoptotic related protein expression than that of mice treated with morin alone.

6.5. CONCLUSION

In research field, *in vivo* testing is often employed over *in vitro* because it is better suited for observing the overall effects of an experiment on a living subject. In the current study the results of the *in vivo* studies were in support of the *in vitro* results, showing that morin treatment could effectively alleviate the carcinogenic characteristics induced by AOM and it further confirms that morin can be regarded as a potential anti-colorectal cancer agent both in the prevention and management aspects. An interesting thing noticed in present study was that, when morin was encapsulated with inulin, the cancer alleviating properties were found to be enhanced and we assume that, in the encapsulated product, morin could reach the target area more safely as well as the fibre content in the encapsulated product may also contributing towards the anticancer efficacy imparted by the spray dried product. These results intern indicate the advantage of using dietary fibre as an encapsulant for the colon targeted delivery of drugs of interest.

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CHAPTER 7

Summary and Conclusion

Cancer is the one of the major and most threatening non-communicable disease with high mortality rate and has become the major cause of death among the population in developed countries as well as in developing countries. It is regarded as a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. The most common types of cancers that appears among males are lung cancer, prostate cancer, colorectal cancer and stomach cancer and among females, the most common types are cervical cancer, lung cancer, colorectal cancer and breast cancer.

Among numerous types of cancers, colorectal cancer is one of the most frequently occurring malignancies worldwide. It is the third most frequently diagnosed cancer after lung and breast cancers and the fourth most common cause of death. GLOBOCAN data reports that there were over 1.8 million new colorectal cancer cases and 881,000 deaths in 2018, accounting for about 1 in 10 cancer cases and deaths. It accounts for more than 10% of all cancer incidence and almost 8% of total cancer deaths. Around 56% of patients with CRC die from their cancer. The prevalence of CRC has amplified steadily in recent years and it is predicted that by the year of 2035 worldwide the number of CRC cases will rise to 1.36 million for men and 1.08 million for women. As the currently applied treatments for colorectal cancer (surgery, chemotherapy and radiotherapy) possess various adverse effects, scientific world is focussing on plant derived anticancer agents with lesser side effects and lesser cost. As dietary fibre can play a significant role in the prevention and management of colorectal cancer, if it is possible to reach both phytochemical and the dietary fibre simultaneously, it can really take part in the fight against colorectal cancer in both prevention as well as management aspect.

Compounds isolated from plants as well as various synthetic derivatives of these compounds from plants, due to their specific structure and wide spectrum of biological activities, are being suggested for treating various types of cancers. It has been reported that 25% to 48% of Food and Drug Administration (FDA) approved anticancer agents are plant origin in nature. In the present thesis work, few phytochemicals selected based on literature survey, were investigated for their anti-colorectal cancer potential. The first chapter gives an overview of colorectal cancer and risk factors associated with it as well as how dietary modifications help in the prevention and management of colorectal cancer.

In chapter 2, we have selected five phytochemicals (morin, zerumbone, luteolin, fisetin and genistein) and screened them for their anticancer efficacy against colorectal cancer and found that all of these phytochemicals are inducing reactive oxygen species mediated apoptosis. Morin and zerumbone exhibited potential activity and were studied further for apoptotic pathway and found that they induced apoptosis through both extrinsic as well as intrinsic pathway of apoptosis. Further to this, we have evaluated anticancer efficacy of morin and zerumbone on more advanced stage of colorectal cancer and found that morin exhibited appreciable anticancer efficacy in advanced stage colorectal cancer compared to zerumbone. Therefore, detailed investigations were carried out on the mechanisms of anticancer effect of morin. It was also found that morin is nontoxic to liver cells (Hep G2).

Accordingly, in chapter 3, morin was studied for its effect on Warburg effect in cancer cells. It is observed that, cancer cells mainly depend on glucose as the major energy source and produce ATP as well as other metabolites by aerobic glycolysis, which further facilitate tumour growth. In our study it was observed that morin could limit entry of glucose into the cells by decreasing GLUT 1 expression leading to energetic

stress in cancer cells as indicated by a drop in ATP level which and further trigger the induction of apoptosis. It was clear from western blotting that morin is bringing out this effect by blocking PI3K/Akt signaling pathway which is further supported by docking studies.

As there exists a close association between colorectal cancer and inflammation, in 4th chapter we have evaluated anti-inflammatory potential of morin in lipopolysaccharide (LPS) stimulated colorectal cancer cells. Pre-treatment with morin followed by LPS exposure to cells, could reduce the nuclear levels of Nrf2, a major chemo-resistance factor against ROS induced apoptosis in cancer cells. NF-κB is usually activated during inflammation and will increase the chemo resistance of cancer cells against various anticancer treatments. In the present study, the NF-κB nuclear translocation assay showed that LPS induced nuclear translocation of NF-κB was inhibited when colorectal cancer cells were pre-treated with morin. A decline in the level inflammatory markers were also noticed. It was also found that the major inflammatory signaling pathway, MAPK signaling pathway was suppressed significantly on treatment with morin.

Diagnosis of most of the colorectal cancer cases occurs after the initiation of metastasis and migration of colorectal cancer cells is one of the major challenge against successful recovery from colorectal cancer. Anticancer agents having anti-migratory potential is regarded as promising strategy towards treatment of colorectal cancer at this stage. In our 5th chapter, we evaluated the anti-migratory potential of morin on colorectal cancer cells and the results suggested that morin inhibits migration of colorectal cancer cells significantly by inhibiting Wnt/β-catein signaling pathway.

In research, *in vivo* testing is often employed over *in vitro* because it is better suited for observing the overall effects of an experiment on a living subject. Based on the

encouraging results on anticancer efficacy of morin against colorectal cancer from *in vitro* studies, further *in vivo* studies were carried out to confirm the results, which form the content of the 6th chapter. For this, we have encapsulated morin with soluble dietary fibre and hence the encapsulated product can impart benefits of morin as well as dietary fibre and we investigated the anticancer potential of morin and encapsulated morin in azoxymethane induced colorectal cancer models (Swiss albino mice). Results from the *in vivo* studies showed that morin as well as fibre encapsulated morin (more effectively) could alleviate the carcinogenic characteristics induced by the selected carcinogen. The *in vivo* studies further confirms that morin can be regarded as a potential anti-colorectal cancer agent both in the prevention and management aspects.

List of Publications

1. **Sithara T**, Arun KB, Syama HP, Reshmitha TR, Nisha P. Morin inhibits proliferation of SW480 colorectal cancer cells by inducing apoptosis mediated by reactive oxygen species formation and uncoupling of Warburg effect. Frontiers in pharmacology. 2017 Sep 12;8:640.
2. **Sithara T**, Dhanya BP, Arun KB, Sini S, Dan M, Kokkuvayil Vasu R, Nisha P. Zerumbone, a cyclic sesquiterpene from Zingiber zerumbet induces apoptosis, cell cycle arrest, and antimigratory effects in SW480 colorectal cancer cells. Journal of agricultural and food chemistry. 2018 Jan 16;66(3):602-12.
3. Arun KB, **Thomas S**, Reshmitha TR, Akhil GC, Nisha P. Dietary fibre and phenolic-rich extracts from Musa paradisiaca inflorescence ameliorates type 2 diabetes and associated cardiovascular risks. Journal of Functional Foods. 2017 Apr 1;31:198-207.
4. Reshmitha TR, **Sithara T**, Geethanjali S, Arun KB, Nisha P. DNA and mitochondrial protective effect of lycopene rich tomato (*Solanum lycopersicum* L.) peel extract prepared by enzyme assisted extraction against H_2O_2 induced oxidative damage in L6 myoblasts. Journal of Functional Foods. 2017 Jan 1;28:147-56.
5. Syama HP, **Sithara T**, Krishnan SL, Jayamurthy P. Syzygium cumini seed attenuates LPS induced inflammatory response in murine macrophage cell line RAW264. 7 through NF-κB translocation. Journal of Functional Foods. 2018 May 31;44:218-26.
6. Arun KB, Madhavan A, Reshmitha TR, **Thomas S**, Nisha P. Musa paradisiaca inflorescence induces human colon cancer cell death by modulating cascades of transcriptional events. Food & function. 2018;9(1):511-24.
7. Arun KB, Madhavan A, Reshmitha TR, **Sithara T**, Nisha P. (2019). Short chain fatty acids enriched fermentation metabolites of soluble dietary fibre from *Musa paradisiaca* drives HT29 colon cancer cells to apoptosis. Plos One. (Accepted)
8. Sini S, **Sithara T**, Lekshmi Krishnan, Sherin D.R, Manoj Kumar T.K, Jayamurthy P. (2019). Essential seed oil component in *Nigella sativa*

ameliorates hyperglycemia induced oxidative stress through the upregulation of Nrf2 in skeletal muscle cells. Journal of food science and technology. (Communicated)

9. B. Prabha, T. R. Reshmitha, M. Madhukrishnan, P. Sasikumar, Sithara T, P. Nisha and K. V. Radhakrishnan. (2019). Isolation and characterization of oligostilbenoids from *Vatica chinensis* L. and their ameliorative effect on H_2O_2 induced oxidative stress in H9c2 cardiomyoblasts. (Communicated)
10. **Sithara T**, Sini S, Arun KB, Nisha P. (2019). Impact of morin on inflammatory status of SW480 human colorectal cancer cells; role on NF-κB nuclear translocation and MAPK signaling pathways (Communicated)
11. **Sithara T**, Arun KB, Nisha P. (2019). Anti-migratory potential of morin on SW480 human colorectal cancer cells and role of Wnt/β-catenin signaling pathway. (Communicated)
12. **Sithara T**, Arun KB, Nisha P. (2019). Encapsulation of morin with dietary fiber (inulin) and *in vivo* evaluation of anticancer efficacy of the encapsulated product. (Communicated)
13. **Sithara T**, Sherin DR, Sini S, Arun KB, Nisha P. (2019). Morin uncouples the Warburg metabolic switch in SW480 human colorectal cancer cells by inhibiting PI3K/Akt signaling pathway. (Communicated)

Conference proceedings

1. A study on antioxidant, antidiabetic and prebiotic activity of jowar (*Soghum bicolor*) grains. International Conference on Food and nutrition challenges: Role of Food science and technology (26th ICFoST), 7-9th December, 2017 at CSIR-IICT, Hyderabad, India. **(Best Poster award)**
2. Anticancer effect of zerumbone, a cyclic sesquiterpene from *Zingiber zerumbet* against SW480 colorectal cancer cells by inducing apoptosis, cell cycle arrest and anti-migratory effect. International Conference on Advances in Degenerative Diseases and Molecular Interventions**,** 23 - 24 November 2017 at Hycinth, Thiruvananthapuram, India. **(Best Poster award)**
3. Ancient grains as a source of prebiotic dietary fiber. Workshop on Food Value Chain: Innovations and Challenges, March 17-18, 2016, organized by Department of Food Science and Technology, National Institute of Food Technology Entrepreneurship and Management (NIFTEM), Haryana, India. (Poster presentation)
4. Anticancer effect of flavonoid Morin on SW480 colorectal cancer cells by inducing apoptosis mediated by reactive oxygen species formation. International Conference on "Nutraceuticals and Chronic Diseases" September 9-11, 2016, Cochin, Kerala, India. (Oral presentation)
5. 8th Annual Meeting of Indian Academy of Biomedical Sciences and Conference on Deliberation on Translation of Basic Scientific Insights into Affordable Healthcare Products, February 25-27, 2019 at Agro-Processing and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram, India.