

**BIOASSAY-GUIDED ISOLATION OF THE ANTI-NEOPLASTIC
COMPOUND FROM *ELAEAGNUS CAUDATA* SCHLTDL.**

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
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DOCTOR OF PHILOSOPHY**

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COMPOUND FROM *ELAEAGNUS CAUDATA* SCHLTDL.

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Submitted
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CERTIFICATE

This is to certify that the thesis entitled “**Bioassay-guided isolation of the anti-neoplastic compound from *Elaeagnus caudata* Schltdl.**” submitted to Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Mr. F. Nghakliana** is a record of the research work carried by him during the period of 2019 – 2025 under my guidance and supervision, and this work has not formed the basis for the award of any other degree, diploma, associateship, fellowship, or other titles in this university or any other university or institution of higher education.

(DR. ZOTHANSIAMA)

DECLARATION

Mizoram University

April, 2025

I, F. Nghakliana, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Zoology.

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CONTENTS

	Page number
<i>Supervisors' Certificate</i>	<i>i</i>
<i>Declaration</i>	<i>ii</i>
<i>Acknowledgement</i>	<i>iii - v</i>
List of figures	1 – 11
List of tables	12
GENERAL INTRODUCTION	13 - 30
Definition and history of Cancer	13
Current scenario of cancer incidence and mortality	14
Hallmarks of Cancer	14
Pathological Classification of Cancer	17
Molecular and genetics of cancer	17
TNM Staging of cancer	18
Treatment modalities of cancer	19
Limitations of existing treatment modalities	20
Role of medicinal plants in chemotherapy	21
<i>Elaeagnus caudata</i> as a source of chemotherapeutic agent	21
The present thesis	22
References	25
CHAPTER 1: Preparation of <i>Elaeagnus caudata</i> extracts, estimation of their phytochemical content, and free radical scavenging activity	
Abstract	31
Introduction	32 – 34
Materials and Methods	35 – 41
Results	42 – 50
Discussion	51 – 54
References	55 – 61

CHAPTER 2: Preliminary screening of the anticancer activity of *Elaeagnus caudata*

Abstract	62
Introduction	63 – 64
Materials and Methods	65 – 69
Results	70 – 76
Discussion	77 – 79
References	80 – 84

CHAPTER 3: Bioassay-guided fractionation of crude extract of *Elaeagnus caudata* and anticancer activity of the active fraction

Abstract	85
Introduction	86 – 87
Materials and Methods	88 – 95
Results	96 – 124
Discussion	125 – 129
References	130 – 136

CHAPTER 4: Isolation, identification and anticancer activity of the lead compound of *Elaeagnus caudata*

Abstract	137
Introduction	138 – 140
Materials and Methods	141 – 147
Results	148 – 166
Discussion	167 – 170
References	171 – 176

CHAPTER 5: Identification of molecular targets of lead compound in colorectal carcinoma using integrated network pharmacology and molecular dynamic simulation

Abstract	177 - 178
Introduction	179 – 181
Materials and Methods	182 – 183
Results	184 – 203
Discussion	204 – 208
References	209 – 213

APPENDICES

Abbreviations	214 – 217
Bio-Data of Candidate	218
Awards & Distinctions	219
Conferences, Seminars and Symposium Attended	220
Workshops and Training Attended	221
Papers presented in Conferences, Seminars and Symposium	222 - 223
Research Publications	224 – 225
Book Chapters Published	226
Particulars of the Candidate	227

LIST OF FIGURES

Figure No.	Description	Page No
1.1.	<i>Elaeagnus caudata</i> Schltdl. (A) Fresh leaves (B) Dried leaves (C) Powdered leaves.	36
1.2.	(A) Soxhlet apparatus (B) Filtration of extracts (C) Dried extract residue.	37
1.3.	Standard graph of gallic acid.	42
1.4.	Phenolic content of extracts of <i>E. caudata</i> determined as gallic acid equivalent (mg/g extract). EC-AE: <i>E. caudata</i> aqueous extract; EC-ME: <i>E. caudata</i> methanolic extract. Values are expressed as mean $\pm$ SEM, n = 3. Different letters indicate significant variations at the highest dose (4 mg/ml).	43
1.5.	Standard graph of quercetin.	43
1.6.	Flavonoid content of extracts of <i>E. caudata</i> determined as quercetin acid equivalent (mg/g extract). EC-AE: <i>E. caudata</i> aqueous extract; EC-ME: <i>E. caudata</i> methanolic extract. Values are expressed as mean $\pm$ SEM, n = 3. Different letters indicate significant variations at highest dose (4 mg/ml).	44
1.7.	Plots of log-doses of (A) Aqueous extract (B) Methanolic extract of <i>E. caudata</i> and (C) the standard ascorbic acid against DPPH inhibition (%) for the calculation of IC ₅₀ .	45
1.8.	IC ₅₀ (μ g/ml) for DPPH of <i>E. caudata</i> extracts and standard ascorbic acid. EC-AE: <i>E. caudata</i> aqueous extract; EC-ME: <i>E. caudata</i> methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean $\pm$ SEM, n=3. Different letters indicate significant variation	45
1.9.	Plots of log-doses of (A) Aqueous extract (B) Methanolic extract of <i>E. caudata</i> and (C) the standard ascorbic acid against ABTS radical inhibition (%) for the calculation of IC ₅₀ .	46

1.10.	IC ₅₀ (µg/ml) for ABTS of <i>E. caudata</i> extracts and standard ascorbic acid. EC-AE: <i>E. caudata</i> aqueous extract; EC-ME: <i>E. caudata</i> methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.	47
1.11.	Plots of log-doses of (A) Aqueous extract (B) Methanolic extract of <i>E. caudata</i> extracts and (C) the standard ascorbic acid against superoxide radical inhibition (%) for the calculation of IC ₅₀ .	48
1.12.	IC ₅₀ (µg/ml) for superoxide radical inhibition of <i>E. caudata</i> extract and standard ascorbic acid. EC-AE: <i>E. caudata</i> aqueous extract; EC-ME: <i>E. caudata</i> methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.	48
1.13	Reducing power of <i>E. caudata</i> extracts. EC-AE: <i>E. caudata</i> aqueous extract; EC-ME: <i>E. caudata</i> methanolic extract; ASA: the standard ascorbic acid. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation at the highest dose (1000 µg/ml)	49
1.14	Anti-haemolytic activity of different concentrations (mg/ml) of <i>E. caudata</i> extract. EC-AE: <i>E. caudata</i> aqueous extract; EC-ME: <i>E. caudata</i> methanolic extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.	50
1.15.	Lipid peroxidation inhibition of different concentrations of <i>E. caudata</i> extracts. EC-AE: <i>E. caudata</i> aqueous extract; EC-ME: <i>E. caudata</i> methanolic extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.	50
2.1.	Plot of log-doses of <i>E. caudata</i> crude extracts against % inhibition of A549 cells after (A) 24 h (B) 48 h, and (C) 72 hr for the calculation of IC ₅₀ . EC-ME: <i>E. caudata</i> methanolic extract; EC-AE: <i>E. caudata</i> aqueous extract.	70

2.2.	IC ₅₀ of the crude extract of <i>E. caudata</i> against A549 cells after 24, 48, and 72 h treatment. EC-ME: <i>E. caudata</i> methanolic extract; EC-AE: <i>E. caudata</i> aqueous extract. *** indicates p < 0.001.	71
2.3.	Acridine orange/Ethidium bromide (Ao/EtBr) dual staining of A549 cells after treatment with 80 µg/ml of methanolic extract of <i>E. caudata</i> for 24 h. Control: A549 cells without treatment. EC-ME: Methanolic extract of <i>E. caudata</i> .	72
2.4.	Percentage of apoptotic cells after treatment of A549 with methanolic extract of <i>E. caudata</i> . Control: A549 cells without treatment. EC-ME: Methanolic extract of <i>E. caudata</i> . Values are expressed as Mean ± SEM. ***p<0.001 compared with the untreated control.	72
2.5.	Effects of methanolic extract of <i>E. caudata</i> (EC-ME; 80 µg/ml) on: (A) glutathione (GSH) level; (B) glutathione-s-transferase (GST) activity; (C) superoxide dismutase (SOD) activity; (D) malondialdehyde (MDA) level to assess lipid peroxidation. Values are expressed as Mean ± SEM. Means not sharing the same letter are significantly different.	73
2.6.	Florescence images of comets observed in control and A549 cells treated with 80 µg/ml of methanolic extract of <i>E. caudata</i> . Control: A549 cells without treatment; EC-ME treated: A549 cells treated with 80 µg/ml of methanolic extract of <i>E. caudata</i> .	74
2.7.	The extent of DNA damage expressed in terms of (A) Tail length and (B) Olive tail moment. Control: A549 cells without treatment; EC-ME: A549 cells treated with 80 µg/ml of methanolic extract of <i>E. caudata</i> . * and ** indicates significant variations at p < 0.05 and p < 0.01 respectively.	75
2.8.	Effects of the methanolic extract of <i>E. caudata</i> on Caspase 6 activity in A549 cells after 24 h treatment. Control: A549 cells without treatment: EC-ME: A549 cells treated with 80 µg/ml of	76

	methanolic extract of <i>E. caudata</i> . ** $p < 0,01$ compared with the untreated control.	
3.1.	Log-dose plotted against % inhibition of different fractions of <i>E. caudata</i> for the calculation of IC_{50} against HCT116 cells (A & B) and A549 cells (C & D).	96
3.2.	IC_{50} of different fractions of <i>E. caudata</i> against HCT116 cells (A) and A549 cells (B). Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	97
3.3.	LC-HRMS chromatogram analysis of B2 fraction of <i>E. caudata</i>	98
3.4.	Molecular structures of compounds identified from the B2 fraction of <i>E. caudata</i>	99
3.5.	Acridine orange/Ethidium bromide (AO/EtBr) dual staining of HCT116 cells after treatment with different doses of B2 fraction for 24 h. Control: Cancer cells without treatment; B2-10, B2-25, and B2-50: Cancer cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of <i>E. caudata</i> respectively; 5FU: Cancer cells treated with 100 $\mu\text{g/ml}$ of 5FU (positive control).	100
3.6.	Acridine orange/Ethidium bromide (AO/EtBr) dual staining of A549 cells after treatment with different doses of B2 fraction for 24 h. Control: Cancer cells without treatment; B2-10, B2-25, and B2-50: Cancer cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of <i>E. caudata</i> respectively; 5FU: Cancer cells treated with 100 $\mu\text{g/ml}$ of 5FU (positive control).	101
3.7.	Apoptotic index of HCT116 cells (A) and A549 cells (B) after treatment with different doses of B2 fraction of <i>E. caudata</i> . Control: Cancer cells without treatment; B2-10, B2-25, and B2-50: Cancer cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of <i>E. caudata</i> respectively; 5FU: Cancer cells treated with 100 $\mu\text{g/ml}$ of 5FU (positive control). Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	101

3.8.	Fluorescent image of comet observed in HCT116 cells treated with different concentrations of B2 fraction of <i>E. caudata</i> . Control: HCT116 cells without treatment; 10, 20, and 50: HCT116 cells treated with 10, 25, and 50 µg/ml of B2 fraction of <i>E. caudata</i> respectively; 5FU: HCT116 cells treated with 100 µg/ml of 5FU.	102
3.9.	The extent of DNA damage induced by B2 fraction of <i>E. caudata</i> in HCT116 cells expressed in terms of tail length (A) and tail olive moment (B). Control: HCT116 cells without treatment; B2-10, B2-25, and B2-50: HCT116 cells treated with 10, 25, and 50 µg/ml of B2 fraction of <i>E. caudata</i> respectively; 5FU: HCT116 cells treated with 100 µg/ml of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.	103
3.10.	Fluorescent image of comet observed in A549 cells treated with different concentrations of B2 fraction of <i>E. caudata</i> . Control: A549 cells without treatment; 10, 20, and 50: A549 cells treated with 10, 25, and 50 µg/ml of B2 fraction of <i>E. caudata</i> respectively; 5FU: A549 cells treated with 100 µg/ml of 5FU.	103
3.11.	The extent of DNA damage induced by B2 fraction of <i>E. caudata</i> in A549 cells expressed in terms of tail length (A) and tail olive moment (B). Control: A549 cells without treatment; B2-10, B2-25, and B2-50: A549 cells treated with 10, 25, and 50 µg/ml of B2 fraction of <i>E. caudata</i> respectively; 5FU: A549 cells treated with 100 µg/ml of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.	104
3.12.	Effects of B2 fraction of <i>E. caudata</i> on the levels and activities of GSH (A), GST (B), SOD (C), and MDA (D) of HCT116 cells. Control: HCT116 cells without treatment; B2: HCT116 cells treated with 25 µg/ml of B2 fraction of <i>E. caudata</i> ; 5FU: HCT116 cells treated with 100 µg/ml of 5FU (positive control). Values are	105

	expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	
3.13.	Effects of B2 fraction of <i>E. caudata</i> on the levels and activities of (A) GSH (B) GST (C) SOD and (D) MDA of A549 cells. Control: A549 cells without treatment; B2: A549 cells treated with 25 μ g/ml of B2 fraction of <i>E. caudata</i> ; 5FU: A549 cells treated with 100 μ g/ml of 5FU (positive control). Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variations.	106
3.14.	Effects of B2 fraction of <i>E. caudata</i> on activities of (A) Caspase-3 in HCT116 cells; (B) Caspase-6 in HCT116 cells; (C) Caspase-3 in A549 cells; and (D) Caspase-6 in A549 cells after 24 h treatment. Control: Cancer cells without treatment; B2: Cancer cells treated with 25 μ g/ml of B2 fraction of <i>E. caudata</i> ; 5FU: Cells treated with 100 μ g/ml of 5FU. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	107
3.15.	Oral Bioavailability chart of selected phytochemicals. The coloured zone is the suitable physicochemical space for oral bioavailability. LIPO: Lipophilicity; SIZE: Molecular size; POLAR: Polarity; INSOLU: Insolubility; INSATU: Insaturation and FLEX: Flexibility	109
3.16.	Venn diagram of proteins involved in NSCLC and CRC targeted by Amorphigenin. NSCLC: Non-Small Cell Lung Cancer; CRC: Colorectal Cancer; APG: Amorphigenin	113
3.17.	Protein-Protein Interaction (PPI) network of 73 protein targets	114
3.18.	The top 10 potential target networks ranked by degree value as generated by Cytoscape 3.10.2.	115
3.19.	Expression of HSP90AA1 in (A) Colorectal Adenocarcinoma (B) Non-Small Cell Lung Cancer	116
3.20.	Expression patterns of HSP90AA1 based on individual cancer stages in (A) Colorectal cancer (B) Non-Small Cell Lung carcinoma	116

3.21.	Expression patterns of HSP90AA1 based on nodal metastasis status in (A) Colorectal cancer (B) Non-Small Cell Lung carcinoma.	117
3.22.	Kaplan-Meier survival plot for HSP90AA1 expression in (A) Colorectal Carcinoma and (B) Non-Small cell lung carcinoma	117
3.23.	3D structure of HSP90 showing binding site (left) and the main residues involved in the HSP90- (A) Amorphigenin and (B) Geldanamycin (control) interaction with bond type and length (right).	119
3.24.	RMSD profile of HSP90-Amorphigenin complex compared to control HSP90-Geldanamycin complex for 100 ns of MD simulation.	121
3.25.	RMSF profile of HSP90 and Amorphigenin complex compared to control HSP90-Geldanamycin complex for 100 ns of MD simulation.	121
3.26.	Number of hydrogen bond formation in HSP90-Amorphigenin complex compared to control HSP90-Geldanamycin complex for 100 ns of MD simulation.	122
3.27.	Solvent Accessible Surface Area between HSP90-Amorphigenin complex compared to control HSP90-Geldanamycin complex for 100 ns of MD simulation.	122
3.28.	gmx_MMPBSA analysis showing time series plot.	123
3.29.	gmx_MMPBSA analysis showing energy decomposition bar plot.	124
4.1.	Log-dose plotted against % inhibition of different sub-fractions of B2 fraction for the calculation of IC ₅₀ against HCT116 cells.	148
4.2.	IC ₅₀ of sub-fractions of B2 fraction against HCT116 cells. Values are expressed as Mean ± SEM. *** indicates p < 0.001.	149
4.3.	Chemical structure of the isolated compound.	151
4.4.	¹ H NMR spectra of the compound isolated.	152
4.5.	¹³ C NMR spectra of the compound isolated.	152

4.6.	Carbon and hydrogen peaks of the isolated compound based on CHNS(O) analysis.	153
4.7.	Oxygen peak of the isolated compound based on CHNS(O) analysis.	153
4.8.	FTIR spectra of the isolated compound.	154
4.9.	Log-dose plotted against % inhibition of 6, 2'-dihydroxyflavone for the calculation of IC ₅₀ against HCT116 cells.	155
4.10.	IC ₅₀ of 6, 2'-dihydroxyflavone against HCT116 cells after 6, 12, and 24 h treatment. 6, 12, 24: HCT116 cells treated with different doses of 6, 2'-dihydroxyflavone for 6, 12 and 24 hours respectively; 5FU-24: HCT116 cells treated with different concentration of 5FU (positive control) for 24 hours. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	156
4.11.	Acridine orange/Ethidium bromide (AO/EtBr) dual staining of HCT116 cells after treatment with different doses of 6, 2'-dihydroxyflavone for 24 h. Control: HCT116 cells without treatment; 0.5 mM, 1.5 mM, and 2.5 mM: HCT116 cells treated with 0.5, 1.5 and 2.5 mM of 6, 2'-dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5FU (positive control) for 24 h.	157
4.12.	Apoptotic index of HCT116 cells after treatment with different doses of 6, 2'-dihydroxyflavone. Control: HCT116 cells without treatment; 0.5, 1.5 and 2.5: HCT116 cells treated with 0.5 mM, 1.5 mM and 2.5 mM of 6, 2'-dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	157
4.13.	Fluorescent image of comet observed in HCT116 cells treated with different concentrations of 6, 2'-dihydroxyflavone. Control: HCT116 cells without treatment; 0.5 mM, 1.5 mM and 2.5 mM:	158

	HCT116 cells treated with 0.5, 1.5 and 2.5 mM of 6, 2'-dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h.	
4.14.	The extent of DNA damage induced by 6, 2'-dihydroxyflavone in HCT116 cells expressed in terms of (A) tail length and (B) olive tail moment. Control: HCT116 cells without treatment; 0.5, 1.5 and 2.5: HCT116 cells treated with 0.5 mM, 1.5 mM and 2.5 mM of 6, 2'-dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	159
4.15.	Effects of 6, 2'-dihydroxyflavone on the levels and activities of (A) GSH, (B) GST, (C) SOD, and (D) MDA of HCT116 cells. Control: HCT116 cells without treatment; 0.5, 1.5 and 2.5: HCT116 cells treated with 0.5 mM, 1.5 mM and 2.5 mM of 6, 2'-dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	161
4.16.	Quantification of the expression levels of pro-apoptotic genes (A) <i>Bax</i> , (B) <i>Bid</i> , (C) <i>p53</i> , and (D) <i>Apaf-1</i> relative to GAPDH in HCT116 cells. Control: HCT116 cells without treatment; 6, 2'-DHF: HCT116 cells treated with 1.5 mM of 6, 2'-dihydroxyflavone for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	162
4.17.	Quantification of the expression levels of anti-apoptotic genes (A) <i>Bcl-X_L</i> , (B) <i>Bcl-2</i> , (C) <i>Survivin</i> , and (D) <i>PARP</i> relative to GAPDH in HCT116 cells. Control: HCT116 cells without treatment; 6, 2'-DHF: HCT116 cells treated with 1.5 mM of 6, 2'-	163

	dihydroxyflavone for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	
4.18.	Effect of 6, 2'-dihydroxyflavone on the activity of Caspase 3 in HCT116 cells. Control: HCT116 cells without treatment; 6,2'-DHF: HCT116 cells treated with 1.5 mM of 6, 2'-dihydroxyflavone for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.	164
4.19.	Effect of 6, 2'-dihydroxyflavone on the activity of Caspase 6 in HCT116 cells. 6,2'-DHF: HCT116 cells treated with 1.5 mM of 6, 2'-dihydroxyflavone for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. ns: statistically not significant.	165
5.1.	Pie-chart representing different classes of proteins that could be targeted by 6, 2'-Dihydroxyflavone	184
5.2.	Venn Diagram of potential colorectal carcinoma genes targeted by 6, 2'-Dihydroxyflavone. 6,2'-HDF – 6, 2'-Dihydroxyflavone, CC – Colorectal Carcinoma	185
5.3.	Compound-target-network Pathway (CTP) of 6,2'-Dihydroxyflavone in targeting colorectal carcinoma	189
5.4.	Protein-Protein Interaction Network of 98 gene targets generated by STRING database.	190
5.5.	The top 10 potential target networks ranked by degree value as generated by Cytoscape 3.10.2 with interconnections.	191
5.6.	Net plot of Gene Ontology (Biological Processes) analysis of core genes.	192
5.7.	Net plot of Gene Ontology (Cellular Components) analysis of core genes.	193
5.8.	Net plot of Gene Ontology (Molecular Functions) analysis of core genes.	193

5.9.	KEGG Pathway enrichment analysis of core genes	195
5.10.	6, 2'-Dihydroxyflavone may exerts its anticancer effect via the EGFR/RAS/ERK and the EGFR/PI3K pathway in colorectal carcinoma	196
5.11.	Docking interaction of 6,2'-Dihydroxyflavone with EGFR.	198
5.12.	Docking interaction of Afatinib (control) with EGFR	198
5.13.	RMSD profile of EGFR-6,2'-Dihydroxyflavone complex compared to control EGFR-Afatinib complex for 100 ns of MD simulation.	199
5.14.	RMSF profile of EGFR-6,2'-Dihydroxyflavone complex compared to control EGFR-Afatinib complex for 100 ns of MD simulation.	200
5.15.	Hydrogen bond formation of EGFR-6,2'-Dihydroxyflavone complex compared to control EGFR-Afatinib complex for 100 ns of MD simulation.	201
5.16.	Solvent Accessible Surface Area of EGFR-6,2'-Dihydroxyflavone complex compared to control EGFR-Afatinib complex for 100 ns of MD simulation.	202
5.17.	gmx_MMPBSA binding free energy of the EGFR-6,2' Dihydroxyflavone complex	203

LIST OF TABLES

Table No	Description	Page No
1.1.	Percentage yield of extracts of <i>E. caudata</i> .	42
3.1.	Compounds identified in the B2 fraction of <i>E. caudata</i> using LC-MS.	98
3.2.	Drug-likeness analysis of drug candidates. ‘Yes’, indicate zero violation (A) 4’- Hydroxyflavone (B) 6,2’-Dihydroxyflavone (C) Aloe Emodin 8-Glucoside (D) Amorphigenin (E) Bergapten (F) Chrysoeriol – 7 – O – Glucoside (G) Genipin (H) Halocynthiaxanthin (I) Harman (J) Pinocembrin (K) Sinapic Acid (L) Vanillic Acid	108
3.3.	Physicochemical properties of phytocompounds based on SwissADME prediction. (A) 4’- Hydroxyflavone (B) 6,2’- Dihydroxyflavone (C) Aloe Emodin 8-Glucoside (D) Amorphigenin (E) Bergapten (F) Chrysoeriol – 7 – O – Glucoside (G) Genipin (H) Halocynthiaxanthin (I) Harman (J) Pinocembrin (K) Sinapic Acid (L) Vanillic Acid.	110
3.4.	ADMET analysis of Genipin and Amorphigenin using PkCSM software	111
4.1.	Primer sequences used for qRT-PCR.	147
4.2.	¹³ C NMR and ¹ H spectral data of isolated compound.	150
5.1.	Mutual genes matched between 6, 2’-Dihydroxyflavone and Colorectal cancer in alphabetical order.	186
5.2.	Top 10 potential target rank and degree values as generated by Cytoscape 3.10.2.	189

GENERAL INTRODUCTION

1. Definition and history of Cancer

Cancer comprises a diverse array of diseases arising from genetic and epigenetic changes in cellular regulation and is characterised by uncontrolled cell growth and division, with the potential to invade surrounding tissues and metastasise to distant sites (Hanahan and Weinberg, 2011). Mutations in oncogenes, tumour suppressor genes, and genes associated with apoptosis facilitate the unregulated growth of cancer cells and the disturbance of cellular homeostasis (Vogelstein *et al.*, 2013). Cellular disruptions are significantly influenced by various factors, including genetic predisposition, exposure to environmental carcinogens, and lifestyle behaviors, rendering it a serious public health concern globally (Weinberg, 2013; Bray *et al.*, 2018)

Cancer has been identified since ancient times, with early references tracing back to around 3000 BCE in Egyptian medical documents like the Edwin Smith Papyrus, which detailed tumour-like formations and their basic treatment methods (Nunn, 2002). The Greek physician Hippocrates (460–370 BCE) coined the name ‘*karkinos*’ (carcinoma) to denote tumours, comparing them to the shape of a crab (Karpozilos and Pavlidis, 2004). Subsequently, Galen (129–216 CE) elaborated on these notions, ascribing the onset of cancer to an excess of black bile, a notion that persisted in significance for centuries (Hajdu, 2011).

Significant advancements in understanding cancer occurred during the Renaissance when the practice of autopsies and microscopic analysis became more widespread. In the 18th and 19th centuries, researchers such as Giovanni Battista Morgagni and Rudolf Virchow laid the groundwork for modern oncology by linking clinical symptoms with pathological findings from autopsies (Wolff and Herlyn, 2015). The 20th century yielded significant advancements with the identification of oncogenes and tumour suppressor genes, enhancing the molecular knowledge of cancer and facilitating the invention of personalised therapeutic strategies (Weinberg, 2013).

In the present day, advancements in precision medicine, immunotherapy, and molecular diagnostics continue to enhance cancer treatment, offering more effective

and personalized therapeutic options (Fitzmaurice *et al.*, 2019). Ongoing research aims to improve early detection, treatment strategies, and prevention efforts, reflecting the continuous evolution of cancer science.

2. Current scenario of cancer incidence and mortality

Cancer represents a significant global health challenge, with its incidence and mortality rates escalating in recent years. According to estimates from the International Agency for Research on Cancer (IARC), there were approximately 19.3 million new cancer cases and 10 million cancer-related deaths worldwide in 2020 (Bray *et al.*, 2024). The most commonly diagnosed cancers were breast, lung, colorectal, and prostate cancers, while lung cancer remained the leading cause of cancer mortality, followed by colorectal and liver cancers (Bray *et al.*, 2024).

In India, cancer poses a substantial public health burden. Data from the National Cancer Registry Programme (NCRP) indicate that the country reported an estimated 1.39 million new cancer cases and approximately 850,000 cancer-related deaths in 2020 (Indian Council of Medical Research, 2020). The most prevalent cancers among Indian men include those of the lung, oral cavity, and oesophagus, whereas among women, breast, cervix uteri, and ovary cancers are the most common. Projections suggest a significant increase in cancer incidence in the coming years; studies forecast a 12.8% rise in cancer cases by 2025 compared to 2020, underscoring the pressing need for enhanced cancer prevention and control strategies in the country (Kulothungan *et al.*, 2022).

3. Hallmarks of Cancer

Cancer is driven by a complex interplay of biological mechanisms that enable uncontrolled cell growth, invasion, and metastasis. Hanahan and Weinberg (2000, 2011) proposed a conceptual framework known as the "Hallmarks of Cancer," which outlines the fundamental traits acquired during tumour development. These hallmarks provide insights into the molecular and cellular alterations that drive cancer progression and have been widely accepted as a basis for understanding tumour biology.

The different hallmarks of cancer may be summarised as follows:

3.1. *Sustaining Proliferative Signalling*

Normal cells tightly regulate growth signals to maintain controlled proliferation. However, cancer cells bypass these controls by acquiring the ability to continuously stimulate their growth. They achieve this by overexpressing growth factors, activating oncogenes such as *RAS*, or disrupting tumour suppressor pathways (Hanahan and Weinberg, 2011). These alterations allow tumours to grow autonomously without the external signals that normal cells require for division.

3.2. *Evading Growth Suppressors*

Under normal conditions, tumour suppressor genes such as *TP53* and *RB* regulate cell cycle progression and prevent excessive cell division. Cancer cells, however, develop mechanisms to evade these regulatory checkpoints, often through genetic mutations or epigenetic modifications that inactivate tumour suppressor pathways (Vogelstein *et al.*, 2013). This allows them to bypass anti-proliferative signals that would otherwise halt their growth.

3.3. *Resisting Cell Death (Apoptosis Evasion)*

Programmed cell death, or apoptosis, is a crucial process that eliminates damaged or unneeded cells. Cancer cells, however, develop resistance to apoptosis by overexpressing anti-apoptotic proteins such as *BCL-2* or by downregulating pro-apoptotic factors like *BAX* (Pistritto *et al.*, 2016). This resistance enables cancer cells to survive despite genetic damage or exposure to cytotoxic agents.

3.4. *Enabling Replicative Immortality*

Most normal cells have a limited number of divisions due to the progressive shortening of telomeres, and protective caps at the ends of chromosomes. Cancer cells overcome this limitation by upregulating telomerase, an enzyme that maintains telomere length and allows indefinite replication (Shay and Wright, 2019). This ability to divide indefinitely contributes to tumour persistence and progression.

3.5. *Inducing Angiogenesis*

Tumours require a constant supply of oxygen and nutrients to sustain their growth. To meet this demand, cancer cells stimulate the formation of new blood vessels (angiogenesis) by overproducing angiogenic factors such as vascular

endothelial growth factor (*VEGF*) (Carmeliet and Jain, 2011). This ensures that the tumour receives sufficient resources to continue expanding.

3.6. *Activating Invasion and Metastasis*

Cancer cells acquire the ability to spread beyond their primary location by altering cell adhesion molecules, such as E-cadherin, and increasing motility through epithelial-mesenchymal transition (EMT) (Lambert *et al.*, 2017). These changes enable them to migrate into surrounding tissues, enter the bloodstream or lymphatic system, and establish secondary tumours in distant organs.

3.7. *Deregulating Cellular Metabolism*

Unlike normal cells, which primarily generate energy through oxidative phosphorylation, cancer cells switch to aerobic glycolysis (known as the Warburg effect) even in the presence of oxygen. This metabolic reprogramming supports rapid cell proliferation by providing essential biosynthetic precursors (Pavlova and Thompson, 2016).

3.8. *Avoiding Immune Destruction*

The immune system has natural mechanisms to recognize and eliminate abnormal cells. However, cancer cells develop strategies to evade immune surveillance by downregulating major histocompatibility complex (MHC) molecules, producing immunosuppressive cytokines, or upregulating immune checkpoint proteins such as PD-L1 (Chen and Mellman, 2017). These adaptations help tumours escape detection and destruction by immune cells.

3.9. *Tumour-Promoting Inflammation*

Chronic inflammation is closely linked to cancer progression, as inflammatory cells within the tumour microenvironment release cytokines and growth factors that support tumour growth and survival (Greten and Grivennikov, 2019). Inflammation also promotes genetic instability, facilitating further mutations that drive cancer evolution.

3.10. *Genome Instability and Mutation*

Cancer cells accumulate mutations at a higher rate due to defects in DNA repair mechanisms. These genetic alterations enable them to adapt and evolve, increasing their survival and resistance to therapy (Negrini *et al.*, 2010). Genomic instability is a

hallmark feature of cancer that contributes to tumour heterogeneity and disease progression.

4. Pathological Classification of Cancer

Cancers are classified based on their tissue of origin and cellular morphology as follows:

4.1. Carcinoma

These malignancies originate from epithelial tissues and account for most cancer cases, including lung, breast, prostate, and colorectal cancers (Weinberg, 2013). Carcinomas are further divided into adenocarcinomas, which arise from glandular epithelial cells, and squamous cell carcinomas, originating from flat epithelial cells (Fletcher *et al.*, 2013).

4.2. Sarcoma

These tumours develop from mesenchymal tissues, such as bone, muscle, and connective tissue. Examples include osteosarcoma (bone cancer) and leiomyosarcoma (smooth muscle cancer) (Fletcher *et al.*, 2013).

4.3. Leukaemia and Lymphomas

Leukaemia involves the malignant proliferation of hematopoietic cells in the bone marrow, leading to abnormal white blood cell production. Lymphomas affect lymphatic tissues and include Hodgkin's and non-Hodgkin's lymphoma (Campo *et al.*, 2011).

4.4. Neuroendocrine Tumours

These originate from hormone-producing cells that exhibit both endocrine and neural properties. Examples include carcinoid tumours and pancreatic neuroendocrine tumours (Modlin *et al.*, 2008).

5. Molecular and genetic basis of cancer

Cancer development is driven by genetic mutations and molecular alterations that disrupt normal cellular functions such as:

5.1. Oncogene Activation:

Mutations in genes such as *KRAS*, *BRAF*, and *MYC* promote uncontrolled cell proliferation and survival, leading to tumorigenesis (Vogelstein *et al.*, 2013).

5.2. Tumour Suppressor Gene Inactivation

Loss of function in genes like *TP53*, *RB1*, and *BRCA1* eliminates crucial regulatory mechanisms, allowing for unregulated growth and genomic instability (Sherr and McCormick, 2002).

5.3. Epigenetic Modifications

Changes such as DNA methylation and histone modifications can silence tumour suppressor genes or activate oncogenes, influencing cancer progression without altering the DNA sequence (Baylin and Jones, 2011).

6. TNM Staging of cancer

The TNM classification, developed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC), is the most widely used cancer staging system (Brierley *et al.*, 2017). Each category is assigned a numerical value, and the combination of T, N, and M designations determines the overall cancer stage. It assesses three primary components:

6.1. T (Tumor Size and Extent)

Describes the primary tumour's dimensions and invasion into adjacent tissues. It is graded from T0 (no evidence of tumour) to T4 (extensive local invasion).

6.2. N (Lymph Node Involvement)

Indicates whether cancer has spread to regional lymph nodes, ranging from N0 (no lymph node involvement) to N3 (extensive lymph node metastasis).

6.3. M (Distant Metastasis)

Determines whether cancer has spread to distant organs, classified as M0 (no metastasis) or M1 (metastatic disease).

7. Cancer Staging Groups

Based on the TNM classification, cancers are categorized into stages (Edge *et al.*, 2010):

7.1. Stage 0 (Carcinoma in situ)

Abnormal cells are confined to their tissue of origin and have not invaded surrounding structures.

7.2. Stage I (Localized Cancer)

The tumour is small and remains within the organ of origin.

7.3. Stage II and III (Locally Advanced Cancer)

The tumour is larger, has invaded nearby tissues, and may involve regional lymph nodes.

7.4. Stage IV (Metastatic Cancer)

Cancer has spread to distant organs, indicating an advanced stage with a poorer prognosis.

8. Treatment modalities of cancer

With the development of anaesthesia in the 19th century, surgery became a viable option for cancer treatment. William Halsted's radical mastectomy, introduced in the late 19th century, was one of the first systematic cancer surgeries, aiming to remove breast cancer along with surrounding lymph nodes (Halsted, 1894). While effective in localized cases, radical surgeries were often disfiguring and did not prevent metastatic spread. Modern advancements have since refined surgical techniques, favouring less invasive approaches, such as lumpectomy and minimally invasive laparoscopic procedures (Fisher *et al.*, 2002).

The discovery of X-rays by Wilhelm Roentgen in 1895 and radium by Marie and Pierre Curie in 1898 paved the way for radiation therapy. Early treatments involved direct exposure to radiation sources, though the lack of precision often led to severe side effects (Tubiana, 2009). Over the 20th century, technological advancements led to more focused radiation techniques, including intensity-modulated radiation therapy (IMRT) and proton therapy, which allow precise tumour targeting while sparing healthy tissues (Suit *et al.*, 2010).

Chemotherapy emerged in the mid-20th century following observations of nitrogen mustard's effects on lymphoma during World War II (Goodman and Gilman, 1946). This discovery led to the development of cytotoxic agents such as alkylating agents, antimetabolites, and anthracyclines, which target rapidly dividing cancer cells (DeVita and Chu, 2008). However, these treatments often cause significant side effects due to their impact on healthy proliferating cells.

The discovery of molecular pathways in cancer led to the development of targeted therapies, which selectively attack cancer cells while minimizing damage to normal tissues. One of the first successful targeted therapies was imatinib (Gleevec), which inhibits the *BCR-ABL* fusion protein in chronic myeloid leukaemia (Druker *et al.*, 2001). Other targeted therapies include monoclonal antibodies like trastuzumab (*HER2* inhibitor in breast cancer) and small-molecule inhibitors like *EGFR* inhibitors for lung cancer (Mok *et al.*, 2009).

Immunotherapy harnesses the body's immune system to fight cancer. Early research on immune responses to tumours led to the development of immune checkpoint inhibitors, such as pembrolizumab and nivolumab, which block *PD-1* and *CTLA-4* to enhance T-cell activity against cancer (Topalian *et al.*, 2012). CAR-T cell therapy, another breakthrough, involves genetically engineering a patient's T cells to target specific cancer antigens, showing remarkable success in hematologic malignancies (June *et al.*, 2018).

Recent cancer treatment strategies focus on personalized medicine, which tailors therapies based on individual genetic profiles (Gambardella *et al.*, 2020). Advances in genomics, artificial intelligence, and nanotechnology are revolutionizing drug development and delivery (Sherani *et al.*, 2024).

9. Limitations of existing treatment modalities

Despite significant advancements in cancer treatment, existing therapies present considerable limitations, including toxicity, resistance, and financial burden (Tran and Zafar, 2018). These challenges highlight the need for innovative treatment approaches that improve efficacy while minimizing adverse effects. Chemotherapy remains the cornerstone of cancer treatment but is associated with severe side effects due to its non-selective nature (Wu *et al.*, 2022). Cytotoxic agents target rapidly dividing cells, affecting both cancerous and normal tissues, leading to adverse effects such as immunosuppression, nausea, neuropathy, and organ damage (DeVita and Chu, 2008). Moreover, drug resistance—either intrinsic or acquired—reduces long-term effectiveness. Cancer cells develop resistance mechanisms such as increased drug efflux, enhanced DNA repair, and evasion of apoptosis, leading to treatment failure

(Holohan *et al.*, 2013). Thus, the discovery of newer treatment modalities is of urgent need.

10. Role of medicinal plants in chemotherapy

Medicinal plants have played a crucial role in the development of cancer chemotherapy, offering a rich source of bioactive compounds with potent anticancer properties (Asma *et al.*, 2012). Many plant-derived molecules have been successfully utilized as chemotherapeutic agents, either in their natural form or as templates for synthetic analogues. These compounds exhibit diverse mechanisms of action, including cell cycle arrest, induction of apoptosis, inhibition of angiogenesis, and modulation of key signalling pathways involved in cancer progression (Cragg and Pezzuto, 2016).

Alkaloids like vincristine and vinblastine from *Catharanthus roseus* disrupt microtubule formation, while camptothecin from *Camptotheca acuminata* inhibits topoisomerase I, leading to DNA damage (Jordan and Wilson, 2004; Pommier, 2006). Taxanes, such as paclitaxel from *Taxus brevifolia*, stabilize microtubules, preventing cell division (Rowinsky and Donehower, 1995). Podophyllotoxins from *Podophyllum peltatum* serve as precursors for etoposide, a topoisomerase II inhibitor (Hande, 1998). Flavonoids and polyphenols, including quercetin and resveratrol, exhibit antioxidant, anti-inflammatory, and pro-apoptotic properties (Jang *et al.*, 1997; Ren *et al.*, 2003). Terpenes like Betulinic acid from *Betula* species and curcumin from *Curcuma longa* modulate multiple signalling pathways, enhancing their anticancer potential (Fulda, 2009; Aggarwal *et al.*, 2007). These plant-derived compounds continue to serve as critical sources for drug development, improving cancer treatment efficacy.

11. *Elaeagnus caudata* as a source of chemotherapeutic agent

The *Elaeagnus* genus has gained attention for its potential anticancer properties due to its rich phytochemical composition, including flavonoids, alkaloids, terpenoids, and phenolic compounds. These bioactive constituents exhibit antioxidant, anti-inflammatory, and cytotoxic effects that can inhibit cancer cell proliferation and induce apoptosis. Phytochemical analysis of the *Elaeagnus* genus reveal a rich composition of bioactive compounds, including flavonoids, terpenoids, and phenolic

acids, which are associated with antioxidant and potential therapeutic properties (Nazir *et al.*, 2020). Species within the *Elaeagnus* genus, such as *Elaeagnus angustifolia* and *Elaeagnus umbellata*, have been recognized for their potential anticancer properties. Extracts from *E. angustifolia* have also shown cytotoxic effects against breast cancer cells, supporting their role in cancer treatment (Zarban *et al.*, 2023). Additionally, molecular docking studies suggest that key phytoconstituents from *E. umbellata* may interfere with cancer-related signalling pathways, further reinforcing its therapeutic potential (Pires *et al.*, 2020). Given these findings, the *Elaeagnus* genus presents a promising source of plant-based compounds for anticancer applications.

Among the *Elaeagnus* genus, *Elaeagnus caudata* remains an unexplored species despite its extensive traditional use. Ethnomedicinal studies have documented its traditional use among the Mizo tribes in Mizoram, India, where mature leaves are utilized for various healthcare purposes (Laldingliani *et al.*, 2022). It is commonly referred to as *Sarzuk* in Mizoram and has been utilized for the management of various health conditions, including the regulation of menstrual cycles, induction of labour, expulsion of retained placenta, and treatment of rheumatoid arthritis. Additionally, it has been traditionally used for addressing various malignancies (Rai and Lalramnghinglova, 2010). While specific pharmacological studies on *E. caudata* are limited, the documented traditional uses and phytochemical profiles of related species suggest potential medicinal applications. Therefore, it represents a promising candidate for the isolation of bioactive compounds with potential anticancer properties.

12. The present thesis

This thesis presents the analysis of the phytochemical composition of *Elaeagnus caudata*, along with a comprehensive investigation of its *in vitro* anticancer and pro-apoptotic activity against the human non-small cell lung carcinoma cell line (A549) and the human colorectal carcinoma cell line (HCT116). Furthermore, a bioassay-guided approach has been employed to facilitate the fractionation of the methanolic extract leading to the isolation of a novel flavonoid compound, which holds potential as a promising candidate for future chemotherapy development.

Additionally, the study explores the molecular targets and signalling pathways involved in the anticancer activity of the isolated compound through *in-silico* analysis.

The various studies conducted in this thesis have been disseminated into different chapters as follows:

Chapter 1: Preparation of *Elaeagnus caudata* extracts, estimation of their phytochemical content, and free radical scavenging activity

The leaves of *E. caudata* were subjected to exhaustive solvent extraction using Soxhlet Apparatus with various solvents in the order of their increasing polarity using Petroleum Ether, Chloroform, Methanol and finally distilled water. Quantitative phytochemical analysis was performed to estimate the total phenolic and flavonoid content in the methanolic and aqueous extracts. The antioxidative potential of the extracts was also assessed using both *in vitro* (DPPH, ABTS, Superoxide radical scavenging activity and Reducing power) and *ex vivo* assays (Inhibition of lipid peroxidation and haemolysis).

Chapter 2: Preliminary screening of the anticancer activity of *Elaeagnus caudata*

This chapter presents a preliminary evaluation of the anticancer and pro-apoptotic potential of the crude extract of *E. caudata* to assess their suitability as a source for the isolation of bioactive compounds with potential anticancer properties. The cytotoxicity of the extracts was analysed using MTT assay against A549 cells and the fraction with the lowest IC₅₀ values was subjected to apoptotic assays such as Ethidium bromide/Acridine orange (EtBr/AO) dual fluorescent staining, Comet Assay, and Caspase 6 activity. Biochemical analysis of the levels and activities of antioxidants such as Glutathione (GSH), Glutathione S-Transferase (GST), Superoxide Dismutase (SOD) and the level of Lipid Peroxidation (LPO) were also performed as per standard protocols.

Chapter 3: Bioassay-guided fractionation of crude extract of *Elaeagnus caudata* and anticancer activity of the active fraction

Based on the findings from Chapter 2, the methanolic extract was fractionated using column chromatography, yielding 17 fractions. Each fraction was evaluated for cytotoxicity against A549 and HCT116 cells using the MTT assay. The most potent

fraction was further analysed for apoptosis-inducing potential through EtBr/AO dual staining and the Comet assay. Additionally, the activities of GSH, GST, SOD, LPO, and caspases 3 and 6 were assessed to confirm apoptosis. Liquid Chromatography-High Resolution Mass Spectroscopy (LC-HRMS) was then employed to identify bioactive components, while *in silico* approaches, including ADMET analysis, network pharmacology, and molecular dynamics simulation, were used to determine potential molecular targets.

Chapter 4: Isolation, identification, and anticancer activity of the lead compound of *Elaeagnus caudata*

In this chapter, a bioassay-guided sub-fractionation of the most potent fraction was performed using column chromatography, resulting in the isolation of a novel flavonoid compound, whose identification and structural elucidation was performed with the help of ^1H and ^{13}C NMR, CHNS(O) analysis and FTIR. The anticancer activity and pro-apoptotic activity of the isolated compound were further investigated using functional assays against HCT116 cells as described in previous chapters. Furthermore, the differential expression of genes involved in apoptosis was also determined using Quantitative Real Time-PCR in the treated cancer cells.

Chapter 5: Identification of molecular targets of a lead compound in colorectal carcinoma using integrated network pharmacology and Molecular dynamic simulation

The final chapter focuses on the integration of network pharmacology and molecular dynamics simulation to elucidate the molecular targets and pathways underlying the anticancer activity of the isolated compound. Molecular targets were identified using SwissTargetPrediction, while genes associated with colorectal carcinoma were retrieved from the GeneCards database. The intersection of these gene sets was determined using the Jvenn tool, and core hub genes were identified through STRING analysis and Cytoscape software. Gene ontology and pathway enrichment analyses were conducted using the Kyoto Encyclopaedia of Genes and Genomes (KEGG). Finally, the inhibitory potential of the isolated compound against the primary molecular target was validated through molecular dynamics simulation.

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CHAPTER 1

Preparation of *Elaeagnus caudata* extracts, estimation of their phytochemical content, and free radical scavenging activity

ABSTRACT

Elaeagnus caudata, a medicinal plant traditionally used in Mizo folk medicine, was investigated for its phytochemical composition and antioxidative properties using various *in vitro* and *ex vivo* assays. Methanolic and aqueous extracts were prepared via Soxhlet extraction, and their phytochemical content were analysed using standard protocols. The methanolic extract exhibited a higher extraction yield (22.24%) than the aqueous extract (18.35%). The quantitative phytochemical analysis revealed significantly higher total phenolic content in the aqueous extract (259.32 ± 5.18 mg GAE/g) than in the methanolic extract (157.92 ± 4.1 mg GAE/g). Likewise, the flavonoid content was also higher in the aqueous extract (1869.25 ± 25.9 mg QE/g) than in the methanolic extract (1000 ± 12.93 mg QE/g). The antioxidative potential of *E. caudata* was also assessed using various assays. The aqueous extract possessed higher scavenging activities than the methanolic extract for DPPH, superoxide anions and ABTS^{•+} radicals with an IC₅₀ of 61.48 ± 1.06 µg/ml, 335.7 ± 0.61 µg/ml, and 372.2 ± 16.17 µg/ml, respectively. Consistently, the aqueous extract was also found to exhibit higher ferric-reducing power than the methanolic extract and the standard ascorbic acid. *Ex vivo* assays further validated the antioxidative effects. Anti-haemolytic activity showed the aqueous extract inhibiting 80.77% of haemolysis, significantly higher than the methanolic extract (43.48%). Lipid peroxidation inhibition in liver homogenates demonstrated an inhibition rate of 81.58% for the aqueous extract and 61.63% for the methanolic extract. These findings indicate that *E. caudata* possesses potent antioxidant properties, likely attributed to its rich phenolic and flavonoid content. The study highlights its potential as a source of natural antioxidants for pharmaceutical and nutraceutical applications. Therefore, further research is warranted to explore its bioactive compounds and other pharmacological significance of *E. caudata*.

1. INTRODUCTION

The utilization of plants for therapeutic purposes is as ancient as humanity itself, and their applications and documentation have progressed significantly throughout time (Petrovska, 2012). The emergence of functional foods and pharmaceutical products derived from medicinal and food plants has then resulted in advancements across many spheres of life (Dixit *et al.*, 2023). These varied therapeutic properties of plants have been attributed to the presence of a group of compounds known as phytochemicals. Phytochemicals are chemical compounds that help plants adapt to and survive various environmental challenges such as infections (Ahmed *et al.*, 2014). They also indirectly regulate important processes related to growth and reproduction (Saxena *et al.*, 2013).

Phytochemicals are widely recognized for their potent antioxidant properties and extensive medicinal potential, exhibiting antibacterial, antidiarrheal, anthelmintic, antiallergic, antispasmodic, anticancer, antiviral activities, etc., (Jaeger and Cuny, 2016; Sharma *et al.*, 2018). Among the various classes of phytochemicals, phenolic compounds represent the predominant group, accounting for approximately 45% of the total phytochemical content in plants, followed by terpenoids and steroids (27%), alkaloids (18%), and other constituents (10%) (Prakash *et al.*, 2011). Therefore, the comprehensive characterization of a plant's phytochemical profile is crucial for elucidating its pharmacological activities and underlying mechanisms.

Phytochemicals extracted and isolated from diverse sources have been significantly utilized in the formulations of functional foods and nutraceuticals, including those for antioxidative purposes (Kumar *et al.*, 2023). Antioxidants are described as chemicals that inhibit, prevent, or eliminate oxidative damage to specific target molecules by interacting with oxidants such as free radicals (Halliwell, 2024). Free radicals such as reactive oxygen species (ROS) originate from either normal metabolic process within the human body or external sources, including X-ray exposure, ozone, cigarette smoke, air pollution, drugs, infections, industrial toxins, etc., (Bagchi and Puri, 1998; Coulibaly *et al.*, 2014). They serve a dual function as both deleterious and advantageous, as they can be either detrimental or beneficial to the organism (Pham-Huy *et al.*, 2008). When present in low to moderate concentrations, reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen

peroxide (H_2O_2), hydroxyl radicals ($\cdot\text{OH}$), and singlet oxygen ($^1\text{O}_2$) are fundamental in modulating various physiological functions such as immune reactions and cellular signalling of the body (Droge, 2002). Normally, these ROS are neutralized by the antioxidant system of the body to maintain redox homeostasis. However, due to their highly reactive property, excessive ROS generation hampers the body's antioxidant defence systems, leading to a condition called 'oxidative stress' ensuing tissue damage (Stadtman, 1992). Oxidative stress is found to be closely associated with various lifestyle-related disorders such as neurodegenerative disorders, coronary heart disease, diabetes, inflammation, arthritis, lung damage, and cancer (Valko *et al.*, 2006; Seifried *et al.*, 2007).

Although cells are naturally equipped with an impressive repertoire of antioxidants, both enzymatic (such as superoxide, catalase, glutathione-s-transferase, and glutathione peroxidase) as well as non-enzymatic antioxidants (such as glutathione, ascorbic acid, and lipoic acid) to counterbalance ROS production, day to day exposure to exogenous ROS-generating agents often hampers the delicate balance of these antioxidants and free radicals (Kurutas, 2016). Therefore, maintaining optimal body function and cell redox homeostasis may sometimes require exogenous antioxidant supplementation. As such, plant-derived natural compounds have been identified as a viable source of potent natural antioxidants.

The genus *Elaeagnus*, commonly known as Silverberries, consists of deciduous evergreen shrubs which are mainly distributed in subtropical regions of Asia, Europe, and North America (Saboonchian *et al.*, 2014). *Elaeagnus caudata*, locally known as *Sarzuk* in Mizoram, has been traditionally used by the Mizo tribe in India to treat various health problems such as stopping menses, induction of labour, the expulsion of retained placenta, rheumatoid arthritis and for treating various malignancies (Rai and Lalramnghinglova, 2010). Among the genus *Elaeagnus*, *E. angustifolia* (Badrhadad *et al.*, 2010), *E. umbellata* (Paudel *et al.*, 2019), *E. pungens* (Zhao *et al.*, 2006), *E. rhamnoides* (Olas *et al.*, 2018) and *E. glabra* (Kim *et al.*, 2020) have been extensively studied and reported to have multi-pharmaceuticals activity such as anti-nociceptive, muscle relaxant, anti-angiogenic, cytotoxic, anti-tumorigenic, and anti-microbial. Bioactive groups including flavonoids (such as rutin, epigallocatechin gallate), phenolic compounds (such as ferulic acid, triterpenoids),

saponins (such as terpengustifol), and alkaloids (such as harmane and tetrahydroharmol) have also been isolated from various species of the *Elaeagnus* genus (Farzaei *et al.*, 2015; Zhu *et al.*, 2018).

Despite the extensive use of *E. caudata* as a traditional medicine by various tribes in different countries, scientific validation of its pharmaceutical properties is still very limited. Therefore, the present study was designed to conduct an investigation into the phytochemical constituents and antioxidative potential of *Elaeagnus caudata* aiming to provide valuable insights into its potential applications in pharmacology and therapeutics.

2. MATERIALS AND METHOD

2.1. Chemicals

Gallic acid, quercetin dihydrate, methanol, ferric chloride, methanol, chloroform, and sodium nitrite were obtained from HiMedia Laboratories Pvt., Ltd. (Mumbai, India). Glacial acetic acid, sulphuric acid, chloroform, and hydrochloric acid, were obtained from Sigma Aldrich Inc (Louis, Germany). Trichloroacetic acid (TCA), folin-ciocalteu's reagent, sodium hydroxide, sodium carbonate, and ascorbic acid were obtained from SD fine-chem Ltd. (Mumbai, India). Aluminium chloride, potassium iodide, bismuth subnitrate, and sodium dihydrogen phosphate were obtained from Merck Specialities Pvt., Ltd. (Mumbai, India).

2.2. Collection, identification and preparation of plant material

Elaeagnus caudata Schltdl., an important plant of the Mizo traditional medicine, was selected for this study. Healthy and non-infected leaves were collected from Serkawn, Lunglei District, Mizoram (22.9081° N, 92.7587° E). The identification and authentication of the plant was conducted by the Department of Horticulture, Aromatic and Medicinal Plants (HAMP), Mizoram University, Aizawl with voucher specimen number MZU/HAMP/23/034. The leaves were washed using distilled water and spread to dry in a well-ventilated dark room at room temperature. Hygienic conditions were followed during the drying process to prevent contamination by insects, fungi, and other foreign substances. Ambient air circulation was also maintained during the whole process. The drying process of all the collected plant material lasted for four months, during which constant monitoring was done to exclude contaminations, decomposition and other damages. The dried leaves were then finely ground into fine powder using an electrical grinder.

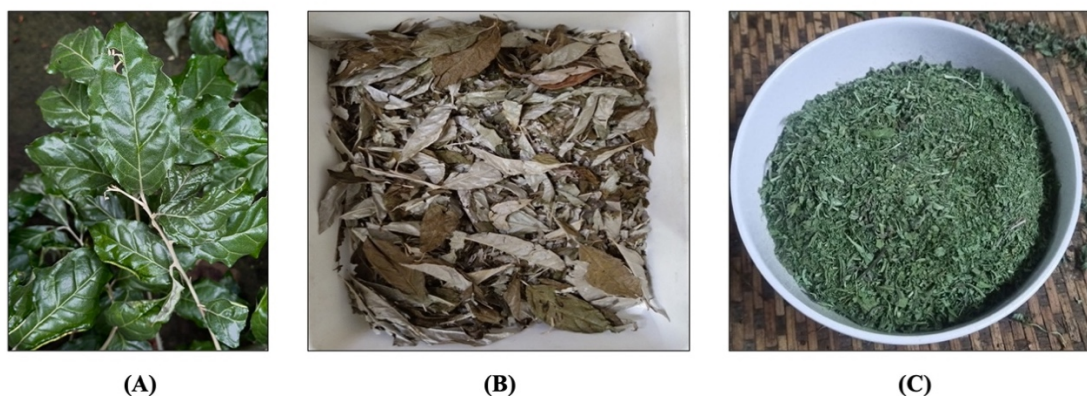


Figure 1.1. *Elaeagnus caudata* Schltdl. (A) Fresh leaves (B) Dried leaves (C) Powdered leaves.

2.3. Extraction of *E. caudata*

Extraction of the dried powdered plant material was done using a Soxhlet apparatus. For each batch, 150 g of the plant material was loaded into the thimble of the Soxhlet apparatus. A round bottom flask was also filled with the appropriate solvent, after which the whole apparatus was assembled. The entire apparatus was then positioned on a temperature-controlled heating mantle. Fat contents of the dried plant material were first washed using petroleum ether while the chlorophyll contents were washed using chloroform for 72 h each. This was followed by sequential extraction using methanol and distilled water as extractants for a minimum of 40 cycles using the Soxhlet apparatus. The liquid extracts were filtered and dried in a rotary evaporator (Buchi, Germany) under reduced pressure at 40°C. The extract residues obtained were then collected and stored at -20°C until use. Henceforth, the methanolic extract and aqueous extract of *E. caudata* will be called EC-ME and EC-AE respectively.

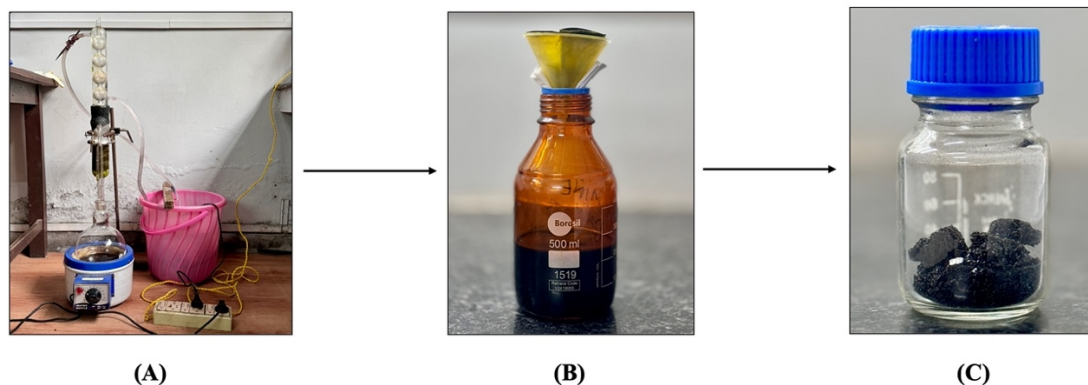


Figure 1.2. (A) Soxhlet apparatus (B) Filtration of extracts (C) Dried extract residue.

2.4. Calculation of extract yield

The total yield of the extracts was calculated using the equation below:

Yield (%) = Weight of the dry extract (residue) obtained/Weight of the powdered leaves before extraction $\times 100$

2.5. Phytochemical analysis

2.5.1. Estimation of total phenolic content

The total phenolic content of *E. caudata* was estimated using the standard method (Khan *et al.*, 2010). Briefly, 5 ml of Folin-Ciocalteu's reagent (diluted ten-fold) was mixed with 1 ml of *E. caudata* extracts (0.25 – 4.0 mg/ml). After 5 min of incubation, 4 ml of sodium carbonate (0.115 mg/ml) was added. The mixture was then incubated in the dark at room temperature for 2 h and the absorbance was recorded at 765 nm. The calibration curve was also prepared by mixing a methanolic solution of gallic acid (1 ml, 0.25 – 4.0 mg/ml) with the reagents above and absorbance was recorded at 765 nm using UV-Visible spectrophotometer (Spectramax M2e, Molecular devices). The experiment was repeated thrice and the total phenolic content was expressed as gallic acid equivalents (GAE) mg/g of the dry extract.

2.5.2. Estimation of total flavonoid content

The total flavonoid content of *E. caudata* extracts was also estimated according to the method of Khan *et al* (2010). Briefly, 0.25 ml of *E. caudata* extracts (0.25 – 4.0 mg/ml) and standard quercetin solution were mixed with 75 μ l of 5% (w/v) sodium nitrite solution and 1.25 ml of distilled water. After the addition of 150 μ l of

10% (w/v) aluminium chloride, the solution was allowed to stand for 5 min followed by the addition of 0.5 ml of 1 M NaOH. The volume of the solution was made up to 2.5 ml using distilled water. The absorbance was recorded immediately at 510 nm. The total flavonoid content of *E. caudata* was expressed in term of quercetin equivalent (mg/g extract).

2.6. Determination of free radical scavenging activity *in vitro*

The ability of the extracts of *E. caudata* to inhibit the generation of free radicals was carried out using various *in vitro* assays in a cell-free system as described below:

2.6.1. DPPH radical scavenging activity

The scavenging activity of *E. caudata* extracts for DPPH radicals was determined according to the method of Leong and Shui (2002) with minor modifications. Briefly, 0.5 ml of extracts of *E. caudata* (0.25 – 1000 µg/ml) was mixed with 1 ml of methanol solution of 0.1 M DPPH followed by 30 min incubation in the dark. The absorbance of the solution at 523 nm was compared with the control. The scavenging activity of the plant extract against DPPH radical was expressed as IC₅₀, which is the concentration (µg/ml) of extract at which 50% of the DPPH radicals were inhibited. Ascorbic acid (ASA) was used as the standard. The test was repeated at all concentrations of each sample in triplicate. The scavenging activity was then calculated based on the percentage of DPPH radicals scavenged using the formula:

$$\text{Scavenging (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

Where, A_{blank} is the absorbance of the control (solution containing all the reagents except the plant extracts) and A_{sample} is the absorbance of the solution containing the plant extract.

2.6.2. Superoxide radical scavenging activity

Superoxide scavenging activity was determined by the Nitroblue tetrazolium (NBT) reduction method with minor modifications (Hyland *et al.*, 1982). In brief, the reaction mixture was prepared using 0.2 ml of NBT (1 mg/ml in DMSO) and 0.6 ml of plant extract (0.25 – 1000 µg/ml). Then, the volume of the mixture was made up to 2.8 ml using 2 ml of alkaline DMSO (1 ml DMSO in 5 mM NaOH). The absorbance of the mixture was recorded at 560 nm and pure DMSO was used as blank. Ascorbic

acid (ASA) served as the standard and the ability of *E. caudata* extracts to scavenge the superoxide radical was calculated.

$$\% \text{ scavenging} = [(A_e - A_o) / A_e] \times 100$$

Where A_o is absorbance without plant extract and A_e is absorbance with the plant extract.

2.6.3. ABTS^{•+} radical scavenging activity

The scavenging activity of *E. caudata* against ABTS^{•+} was determined using the method of Khan *et al.*, 2010. Briefly, 5 ml each of 7 mM ABTS and 2.45 mM potassium persulfate were mixed to prepare a stock solution which was then incubated in the dark at room temperature for 12 h to yield a dark-coloured solution that contains ABTS^{•+} radicals. A freshly prepared working solution consists of a stock solution diluted with 50% methanol having an initial absorbance of 0.70 ± 0.02 at 745 nm. The ABTS^{•+} radical scavenging activity was then assessed by mixing 150 μ l of different concentrations of *E. caudata* extracts (0.25 – 1000 μ g/ml) with 1.5 ml of ABTS^{•+} working solution. The decrease in absorbance was measured immediately at 745 nm. The test was repeated at all concentrations of each sample in triplicate. Ascorbic acid (ASA) served as the standard. The scavenging activity of the plant extract was then calculated using the formula:

$$\text{Scavenging (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

Where A_{blank} is the absorbance of the control (solution containing all the reagents except the plant extracts) and A_{sample} is the absorbance of the solution containing the plant extract.

2.6.4. Ferric Reducing Assay

The reducing power of *E. caudata* extracts was estimated using standard method with minor modifications (Adjimani & Asare, 2015). Briefly, 2.5 ml each of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide solution were mixed with *E. caudata* extracts (25 – 1000 μ g/ml). After incubation of the mixture at 50°C for 20 min, 2.5 ml of 10% TCA was added. The mixture was then centrifuged at 3000 rpm for 10 min. An equal volume of distilled water was added to the supernatant

followed by 0.5 ml of 1% ferric chloride solution. The absorbance of the mixture was measured at 700 nm. The increase in absorbance indicated increasing the reducing power of the extract.

2.7. Determination of antioxidant activity *ex vivo*

2.7.1. Animals

A colony of inbred Swiss albino mice is being maintained under standard environmental conditions of temperature ($22^{\circ}\text{C} \pm 5^{\circ}\text{C}$) and light (12 h of light and dark, respectively) (Frontier Euro Digital Timer, Taiwan) at the Animal House, Department of Zoology, Mizoram University, India. The animals had free access to food and water. Caring and handling of animals were carried out based on the guidelines given by WHO, Geneva, Switzerland. The study was also approved by the Institutional Animal Ethical Committee, Mizoram University, India (No. MZU-IAEC/2018/09) and CPCSEA, New Delhi, India (Registration No. 1999/GO/ReBi/S/18/CPCSEA).

2.7.2. Anti-haemolytic activity

The inhibition of haemolysis in mice by *E. caudata* extracts was measured to determine their antioxidative potential using the standard protocol (Zhou *et al.*, 2014). Blood was collected from Swiss albino mice of the same age group (10 – 12 weeks) and body weights (25 – 30 g) by heart puncture in a heparinized tube. Haemolysis was induced with H_2O_2 which serves as a free radical initiator. A mixture was prepared by adding 0.5 ml of 5 % (v/v) suspension of RBC in PBS, 0.4 ml (0.5 mg/ml) of different extracts of *E. caudata* and 100 μl of 1 mol/L H_2O_2 . The reaction mixture was incubated at 37°C for 3 h. It was then diluted with 4 ml of PBS and centrifuged at 2000 rpm for 10 min. The supernatant was collected and absorbance was recorded at 540 nm. The rate of inhibition of haemolysis was then calculated using the formula:

$$\text{Inhibition rate (\%)} = [1 - (A_1 - A_2) / A_0] \times 100$$

Where A_0 is the absorbance of control, A_1 is the absorbance of the solution containing the plant extract and A_2 is the absorbance without RBC.

2.7.3. Inhibition of lipid peroxidation

The lipid peroxidation inhibitory potential of *E. caudata* extracts was measured according to the method of Rajinder *et al.*, (2010). A fresh liver was excised from a healthy Swiss albino mouse and 1% liver homogenate was prepared by weighing the required amount of liver tissue and pulverized in a tissue homogenizer with PBS. The mixture was then centrifuged at 3000 rpm at 4°C for 10 min. 0.5 ml of supernatant was mixed with 0.5 ml (0.5 mg/ml) of *E. caudata* extracts, 0.25 ml each of 0.5 mol/l FeCl₂ and H₂O₂ and incubated at 37°C for 1 h. Absorbance was measured at 535 nm and the rate of inhibition of lipid peroxidation was calculated using the formula:

$$\text{Inhibition rate (\%)} = [1 - (A_1 - A_2) / A_0] \times 100$$

Where, A₀ is the absorbance of control, A₁ is the absorbance of the solution containing the plant extract and A₂ is the absorbance without liver homogenate.

2.8. Statistical analysis

Results are expressed as mean ± standard error of mean. Student t-test was employed for comparison total phenolic content, total flavonoid content, anti-haemolytic and lipid peroxidation inhibition assay. One-way analysis of variance (ANOVA) followed by Tukey multiple comparisons of means was also performed to test the significant variations on the free radical scavenging activities using SPSS ver.20.0 software (SPSS Inc, Chicago, Illinois, USA). The IC₅₀ values were calculated by plotting the % inhibition against the log doses using GraphPad Prism software version 8.0. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Extract yield

Of the two solvents used for extraction, the methanolic extract showed a higher yield than the aqueous extract as presented in Table 1.1.

Table 1.1. Percentage yield of extracts of *E. caudata*.

Solvent	Powdered Sample	Residue obtained	% Yield
Methanol	3.32 kg	804.9 g	22.24 %
Aqueous	2.2 kg	403.7 g	18.35 %

3.2. Quantitative estimation of phytochemicals

3.2.1. Total Phenolic Content

The total phenolic content was estimated using a standard graph of gallic acid (Figure 1.3). The total phenolic content of the extracts revealed a dose-dependent increase in the phenolic content of both EC-AE and EC-ME (Figure 1.4). At 4 mg/ml, EC-AE has significantly higher ($p < 0.001$) total phenolic content (259.32 ± 5.18 mg GAE/g of dry extract) than that of EC-ME (157.92 ± 4.1 mg GAE/g of dry extract).

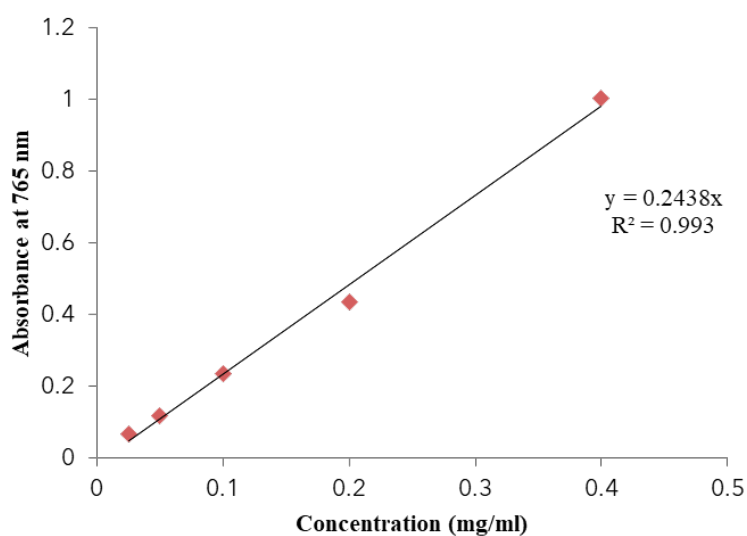


Figure 1.3. Standard graph of gallic acid.

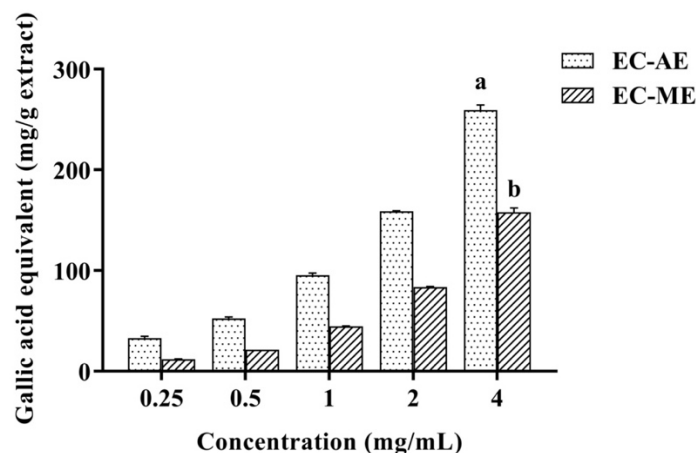


Figure 1.4. Phenolic content of extracts of *E. caudata* determined as gallic acid equivalent (mg/g extract). EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract. Values are expressed as mean $\pm$ SEM, $n = 3$. Different letters indicate significant variations at the highest dose (4 mg/ml).

3.2.2. Total flavonoid content

The total flavonoid content was also calculated using standard graph of quercetin (Figure 1.5). The total flavonoid content of *E. caudata* increases with an increase in the dose of *E. caudata* extracts (Figure 1.6). In comparison, at 4 mg/ml, the aqueous extract showed significantly higher ($p < 0.001$) total flavonoid content (1869.25 ± 25.9 mg QE/g of dry extract) compared to that of the methanolic extract (1000 ± 12.93 mg QE/g of dry extract).

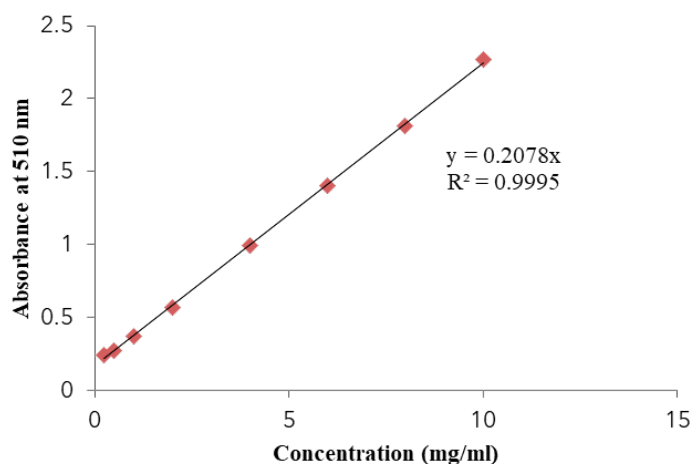


Figure 1.5. Standard graph of quercetin.

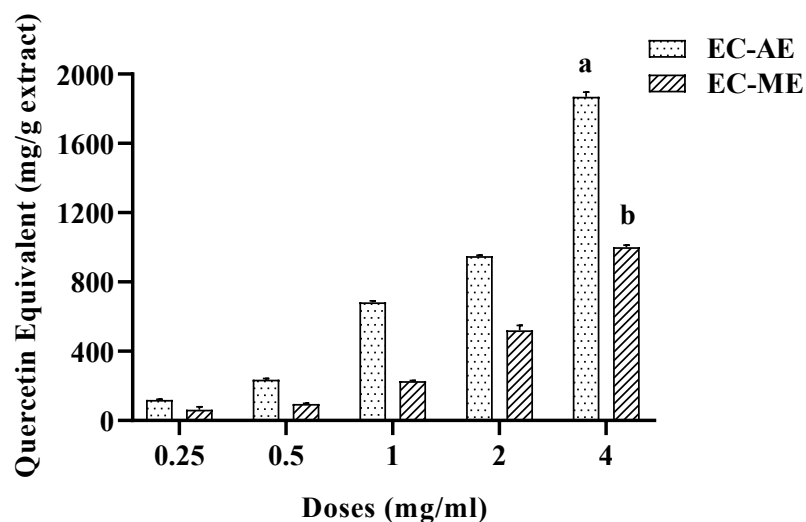


Figure 1.6. Flavonoid content of extracts of *E. caudata* determined as quercetin acid equivalent (mg/g extract). EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract. Values are expressed as mean $\pm$ SEM, $n = 3$. Different letters indicate significant variations at highest dose (4 mg/ml).

3.3. *In vitro* antioxidant assays

3.3.1. DPPH Radical Scavenging Assay

E. caudata extracts exhibited a dose-dependent increase in DPPH radicals scavenging activity as indicated by the discoloration of DPPH with the increase in the dose of *E. caudata*. Maximum scavenging was observed at the dose of 200 μ g/ml for both methanolic and aqueous extracts that declined thereafter. Log-doses of *E. caudata* extracts and the standard ascorbic acid (ASA) were plotted against DPPH inhibition (%) to determine their IC_{50} (Figure 1.7A-C). EC-AE possessed a higher scavenging activity than that of EC-ME. However, the standard ascorbic acid still possessed significantly higher scavenging activity ($p < 0.001$) than that of the *E. caudata* extracts (Figure 1.8).

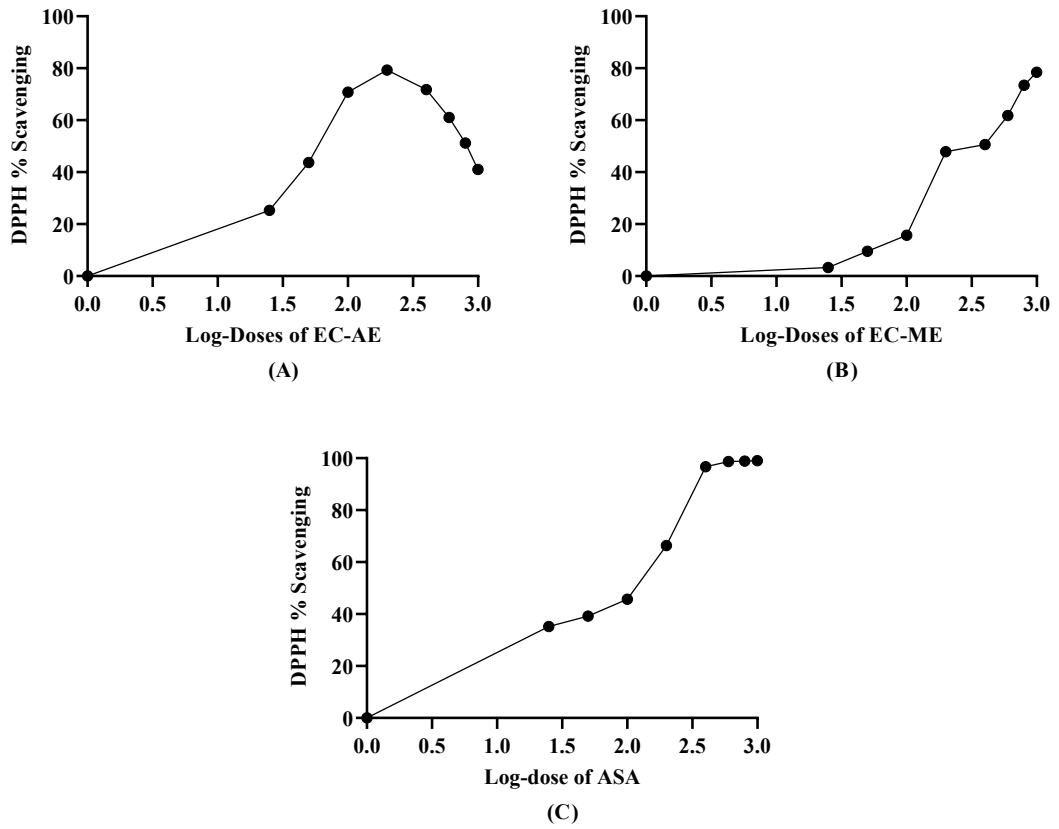


Figure 1.7. Plots of log-doses of (A) Aqueous extract (B) Methanolic extract of *E. caudata* and (C) the standard ascorbic acid against DPPH inhibition (%) for the calculation of IC_{50} .

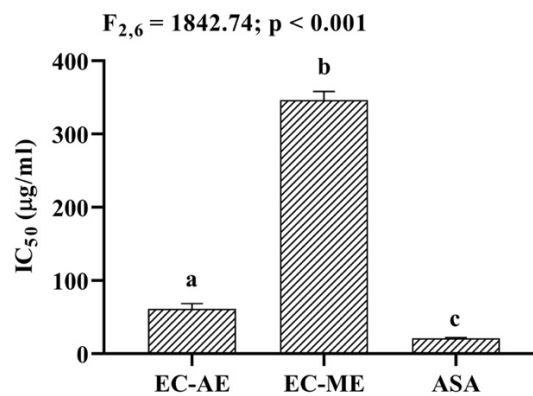


Figure 1.8. IC_{50} ($\mu\text{g/ml}$) for DPPH radicals scavenging activity of *E. caudata* extracts and standard ascorbic acid. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean $\pm$ SEM, $n=3$. Different letters indicate significant variation.

3.3.2. ABTS^{•+} radical scavenging activity

ABTS^{•+} radical scavenging activity of *E. caudata* extracts also increased dose-dependently as indicated by discoloration of the ABTS^{•+}, which was measured spectrophotometrically at 745 nm. Maximum scavenging activity was observed at 1000 $\mu\text{g/ml}$ dose for aqueous extract. The log-doses of *E. caudata* extracts and the standard ascorbic acid (ASA) were plotted against ABTS^{•+} inhibition (%) to determine their IC₅₀ (Figure 1.9A-C). EC-AE was observed to possess higher ABTS^{•+} scavenging activity than the EC-ME (Figure 1.10).

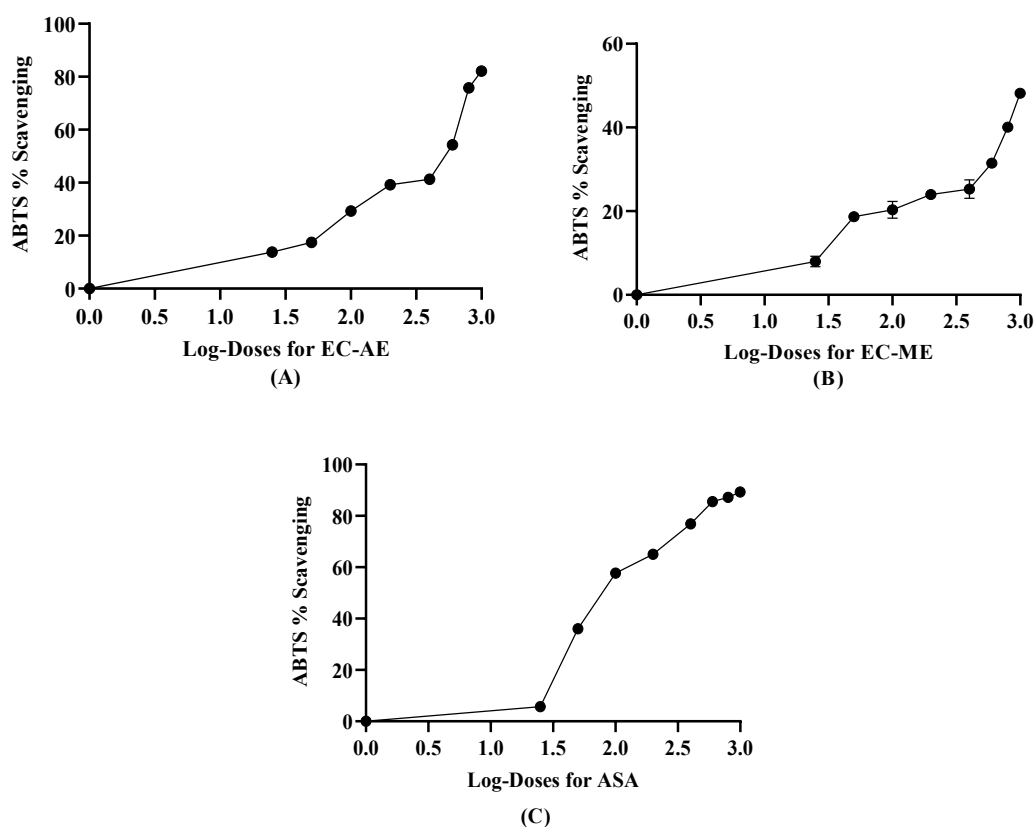


Figure 1.9. Plots of log-doses of (A) Aqueous extract (B) Methanolic extract of *E. caudata* extracts and (C) the standard ascorbic acid against ABTS radical inhibition (%) for the calculation of IC₅₀.

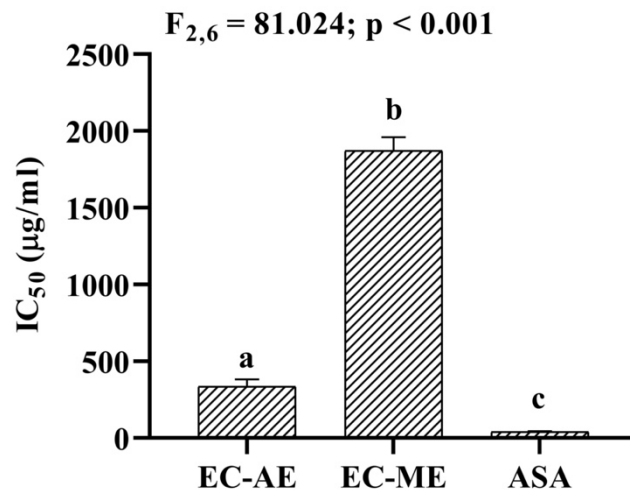


Figure 1.10. IC₅₀ (µg/ml) for ABTS^{•+} scavenging activity of *E. caudata* extracts and standard ascorbic acid. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

3.3.3. Superoxide Radical Scavenging Assay

E. caudata extracts also showed a dose-dependent inhibition of superoxide radical (O₂^{•-}) generation. Maximum O₂^{•-} scavenging activity was observed at a concentration of 1000 µg/ml for EC-AE. Log doses of *E. caudata* extracts and the standard ascorbic acid (ASA) were plotted against O₂^{•-} inhibition (%) for the calculation of IC₅₀ (Figure 1.11A-C). EC-AE possessed a higher scavenging activity than that of EC-ME. Moreover, the scavenging activity of ASA was not significantly different from the aqueous extract of *E. caudata* (Figure 1.12).

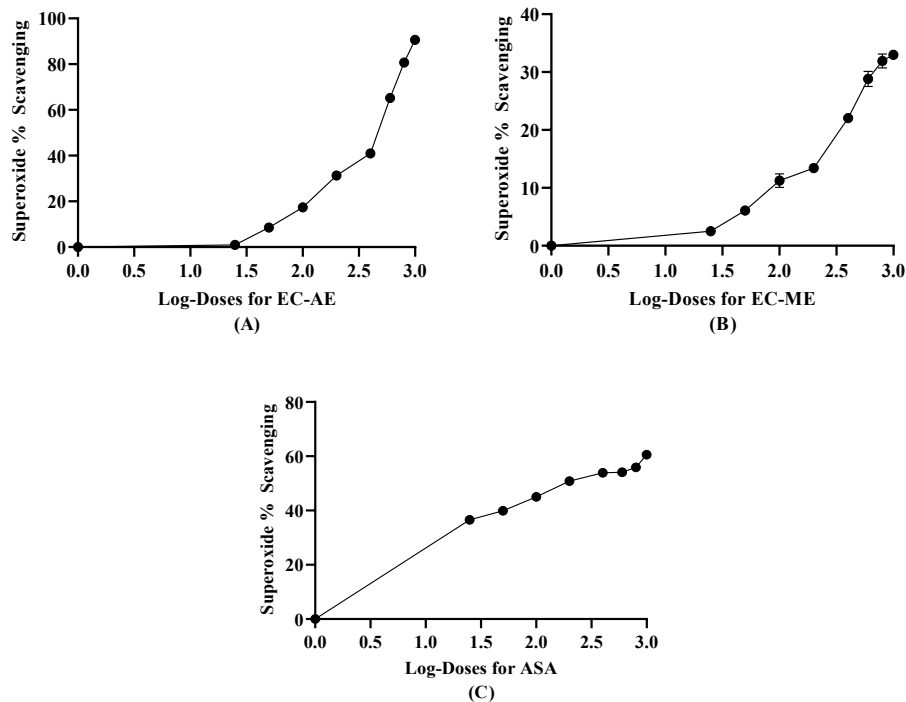


Figure 1.11. Plots of log-doses of (A) Aqueous extract (B) Methanolic extract of *E. caudata* extracts and (C) the standard ascorbic acid against superoxide radical inhibition (%) for the calculation of IC_{50} .

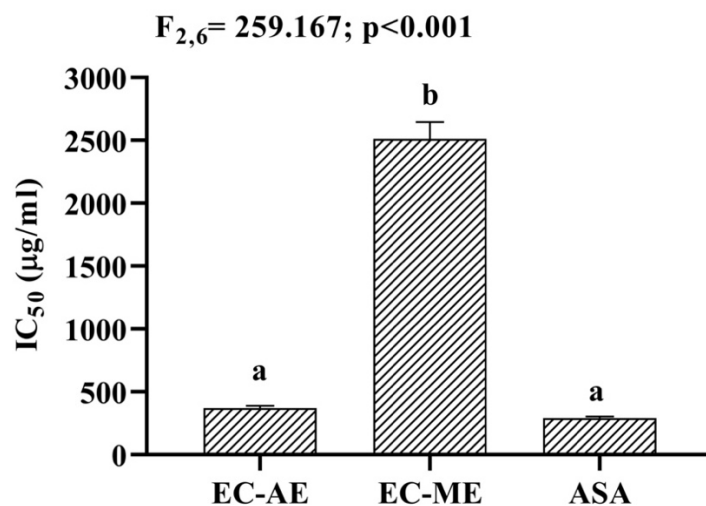


Figure 1.12. IC_{50} (µg/ml) for superoxide radical inhibition of *E. caudata* extracts and standard ascorbic acid. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean $\pm$ SEM, $n=3$. Different letters indicate significant variation.

3.3.4. Ferric Reducing power

The reducing power of *E. caudata* extracts was determined by measuring the transformation of Fe^{3+} to Fe^{2+} . The reducing activity of *E. caudata* extracts increased in a concentration-dependent manner (Figure 1.13). At 1000 $\mu\text{g/ml}$, EC-AE (2.291 ± 0.044) showed a significantly higher ($p < 0.001$) reducing activity than that of EC-ME (0.671 ± 0.021).

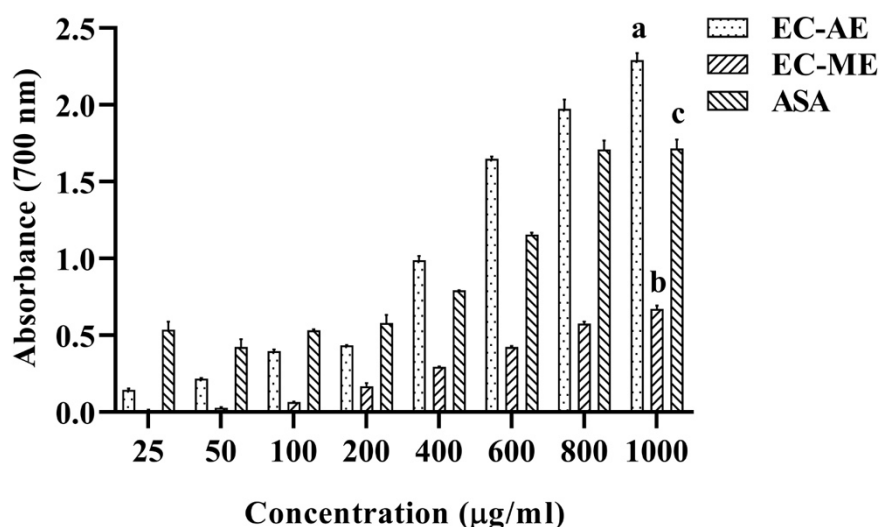


Figure 1.13. Reducing power of *E. caudata* extracts. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract; ASA: the standard ascorbic acid. Values are expressed as Mean $\pm$ SEM, $n=3$. Different letters indicate significant variation at the highest dose (1000 $\mu\text{g/ml}$).

3.4. Ex-vivo antioxidant assay

3.4.1. Anti-haemolytic activity

The anti-haemolytic activity was determined using 0.5 mg/ml of *E. caudata* extracts. Significant variation was observed in the anti-haemolytic activity of the two extracts ($p < 0.005$). EC-AE showed a significantly higher inhibitory activity against haemolysis with an inhibition rate of 80.77% compared to that of EC-ME (43.48%) at the dose of 1 mg/ml (Figure 1.14).

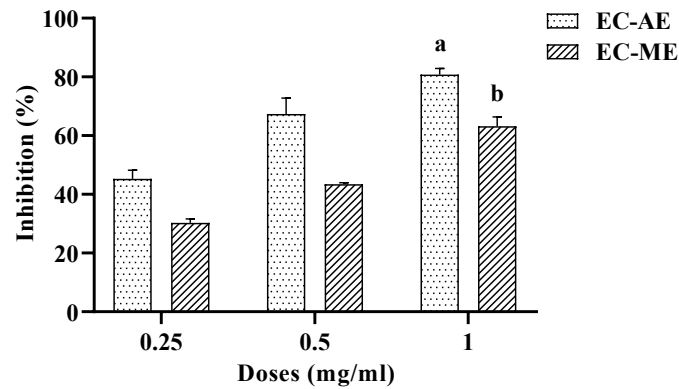


Figure 1.14. The anti-haemolytic activity of different concentrations (mg/ml) of *E. caudata* extracts. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract. Values are expressed as Mean $\pm$ SEM, n=3. Different letters indicate significant variation.

3.4.2. Lipid peroxidation inhibition

The lipid peroxidation inhibition potential of *E. caudata* extracts was estimated in mice liver homogenate. Significant variation was observed also between the two extracts in their inhibitory activity against lipid peroxidation ($p < 0.001$). EC-AE possessed a higher inhibitory activity with an inhibition rate of 81.58% compared to that of EC-ME at the highest dose, i.e., 1 mg/ml (61.63%) (Figure. 1.15).

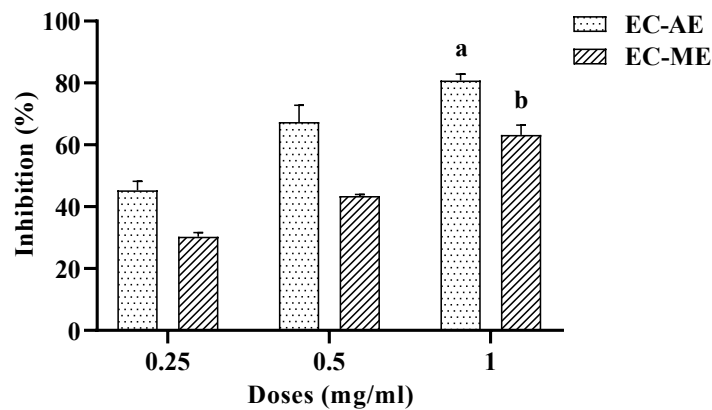


Figure 1.15. Lipid peroxidation inhibition of different concentrations of *E. caudata* extracts. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract. Values are expressed as Mean $\pm$ SEM, n=3. Different letters indicate significant variation.

4. DISCUSSION

The recent advancements in the understanding of free radicals in biology are catalysing a medical revolution that heralds a new era of health and disease management (Leonel *et al.*, 2024). Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in our body by various exogenous and endogenous factors (Chandimali *et al.*, 2025). A balance between these free radicals and antioxidants is necessary for maintaining the proper physiological functions of the body as the excessive generation of these radicals leads to a condition called ‘oxidative stress’ (Lobo *et al.*, 2010). Free radicals thus adversely alter various biomolecules including lipids, proteins, and DNA triggering certain human diseases such as cancer (Phaniendra *et al.*, 2015).

Accumulating research has shown that nutrition is essential in preventing chronic diseases, as many are linked to dietary habits (Neuhouser, 2019). Functional food encompasses the notion of viewing food as essential not only for sustenance but also as a means of promoting mental and physical well-being, aiding in the prevention and mitigation of disease risk factors, or increasing specific physiological processes (Obayomi *et al.*, 2024). Therefore, nutraceutical research has been focused on plants in search of suitable candidates with potent antioxidant activities.

Phytochemicals are biologically active, naturally occurring compounds found in plants, providing certain health benefits for humans more than those attributed to macronutrients and micronutrients (Hasler & Blumberg, 1999). The present study revealed the presence of significant amounts of phenolic and flavonoid compounds in *E. caudata* extracts. Phenolic compounds have been reported to show antioxidant activity by scavenging or stabilizing free radicals due to their conjugated ring structures and presence of hydroxyl groups (Seyoum *et al.*, 2006). Phenolic compounds have also been reported to exhibit antiallergenic, antimicrobial, antiatherogenic, antithrombotic, antiinflammatory, vasodilatory, and cardioprotective effects (Alpinar *et al.*, 2009). Similarly, flavonoids are also reported to have antioxidative action through scavenging or chelating processes (Hassanpour and Doroudi, 2023). In addition, flavonoids have been reported to serve as health-promoting compounds and protect humans against several diseases like atherogenesis, carcinogenesis, hepatotoxicity, and thrombosis (Tiwari, 2001).

This chapter mainly focuses on the elucidation of the antioxidative potential of *E. caudata* using various *in vitro* and *ex vivo* assays. The reduction of methanolic DPPH solution to its non-radical form, DPPH-H, is a well-established method for assessing the antioxidative properties of various compounds and plant extracts (Baliyan *et al.*, 2022). The extracts of *E. caudata* effectively reduced the stable DPPH radical to its yellow-coloured diphenyl-picrylhydrazine form, likely due to the presence of bioactive compounds capable of donating hydrogen atoms to neutralize free radicals by eliminating unpaired electrons. Cysteine, glutathione, ascorbic acid, tocopherol, and poly-hydroxyl aromatic compounds have been reported to reduce DPPH due to their hydrogen-donating ability (Kedare and Singh, 2011). The antioxidant potential of *E. caudata* extracts was further evaluated by assessing their ability to reduce the blue-coloured ABTS^{•+}, generated through the interaction of ABTS and potassium ferricyanide, to its neutral ABTS form. The efficiency of this conversion is influenced more by the molecular weight of phenolic compounds, the number of aromatic rings, and the nature of hydroxyl group substitution rather than the presence of specific functional groups (Hagerman, 1994). This study demonstrates the aqueous extract of *E. caudata* as a potent antioxidant and the scavenging activity of ABTS^{•+} could be due to their high phenolic contents.

Superoxide (O₂^{•-}) radical produced as a result of incomplete metabolism of oxygen serves as a precursor of more reactive oxygen species, contributing to tissue damage and various diseases (Bardaweel *et al.*, 2018). Superoxide can decompose to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, which are very harmful to the cellular components and initiate lipid peroxidation (Fujii *et al.*, 2022). Thus, neutralization of superoxide radicals is necessary to inhibit the chain of ROS generation and protect the cells from oxidative stress. It has also been observed that the antioxidant activities of certain flavonoids are efficient, primarily through the scavenging of superoxide anion radicals (Robak and Gryglewski, 1988). Thus, the presence of significant amounts of flavonoids in *E. caudata* extracts might be responsible for their scavenging activity against superoxide radicals. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. Therefore, the reducing ability of *E. caudata* extracts was also assessed by estimating the conversion of Fe³⁺ to Fe²⁺. The dose-dependent increase in the reducing power of the *E. caudata* extracts further supported their potent antioxidant activity.

Haemolysis can occur due to membrane damage caused by the activity of free radicals on erythrocytes, the major target of free radicals (Baik *et al.*, 2017). Lipid peroxidation generates certain degradation products such as malondialdehyde (MDA) that are considered to be an important cause of cell membrane destruction and cell damage (Ayala *et al.*, 2014). In the present study, lipid peroxidation in the mouse liver homogenate was induced by FeCl₂-H₂O₂. Formation of MDA is used as an indicator of lipid peroxidation, and subsequently oxidative stress. *E. caudata* extracts showed significant anti-haemolytic activity and lipid peroxidation inhibition potential which could be due to the presence of numerous amounts of phenols and flavonoids. Certain phenolic compounds have been reported to participate in the cell membrane, hindering the diffusion of free radicals and consequently decreasing the chain reaction of free radicals (Singh and Rajini, 2008). Flavonoids have also been reported to inhibit lipid peroxidation in the erythrocyte membrane and improve their integrity against lysis by binding to the membrane (Chaudhuri *et al.*, 2007).

The association between oxidative stress and the development of many disease disorders is not novel knowledge (Pizzino *et al.*, 2017). The onset and progression of cancer is linked to chromosomal abnormalities and the activation of oncogenes caused by free radicals (Valko *et al.*, 2017). There is substantial scientific evidence that supports the dominant role of oxidative stress as the cause of many cardiovascular diseases such as atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy, and congestive heart failure (Pham-Huy *et al.*, 2008). The synthesis of β -amyloid, a deleterious peptide frequently observed in the brains of Alzheimer's patients, is attributable to oxidative stress and significantly contributes to neurodegenerative mechanisms (Butterfield, 2002). Several research has implied that blood concentrations of lipid peroxidation products (F₂-isoprostanes, Malondialdehyde) are elevated in pre-eclamptic pregnancies and intrauterine growth restriction, indicating a potential role of reactive oxygen species/reactive nitrogen species in the etiology of certain gynecological conditions (Braekke *et al.*, 2006; Biri *et al.*, 2007; Hracsko *et al.*, 2008).

Therefore, the pivotal role of antioxidants in the chemoprevention of numerous diseases has been increasingly recognized, as oxidative stress is a major contributor to the pathogenesis of various chronic and degenerative conditions (Muscolo *et al.*, 2024). Natural sources of antioxidants, particularly those rich in bioactive compounds such as

phenolics and flavonoids, have garnered considerable interest due to their potential health benefits (Tungmunthum *et al.*, 2018). *E. caudata*, characterized by its high phenolic and flavonoid content, exhibits significant free radical scavenging activity, effectively inhibiting free radical-induced haemolysis and lipid peroxidation. These properties suggest that *E. caudata* may serve as a promising candidate for the development of natural antioxidant-based therapeutic interventions. However, despite its demonstrated antioxidative potential, an inclusive pharmacological evaluation is necessary to further elucidate its mechanisms of action, bioavailability, and potential clinical applications. Future research should focus on in-depth molecular and biochemical analyses to validate its efficacy and explore its role in the prevention and management of oxidative stress-related diseases such as cancer.

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CHAPTER 2

Preliminary screening of the anticancer activity of *Elaeagnus caudata*

ABSTRACT

Elaeagnus caudata has been extensively used to treat various ailments including cancer in the traditional medicine of North-East India. Additionally, several bioactive compounds with potent anticancer properties have been isolated from other species of the *Elaeagnus* genus, suggesting that *E. caudata* may also contain similar or novel anticancer compounds. Therefore, a preliminary study was conducted to determine the anticancer activity of *E. caudata* using various *in vitro* assays. MTT assay revealed a dose and time-dependent cytotoxic effect of *E. caudata* extract with the methanolic extract (EC-ME) possessing stronger cytotoxicity as compared to the aqueous extract. Fluorescent AO/EtBr staining confirmed apoptotic induction, with EC-ME-treated cells exhibiting a significantly higher apoptotic index (86.7%) compared to the untreated control (1.2%). Antioxidant assays demonstrated a significant increase in the level of glutathione (GSH) and activities of glutathione S-transferase (GST), and superoxide dismutase (SOD) levels, while lipid peroxidation, measured via malondialdehyde (MDA) level, was significantly reduced. The genotoxic potential of EC-ME was also assessed using the Comet assay, which showed increased DNA damage, indicated by enhanced comet tail length and olive tail moment. Further, caspase 6 activity was significantly elevated (2.3 fold increase) in EC-ME-treated cells, confirming apoptotic cell death. As a preliminary study, these findings suggest that *E. caudata* exhibits promising anticancer properties through induction of apoptosis, modulation of antioxidant levels, and induction of DNA damage in A549 cells. However, further in-depth studies, including compound isolation are necessary to fully elucidate its therapeutic potential and mechanisms of action.

1. INTRODUCTION

The treatment of cancer using chemotherapeutic approaches has seen significant development over the past decades (Gach-Janczak *et al.*, 2024). Despite the increasing utilization of advanced techniques like immunotherapy and targeted therapy, chemotherapy has remained an essential component in cancer treatment, especially in underdeveloped and developing countries where advanced technologies are very much still inaccessible (Koper *et al.*, 2023). Chemotherapy may serve as a standalone therapeutic approach or in conjunction with other oncological treatments such as surgical procedures and radiotherapy (Me *et al.*, 2023). The concept of chemotherapy, which employs toxic agents and pharmaceuticals to eradicate malignant cells, emerged following the observations of mustard gas's lethal effects on lymphatic tissues and bone marrow in the 1940s (Goodman and Wintrobe, 1946). The results were subsequently confirmed in mice utilizing an effective derivative of the gas (nitrogen mustard) that demonstrated significant regression of lymphoma tissues (Needham *et al.*, 1947; Ghanei, 2004; Chen *et al.*, 2018). Since then, more chemotherapy agents have been identified and developed.

Nonetheless, despite significant advancements in chemotherapy development, chemotherapy drugs share a cohesion in their side effects and complications. The common side effects of anticancer therapy include secondary malignancies, nephrotoxicity, hepatotoxicity, neurotoxicity, cardiotoxicity, and ototoxicity (van den Boogaard *et al.*, 2022). Therefore, though the clinical practice of cancer chemotherapy has attained considerable accomplishments, yet, there is potential for enhancement and development, especially regarding the efficacy and safety of the administered chemotherapeutic drugs. Thus, there is still a pressing necessity to discover novel drugs that are as efficacious, or even more efficacious than the current chemotherapeutic drugs, however with minimized side effects and complications.

Natural products, including plants, have remained a prominent source of anticancer agents due to their safety, efficacy, and lesser side effects (Thillaivanan and Samraj, 2014; Anand *et al.*, 2023). There is increasing evidence for the potential of plant-derived compounds as inhibitors of various stages of tumorigenesis and associated inflammatory processes, thereby indicating the importance of plant products in cancer prevention and therapy. Plants such as *Artemisia annua* L.,

Camptotheca acuminata Decne., *Ginkgo biloba* L., *Curcuma longa* L., *Podophyllum peltatum* L., *Taxus brevifolia*, *Taxus baccata*, *Combretum caffrum*, *Euphorbia peplus*, etc., have continued to prevail in modern medicine, as plant-derived compounds constitute a significant proportion of current pharmaceutical agents, particularly in the domains of antibiotherapy and chemotherapy (Cragg and Pezzuto, 2016). Over 60% of current chemotherapy is either isolated or derived from plant products (Asma *et al.*, 2022). Consequently, the pursuit of a novel chemotherapeutic drug with lessened side effects and complications has been focusing on the screening of plants over the decades.

One such plant with extensive traditional use and a good prospect of harbouring a novel compound with anticancer activity is the silverberry, *Elaeagnus caudata*. In North-East India, *E. caudata* has been used in traditional medicine by the Dimasa tribe of Assam and the Mizo tribe of Mizoram for the treatment of different ailments including a variety of cancer (Rout *et al.*, 2012, Sharma *et al.*, 2001; Lalfakzuala *et al.*, 2007; Rai and Lalramnghinglova, 2010; Lalzarzovi, 2016). Many bioactive compounds with strong anticancer activity such as rutin, epigallocatechin gallate, ferulic acid, triterpenoids, terpengustifol, harmane, and tetrahydroharmol have been isolated from other members of the same genus (Nazir *et al.*, 2020).

Thus, given the extensive medicinal use of *E. caudata* and the isolation of many anticancer compounds from other species of the same genus, we hypothesize that *E. caudata* could also be a reservoir of compounds possessing significant anticancer potential. Thus, this chapter focuses on the initial assessment of the anticancer and pro-apoptotic properties of *E. caudata* before conducting extensive research to isolate compounds.

2. MATERIALS AND METHODS

2.1. Chemicals

Trypsin-EDTA, Minimum Essential Medium (MEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), sodium bicarbonate, bovine serum albumin (BSA), glutathione (GSH) reduced, nicotinamide adenosine dinucleotide (NADH), nitroblue tetrazolium (NBT), Folin-ciocalteu's reagent, n-butanol, thiobarbituric acid (TBA), potassium chloride (KCl), sodium chloride (NaCl), Triton X-100, acridine orange, ethidium bromide, phenazine methosulphate (PMS), dimethyl sulphoxide (DMSO), 1-chloro-2,4 dinitrobenzene (CDNB) and 5, 5' dithio 2-nitrobenzoic acid (DTNB) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). L-Glutamine, phenol red, agarose (low gelling temperature), ethylenediamine tetra-acetic acid (EDTA), Trizma base, Trizma hydrochloride, and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co., Bangalore, India. 5-Fluorouracil (5FU) was obtained from GLS Pharma Ltd. (Hyderabad, India). The remaining chemicals were purchased from Merck Specialities Pvt., Ltd. (Mumbai, India).

2.2. Preparation of *E. caudata* extracts

The methanolic and aqueous extract of *E. caudata* were prepared as described in Chapter 1. In brief, the Soxhlet apparatus was utilized to perform various exhaustive solvent extractions of *E. caudata* leaves. Defatting was done using petroleum ether while chlorophyll washing was done using chloroform. Extraction was then proceeded with methanol followed by distilled water to yield the methanolic and aqueous extracts of *E. caudata* respectively. The extracts were then concentrated using a rotary evaporator and the dried residue was stored at -20°C.

2.3. Cell culture

Human non-small cell lung carcinoma cell line (A549) was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were then cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS and 1% L-glutamine with loosened caps at 37°C in an incubator containing 5% CO₂ (Eppendorf AG, Hamburg, Germany)

2.4. MTT assay

The cytotoxic effect of *E. caudata* extracts was estimated by 3-(4, 5-dimethylthiazol 2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay (Mossman, 1983). Briefly, 1×10^4 cancer cells were seeded in a 96 well plate containing 100 μ l MEM per well. The cells were allowed to adhere for 24 h and treated with different concentrations of *E. caudata* extracts (25 – 200 μ g/ml) for 24, 48 and 72 h. After treatment, 10 μ l of MTT (5 mg/ml) was added to each well and incubated for 2 h. The drug-containing media were removed, and cells were washed with serum-free media. Then, the formazan crystals were dissolved in 100 μ l of DMSO followed by incubation for 30 min after which the absorbance was measured at 560 nm using a microplate reader (Spectramax m2e, Molecular Devices). Three independent experiments consisting of three replicates were carried out for each treatment. Cytotoxicity was expressed as inhibition (%) which was calculated by the formula given below:

$$\% \text{ inhibition} = (\text{Control-Treatment/Control}) \times 100.$$

The methanolic extract (EC-ME) being superior in its cytotoxicity as indicated by the lower IC₅₀ against A549 was chosen for further analysis.

2.5. Cell morphology analysis by fluorescent staining (Apoptotic assay)

The mode of cell death induced by EC-ME was determined by dual fluorescent staining using Acridine orange/Ethidium bromide (AO/EtBr). Briefly, 1×10^5 A549 cells were seeded in a 6 well plate containing 5 ml of media. Cells were allowed to adhere overnight and treatment was given for 24 h with 80 μ g/ml EC-ME. After treatments, cells were washed with sterile PBS and detached with trypsin EDTA. The cells were pelleted and resuspended in 100 μ l of FBS-free media. Subsequently, 25 μ l cell suspension was stained with 2.5 μ l each of acridine orange (100 μ g/ml) and ethidium bromide (100 μ g/ml) in a ratio of 1:1 for 2 min followed by gentle mixing. The morphology of apoptotic cells were then examined on a slide under a fluorescent microscope (Thermo Fisher Scientific, EVOSR Fluorescence Imaging, AMEP-4615).

A minimum of 300 cells were scored and the apoptotic index was calculated as follows:

$$\text{Apoptotic index (\%)} = \frac{\text{Number of apoptotic cells scored}}{\text{Total number of cells counted}} \times 100$$

2.6. Antioxidant assays

For the estimation of antioxidant enzyme activities and lipid peroxidation, 3×10^6 A549 cells were seeded in T-25 flasks containing 5 ml media. The cells were treated with 80 $\mu\text{g/ml}$ of EC-ME for 24 h. After treatment, the drug-containing media were discarded, and the cells were washed with sterile PBS and harvested with trypsin EDTA. The cells were pelleted, and sonicated in a sonicator (PCI Analytics Pvt. Ltd., Mumbai, India) and 5% homogenate was prepared using cold sterile PBS (pH 7.4) and used for the biochemical estimations. Total protein contents were determined using Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as standard.

2.6.1. Glutathione (GSH)

The concentration of GSH was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm (Moron *et al.*, 1979). Briefly, 1.8 ml of 0.02 M Na_2HPO_4 (pH 8.0) and 40 μl of 10 mM DTNB were mixed with 160 μl of cell homogenate and incubated for 2 min at room temperature. The absorbance of the sample was read against a blank, which consisted of distilled water instead of cell homogenate, at 412 nm and the concentration of GSH was calculated from the standard curve and expressed in $\mu\text{mol/mg}$ of total protein.

2.6.2. Glutathione S-transferase (GST)

The activity of GST was measured following the standard method (Beutler, 1984). Briefly, 850 μl of phosphate buffer (pH 6.5) was mixed with 50 μl of 20 mM CDNB, incubated for 10 min at 37°C followed by the addition of 50 μl each of cell homogenate and 20 mM GSH. The absorbance of the blank and sample was measured at 360 nm and the enzyme activity was expressed in units/mg of total protein.

2.6.3. Superoxide dismutase (SOD)

The SOD activity was estimated using the standard method with minor modifications (Fried, 1975). Briefly, 100 μl each of cell homogenate and 186 μM PMS

were mixed with 300 μ l of 3.0 mM NBT and 200 μ l of 780 μ M NADH. The mixture was incubated for 90 sec at 30°C and 1 ml of acetic acid and 4 ml of n-butanol were added to stop the reaction. The absorbance of the sample and blank was measured at 560 nm, and the enzyme activity was expressed in units (1 SOD Unit = 50% inhibition of NBT reduction)/mg protein.

$$\% \text{ inhibition} = (\text{OD of blank} - \text{OD of test} / \text{OD of blank}) \times 100$$

$$\text{SOD unit} = 1/50 \times \% \text{ inhibition.}$$

2.6.4. Lipid peroxidation (LPO) assay

Malondialdehyde (MDA) formed by the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction of lipids. MDA has been identified as the product of lipid peroxidation (LPO) that reacts with TBA to give a red product. LPO was estimated using the standard method (Beuege and Aust, 1978). Briefly, cell homogenate was added to a mixture containing 10% TCA, 0.8% TBA, and 0.025 N HCl in a 1:2 ratio. The mixture was boiled for 10 min in a boiling water bath. After centrifugation, the supernatant was collected and its absorbance was read at 535 nm against the blank. The malondialdehyde (MDA) concentration of the sample was calculated using the extinction coefficient of $1.56 \times 10^{-6} \text{ M}^{-1} \text{ cm}^{-1}$.

2.7. Assessment of genotoxicity of EC-ME using Comet assay

The genotoxic effect of EC-ME in A549 cells was determined using the alkaline single-cell gel electrophoresis (Comet Assay) (Singh *et al.*, 1988). Briefly, 2×10^4 A549 cells treated with 80 μ g/ml of EC-ME for 24 h along with the untreated control were suspended in 75 μ l of 0.5% low-melting point agarose (LMPA) prepared in PBS and spread onto a frosted slide precoated with 1% normal-melting point agarose (NMPA) and covered with a coverslip. Once the gel solidified, the coverslip was gently removed and the third layer of 90 μ l 0.5% LMPA was added. The slides were then incubated for 2 h in a freshly prepared lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Trizma base, 1% Triton X-100, and 10% DMSO, pH 10). After lysis, slides were placed on a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13)

for 20 min to allow unwinding of DNA. Electrophoresis was then carried out for 30 min at 24 V and 300 mA. The slides were then neutralized by washing with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min. After neutralization, slides were washed with d.H₂O and then stained with ethidium bromide (EtBr) solution (2 µg/ml) for 5 min. Each slide was prepared in triplicate and 100 randomly selected cells from each slide were examined using a fluorescence microscope with a magnification of 200x. Image captured were analysed with Image J software.

2.8. Caspase 6 activity assay

To further assess the apoptotic-inducing effect of EC-ME, the activity of caspase 6 was determined using a commercially available kit (BioVision Incorporated, USA). Briefly, 5×10^5 A549 cells were treated with 80 µg/ml of EC-ME for 24 h in a 6-well plate along with the untreated control. After treatment, cells were washed and lysed in 50 µl of chilled lysis buffer followed by 10 min incubation on ice. The cell lysates were centrifuged at $15,000 \times g$ for 1 min at 4°C, and the supernatant was collected. The assay was performed in a total volume of 100 µl in 96 well plates. 150 µg of protein from each sample was assayed for caspase 6 activity against its specific colorimetric substrate VEID-pNA. The mixture was incubated for another 2 h at 37°C and absorbance of free p-nitroanilide (pNA) produced via cleavage from specific substrates by activated caspase 6 was measured at 405 nm using a microplate reader.

2.9. Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean. Student t-test was performed for comparison of cytotoxicity, DNA damage, caspase activity, antioxidant/oxidant status using SPSS ver.20.0 software (SPSS Inc, Chicago, Illinois, USA). The IC₅₀ values were calculated by plotting the % inhibition against the log doses using GraphPad Prism software version 8.0. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Cytotoxic effects of *E. caudata* extracts

The cytotoxic effect of *E. caudata* against A549 cells at different treatment durations were expressed as % inhibition and were plotted against their log doses for the calculation of their respective IC_{50} (Figure 2.1). The treatment of A549 cells with the extracts of *E. caudata* showed a dose and time-dependent increase in cytotoxicity (Figure 2.2). It was also observed that for each treatment duration, the IC_{50} of EC-ME was significantly lower than that of EC-AE.

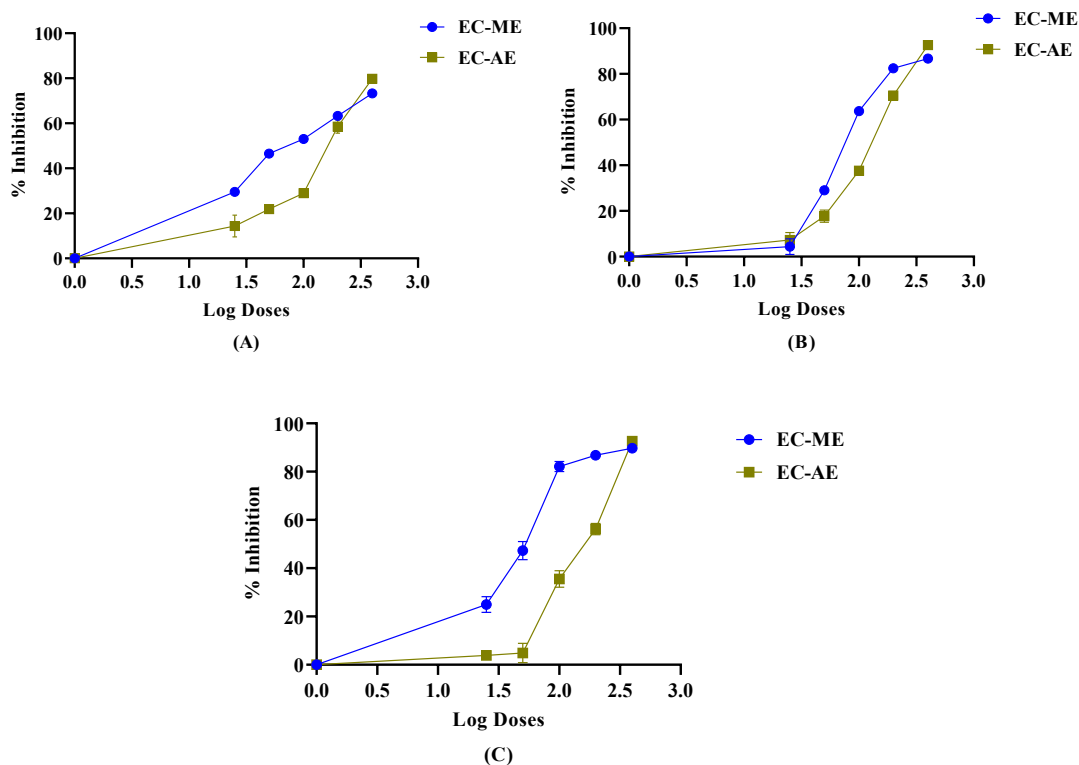


Figure 2.1. Plot of log-doses of *E. caudata* crude extracts against % inhibition of A549 cells after (A) 24 h (B) 48 h, and (C) 72 hr for the calculation of IC_{50} . EC-ME: *E. caudata* methanolic extract; EC-AE: *E. caudata* aqueous extract.

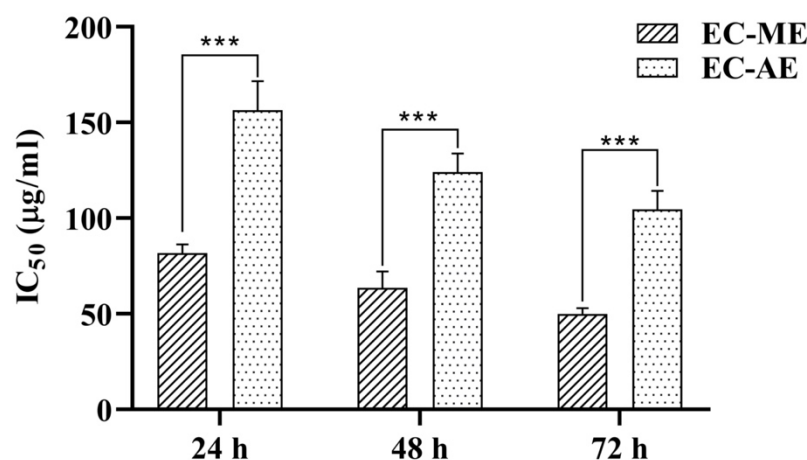


Figure 2.2. IC₅₀ of the crude extract of *E. caudata* against A549. EC-ME: *E. caudata* methanolic extract; EC-AE: *E. caudata* aqueous extract. Values are expressed as Mean $\pm$ SEM (n = 3). *** indicates $p < 0.001$.

3.2. Morphological evidence of apoptosis induced by EC-ME

Acridine orange is nucleic acid fluorescent dye that permeates both live and dead cells, and makes the nuclei appear green. Ethidium bromide is taken up only by dead cells whose cytoplasmic membrane integrity is lost and stains the nuclei yellowish orange. For this reason, live cells have green nuclei whereas apoptotic cells that incorporated ethidium bromide exhibit condensed and fragmented orange chromatin (Figure 2.3). Dual AO/EtBr staining revealed that exposure of A549 cells to 80 $\mu\text{g/ml}$ of EC-ME for 24 h resulted in a significant increase in the number of apoptotic cells. Fluorescence microscopic image shows that EC-ME was able to induce morphological alterations in A549 cells such as membrane blebbing, nuclear condensation, and nuclear fragmentation which are the distinct characteristics of apoptotic cells. The percentage of dead cells in EC-ME treated A549 cells was 86.7 % while it was only 1.2 % in the untreated control (Figure 2.4).

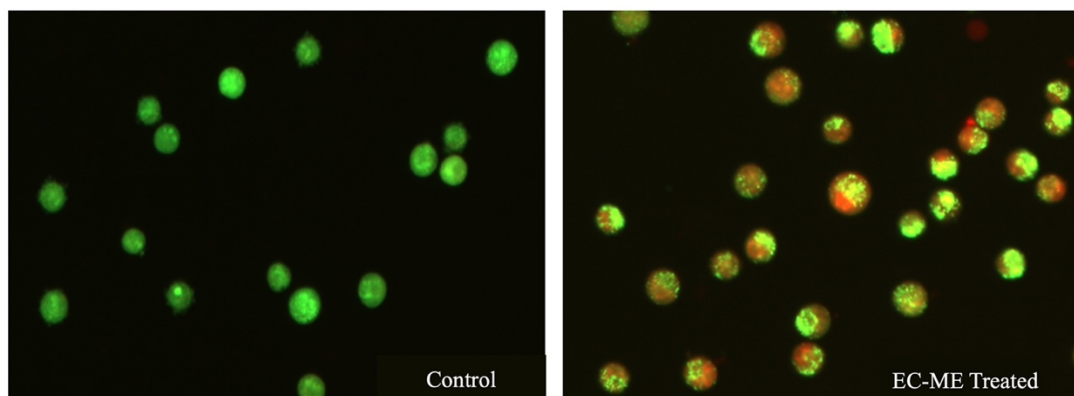


Figure 2.3. Acridine orange/Ethidium bromide (AO/EtBr) dual staining of A549 cells after treatment with 80 $\mu\text{g/ml}$ of methanolic extract of *E. caudata* for 24 h. Control: A549 cells without treatment. EC-ME: Methanolic extract of *E. caudata*.

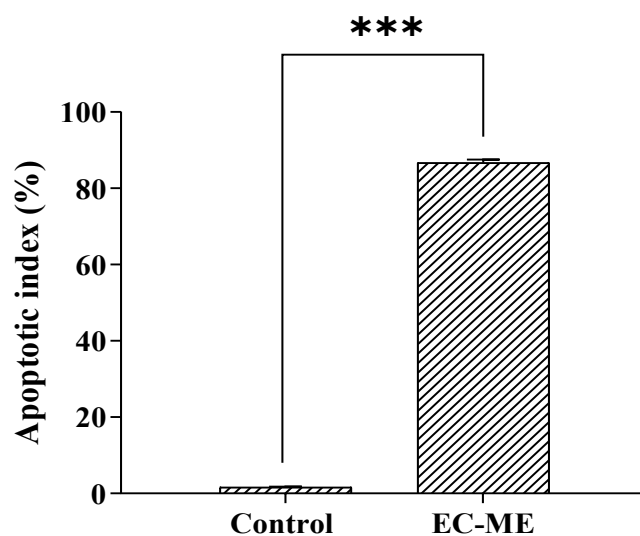


Figure 2.4. Percentage of apoptotic cells after treatment of A549 with methanolic extract of *E. caudata*. Control: A549 cells without treatment. EC-ME: Methanolic extract of *E. caudata*. Values are expressed as Mean $\pm$ SEM ($n = 3$). *** $p < 0.001$ compared with the untreated control.

3.3. Effects of EC-ME on antioxidants/oxidant status of A549 cells

To assess whether *E. caudata* alter the antioxidant levels in A549 cells, cells were treated with 80 µg/ml of EC-ME and the levels of GSH, GST and SOD were assessed. Glutathione content of A549 cells in treatment group was significantly ($p < 0.01$) higher than the untreated control (Figure 2.5A). A549 cells treated with EC-ME for 24 h also resulted in significant increase in GST and SOD activities when compared to untreated control (Figure 2.5B & C). To investigate whether EC-ME treatment induces rise in intracellular oxidant level, the level of lipid peroxidation (LPO) as a biomarker of oxidative stress was assessed. Treatment of A549 cells with 80 µg/ml of EC-ME resulted in reduction of lipid peroxidation (Figure 2.5D).

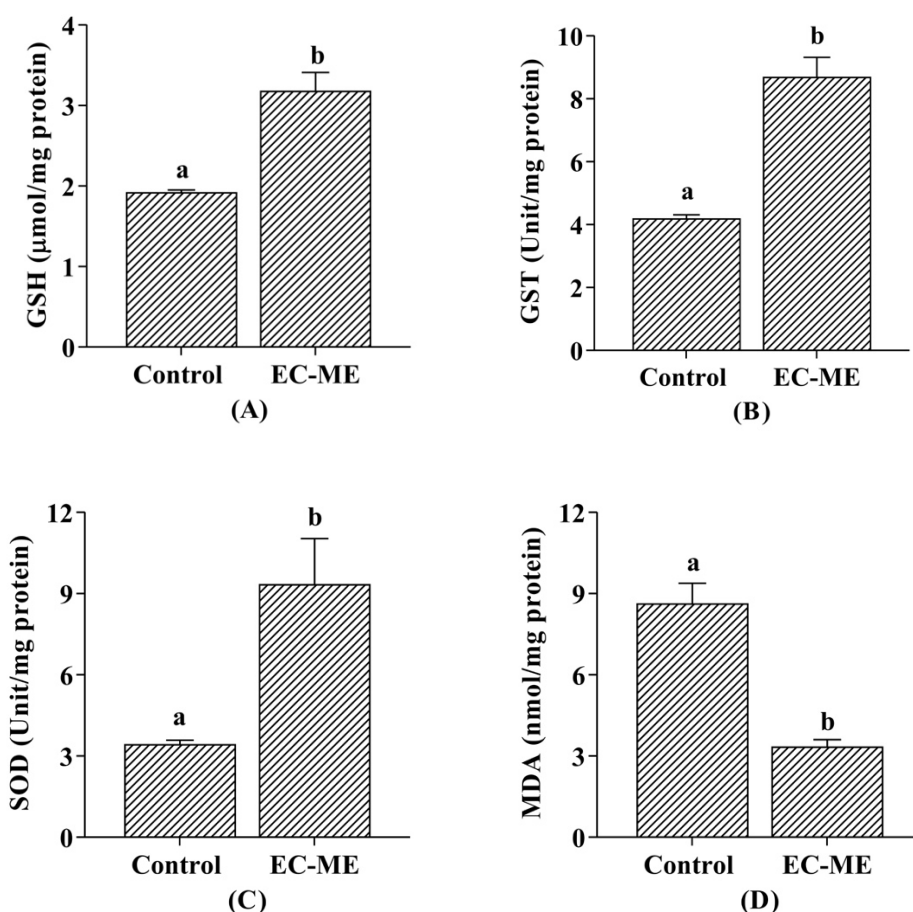


Figure 2.5. Effects of methanolic extract of *E. caudata* (EC-ME; 80 µg/ml) on: (A) glutathione (GSH) level; (B) glutathione S-transferase (GST) activity; (C) superoxide dismutase (SOD) activity; (D) malondialdehyde (MDA) level to assess lipid

peroxidation. Values are expressed as Mean $\pm$ SEM ($n = 3$). Means not sharing the same letter are significantly different.

3.4. Induction of DNA strand breaks by EC-ME

DNA damaging effect of EC-ME in A549 cells was assessed by employing the alkaline comet assay. Treatment of A549 cells with 80 $\mu\text{g/ml}$ of EC-ME induced significant DNA damage (Figure 2.6) as indicated by increased tail length and olive tail moment in the EC-ME treated group when compared to the untreated control (Figure 2.7A & B).

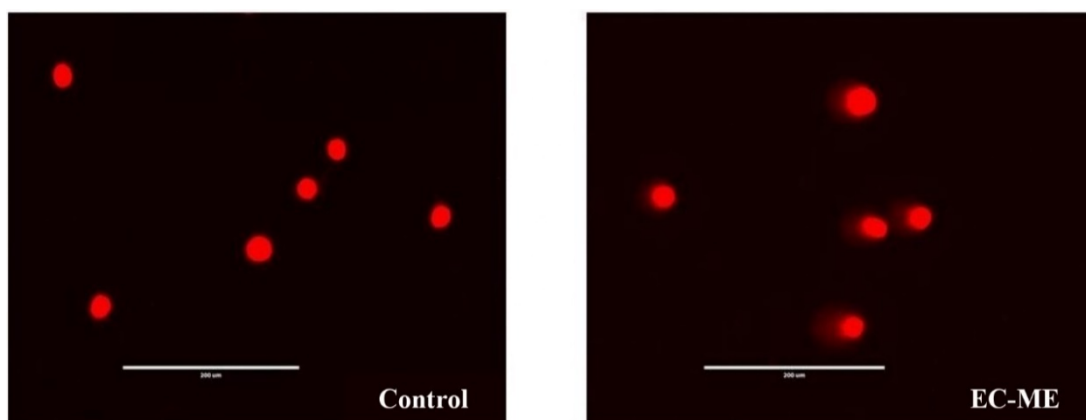


Figure 2.6. Fluorescence images of comets observed in control and A549 cells treated with 80 $\mu\text{g/ml}$ of methanolic extract of *E. caudata*. Control: A549 cells without treatment; EC-ME treated: A549 cells treated with 80 $\mu\text{g/ml}$ of methanolic extract of *E. caudata*.

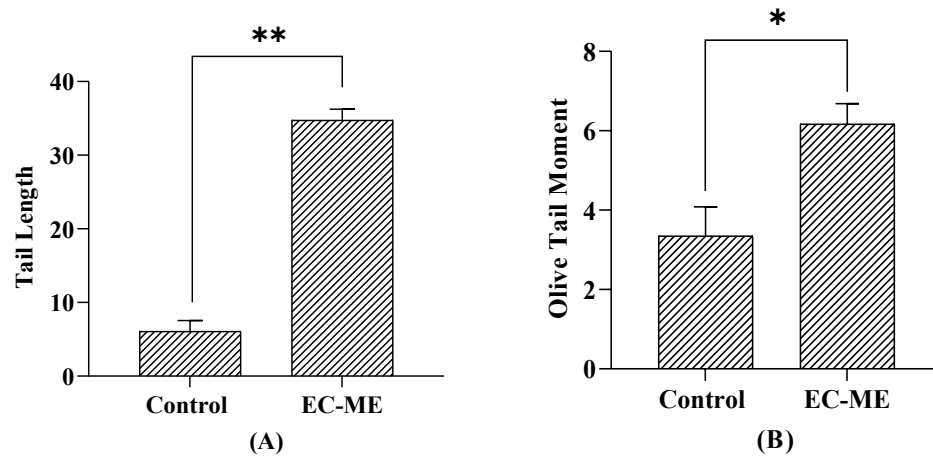


Figure 2.7. The extent of DNA damage expressed in terms of (A) Tail length and (B) Olive tail moment. Control: A549 cells without treatment; EC-ME: A549 cells treated with 80 $\mu\text{g/ml}$ of methanolic extract of *E. caudata*. Values are expressed as Mean $\pm$ SEM ($n = 3$). * and ** indicates significant variations at $p < 0.05$ and $p < 0.01$ respectively.

3.5. Activation of caspase 6 by EC-ME on A549 cells

Caspase 6 plays an important role in the execution of apoptosis in cancer cells. The effect of EC-ME on A549 cellular death was assessed by caspase 6 activity. Treatment of A549 cells with 80 µg/ml of EC-ME for 24 h resulted in a 2.3-fold increase in the activity of caspase 6 when compared to the untreated control (Figure 2.8).

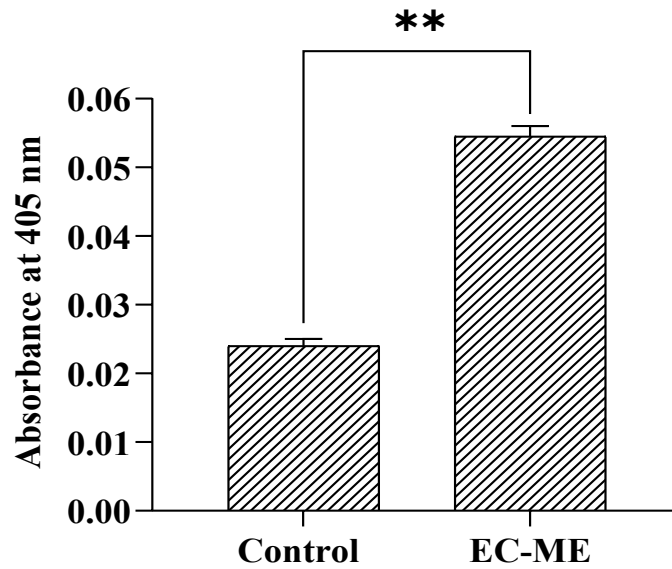


Figure 2.8. Effects of the methanolic extract of *E. caudata* on Caspase 6 activity in A549 cells after 24 h treatment. Control: A549 cells without treatment; EC-ME: A549 cells treated with 80 µg/ml of methanolic extract of *E. caudata*. Values are expressed as Mean ± SEM (n = 3). ** p < 0.01 compared with the untreated control.

4. DISCUSSION

Recent studies have focused on utilizing medications from traditional medicine to improve the efficacy of cancer treatment (Singh *et al.*, 2016). Despite the availability of numerous reports on the medicinal benefit of *E. caudata*, investigations that look into the scope of *E. caudata* for cancer therapy are still limited. Therefore, this study was undertaken to examine the growth inhibitory and cytotoxic effects of *E. caudata* in human lung adenocarcinoma A549 cells.

The primary objective of targeting cell proliferation in cancer therapy is to induce cell death or halt cell cycle progression through the use of cytotoxic compounds. The MTT assay is a widely utilized and standardized method for assessing the cytotoxicity of pharmacological compounds in various cultured cells, wherein the reduction of MTT occurs exclusively in metabolically active cells. Treatment of A549 cells with the extracts of *E. caudata* showed a dose and time-dependent cytotoxicity and the methanolic extract (EC-ME) was found to possess stronger cytotoxicity as compared to the aqueous extract (EC-AE) (Figure 2.2). Therefore, the methanolic extract with its IC₅₀ of 80 µg/ml and the optimal treatment duration of 24 h was selected for further analysis.

Understanding the precise mechanisms through which an anticancer agent exerts its effects is a crucial aspect of evaluating and developing novel anticancer. Apoptosis is a fundamental and tightly regulated mechanism of cell death that facilitates the removal of damaged or dysfunctional cells while preserving surrounding normal cells. Dysregulation of this process contributes to the pathogenesis of various diseases, including cancer (Elmore, 2007). Any compound capable of inducing apoptosis is regarded as a potential candidate for cancer chemotherapeutic treatment (Tor *et al.*, 2015). To investigate whether methanolic extract-induced cytotoxicity of A549 cells is via apoptosis, AO/EtBr dual staining was used to identify and quantify the apoptotic morphology. A549 cells treated with 80 µg/ml of EC-ME exhibited characteristic apoptotic morphology with brightly orange-red and condensed nuclei compared to the untreated control cells which showed round and intact green nuclei representing the live cells (Figure 2.3). Several plant-derived anticancer drugs have been reported to show similar effects in various cancer types (Fattahi *et al.*, 2013; Bhagat *et al.*, 2015; Arvind *et al.*, 2016; Madunić *et al.*, 2018).

Maintenance of redox balance is important as excessive ROS production has been associated with the pathophysiology of various diseases including cancer (Reczek and Chandel, 2017). Many cancer cells are shown to have higher ROS levels as compared to their normal counterpart due to enhanced metabolism and mitochondrial dysfunction (Tafari *et al.*, 2015). Since high ROS levels have a close association with tumour initiation, angiogenesis, cell invasion, metastasis, and chemoresistance in different cancer models, the use of antioxidants or agents that enhance the antioxidant system may provide an opportunity to reduce intracellular ROS-mediated tumorigenesis and cancer progression (Galadari *et al.*, 2017). Natural products including plant extracts have demonstrated antioxidant efficacy such as sesamol, curcumin, ascorbic acid, vitamin E, and melatonin for cancer treatment both *in vitro* and *in vivo* (Zhang *et al.*, 2023). Consistently, in the present study, augmentation of GSH level and activities of GST and SOD, and decreased lipid peroxidation as evidenced by the significant decrease in MDA levels after EC-ME treatment demonstrates its antioxidant nature which may be responsible for its anticancer activity in A549 cell. Over-expression of antioxidant enzymes such as SOD1, SOD2, SOD3, GPx3, and Prx6 has been reported to induce cell death, decrease survival time, and suppress metastasis in various cancer cells (Galadari *et al.*, 2017). Plants such as *Hypericum hookerianum*, *Cyathula prostrata*, and *Cocculus hirsutus* have been reported to possess anticancer activities via the elevation of the antioxidant defense system and reduction of lipid peroxidation (Dongre *et al.*, 2008; Mayakrishnan *et al.*, 2014; Thavamani *et al.*, 2014).

DNA damage in response to anticancer agents is an important factor in anticancer therapy. To check for DNA strand breaks as a result of EC-ME treatment, a single-cell gel electrophoresis assay (Comet assay) was performed. Our results showed that EC-ME possessed a DNA-damaging effect as evidenced by the increased comet tail length and olive tail moment in A549 cells treated with EC-ME (Figure 2.7). Several plant-derived anticancer drugs have also been reported to show similar effects in various cancer types (Madunic *et al.*, 2018; Moorthy *et al.*, 2018).

Caspase 6 is one of the executor caspases that plays a central role in the execution of both the intrinsic (the mitochondrial-mediated) and extrinsic (the death receptor-mediated) apoptotic pathways by cleaving several key proteins including

intra-nuclear proteins (McIlwain *et al.*, 2013). This cleavage mediates the disassembly of the cell into the apoptotic morphological changes including cell shrinkage, chromatin condensation, and nuclear fragmentation (Elmore, 2007). Thus, activation of caspase 6 is a strong biomarker for cells undergoing apoptosis. Intense upregulation of caspase 6 has been demonstrated to be an important phenomenon associated with lung carcinoma cells undergoing apoptosis (Törmänen-Näpänkangas *et al.*, 2001). Increased caspase 6 activity in A549 cells following EC-ME treatment strongly indicated that EC-ME-induced apoptosis was executed through a caspase-dependent pathway.

Our preliminary study demonstrates a novel finding showing the role of the methanolic extract of *E. caudata* as a promising anticancer agent. The extract exhibited significant cytotoxicity, probably through apoptosis, as evident from the elevated activity of Caspase 6 and the subsequent induction of DNA damage, and enhanced antioxidant defence mechanisms. Thus, these results are suggestive that the methanolic extract of *E. caudata* could be a prospective candidate for further in-depth analysis and may warrant further refinement towards the identification and isolation of a new lead compound for future cancer chemotherapy.

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CHAPTER 3

Bioassay-guided fractionation of crude extract of *Elaeagnus caudata* and anticancer activity of the active fraction

ABSTRACT

Despite its extensive traditional use in treating various illnesses, including cancer, *E. caudata* remains neglected and relatively unexplored in terms of its pharmacological potential. The methanolic extract of *E. caudata* leaves was fractionated using column chromatography, yielding 17 fractions. The B2 fraction was identified as the most cytotoxic, with the lowest IC₅₀ against A549 and HCT116 cells. A higher apoptotic index further supported the B2 fraction activities, increased caspase 3/6 activity, DNA damage, and elevated lipid peroxidation with decreasing antioxidant levels. LC-HRMS analysis revealed 12 major compounds in the B2 fraction, with Amorphigenin emerging as a promising drug candidate based on the SwissADME and pkCSM analysis. Network pharmacology identified HSP90 as the primary target of Amorphigenin, with a binding affinity of -11.11 kcal/mol, which is stronger than the control Geldanamycin. Molecular dynamic simulation reveals an average RMSD of 1.0 nm and lower RMSF value with the generation of a significant amount of hydrogen bonds indicating a more stable binding conformation of the Amorphigenin-HSP90 complex than with the control. The Amorphigenin complex also exhibited elevated SASA values than the control oscillating between 110 and 115 nm². The gmx_MMPBSA calculation reveals the binding energy of the complex varies between -35 and -17 kcal/mol, with an average of about -25 kcal/mol. Thus, our findings suggest that *E. caudata* and its bioactive compounds, particularly Amorphigenin, hold potential for the development of novel targeted therapies for non-small cell lung carcinoma and colorectal cancer.

1. INTRODUCTION

Despite ongoing advances in theranostics, cancer has remained a major health concern with an incidence of 19.9 million cases and 9.7 million deaths worldwide in 2022 (Bray *et al.*, 2024). Further exacerbating this burden is the rise in the number of new cancer cases, which has been predicted to increase by 47% in 2040 (Sung *et al.*, 2021). Among various cancers, lung cancer constitutes the highest mortality worldwide (18.7%) followed by colorectal cancers (9.3%) (Bray *et al.*, 2022). India recorded 1.5 million cancer cases in 2022, making it the third-highest country in terms of worldwide cancer incidence, following China and the United States. The cases are expected to rise by 12.8% in 2025 (Satishkumar *et al.*, 2022). Globally, the World Health Organization (WHO) has also predicted that cancer will surpass ischemic heart disease as the top cause of death by 2060 (Mathers *et al.*, 2016).

Although cancer prognosis has improved recently due to radical therapies such as organ transplantation, chemotherapy, radiotherapy, immunotherapy, and surgical resection, cancer treatment has remained persistently hindered by the non-specificity and toxic side effects of these treatment modalities. Furthermore, cancer therapies have continued to face considerable problems as resistance development and recurrence occur in many patients (Choi *et al.*, 2023; Nors *et al.*, 2024). The introduction of pathway-targeted therapies in the past few years has resulted in an evolution toward personalized medicine, in which treatment regimens are tailored to the specific genetic and molecular characteristics of cancers (Zhong *et al.*, 2016). However, the plasticity of tumours has challenged these targeted therapies, which results in the triggering of resistance mechanisms that include target mutations, and reactivation of inhibited pathways as well as changes in tumour microenvironment interactions (Choi and Chang, 2023). Thus, despite all the advancements in cancer therapeutics, limitations exhibited by these existing conventional treatments have continued to prompt the search for more precise and less toxic therapeutic drugs and techniques.

Over the past few decades, the pharmaceutical industry has redirected its focus to natural products in its quest for better drugs for cancer treatment owing to their effectiveness and lesser toxicity. Throughout history, humanity has consistently relied on nature to address a wide range of illnesses, including cancer. WHO has estimated

that 80% of the world's population is reliant on plant-derived drugs and formulations for the management of various diseases (Chaudhary *et al.*, 2015). Several researchers have also indicated that the incorporation of phytomedicines into traditional cancer therapy protocols has lowered mortality and lessened adverse drug effects (Lalremruati *et al.*, 2019). Simultaneously, there is substantial evidence that bioactive compounds produced by plants can suppress several stages of carcinogenesis and related inflammatory processes, thereby highlighting the importance of these natural products in the prevention and treatment of cancer (Solowey *et al.*, 2014).

Among such plants with wide traditional use is the silverberry, *Elaeagnus caudata*, which is locally known as 'Sarzuk' by the Mizo tribe of India. The roots and leaves have been traditionally used by the Mizo tribe to treat several illnesses including rheumatoid arthritis, induction of labour, the expulsion of the retained placenta as well as for various malignancies (Rai and Lalramnghinglova, 2010). Various pharmacological properties including anti-nociceptive, muscle relaxant, anti-angiogenic, cytotoxic, anti-tumorigenic, and anti-microbial activity have all been well investigated and documented among various species of the genus *Elaeagnus* such as *E. angustifolia*, *E. umbellata*, *E. pungens*, *E. rhamnoides* and *E. glabra* (Nazir *et al.*, 2020). Despite its extensive traditional use, *E. caudata* remains an unexplored species of its genus.

Preliminary studies of the crude extract of *E. caudata* in Chapter 2 reveal the methanolic extract to possess potent cytotoxicity and apoptosis-inducing potential. Therefore, in this chapter, we aim to further validate the anticancer activity of *E. caudata* by dissecting the bioassay-guided fractionation of the plant and identifying the key active compounds. We also aim to identify the molecular target(s) of the *E. caudata* active fraction and analyse their interaction using *in silico* approaches including network pharmacology, molecular docking, and molecular dynamics simulation. Thus, our study aspires toward the identification of a lead compound in a prospective anticancer drug discovery path.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Minimum essential medium (MEM), Trypsin-EDTA, fetal bovine serum (FBS), bovine serum albumin (BSA), 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), sodium bicarbonate, glutathione (GSH) reduced, Folin-ciocalteu's reagent, nicotinamide adenosine dinucleotide (NADH), nitroblue tetrazolium (NBT), n-butanol, thiobarbituric acid (TBA), potassium chloride (KCl), 5, 5' dithio 2-nitrobenzoic acid (DTNB), sodium chloride (NaCl), phenazine methosulphate (PMS), ethidium bromide, dimethyl sulphoxide (DMSO), 1-chloro-2, 4 dinitrobenzenes (CDNB), methanol, petroleum ether, chloroform, and Triton X-100 were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Ethylenediamine tetra-acetic acid (EDTA), L-glutamine, agarose (low gelling temperature), phenol red, trichloroacetic acid (TCA), silver nitrate, Trizma base, and Trizma hydrochloride were purchased from Sigma Chemical Co., Bangalore, India. 5-Fluorouracil (5FU) was purchased from GLS Pharma Ltd. (Hyderabad, India). The remaining chemicals were procured from Merck Specialities Pvt., Ltd (Mumbai, India).

2.2. Plant material and preparation of *E. caudata* extracts

The extracts of *E. caudata* were prepared as described in Chapter 1. The methanolic extract (EC-ME) was chosen for subsequent study following the preliminary cytotoxicity and apoptotic investigations conducted in Chapter 2.

2.3. Fractionation of methanolic extract of *E. caudata* using column chromatography

A column with a length of 100 cm and a bore size of 4.5 cm was affixed to a pre-assembled retort stand. Activated 60 – 120 mesh silica gel was packed up to two-thirds by the wet packing method using hexane. It was then left untouched for approximately one hour. The methanolic extract of *E. caudata* was mixed with silica gel and then packed above the silica gel. A cotton pad was placed on the top of the silica bed to maintain a uniform layer. Elution was performed with a stepwise gradient of Hexane: Ethyl Acetate (v/v) (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 90:10 and 0:100) and Ethyl Acetate: Methanol (90:10, 80:20 and 70:30). The

fractions were collected at 50 drops per minute. Each fraction was monitored with thin-layer chromatography (TLC) and elutes with similar spots were pooled into one fraction. A total of 17 fractions (A1, A2, B1, B2, B3, C1, C2, D1, E1, F1, G1, H1, H2, H3, I1, J1 and K1) were collected.

2.4. Cell lines and cell culture

Human non-small cell lung carcinoma cell line (A549), human colorectal carcinoma cell line (HCT116), and normal murine fibroblast cell line (L929) were procured from the National Centre for Cell Science (NCCS), Pune, India. All cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS and 1% L-Glutamine in a humidified incubator with 5% CO₂ at 37°C (Eppendorf, Hamburg, Germany).

2.5. Cytotoxicity assay

The cytotoxicity of the different fractions of methanolic extract of *E. caudata* was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983). Briefly, 1×10^4 cells were seeded in a 96 well plate. The cells were treated with different concentrations (10, 20, 40, 60, 80, and 100 µg/ml) of each fraction for 24 h after which the drug-containing media was discarded and replaced with serum-free media. 10 µl of MTT (5 mg/ml) was then added and incubated for another 2 h at 37°C in a CO₂ incubator. Following this, 100 µl of DMSO was used to dissolve the insoluble purple formazan crystals. After 30 min of incubation, the absorbance of the solution was measured at 560 nm using a microplate reader (Spectramax m2e, Molecular Devices). For each treatment, three independent experiments with three replicates were conducted. Cytotoxicity was expressed as inhibition (%) which was calculated by the formula given below:

$$\% \text{ inhibition} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100.$$

For subsequent studies, the fraction with the lowest IC₅₀ (i.e., B2) and the optimal inhibition time of 24 h was chosen.

2.6. Liquid Chromatography – High Resolution Mass Spectroscopy

Liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) was employed to determine the bioactive compounds present in the best active fraction of *E. caudata* (B2 fraction). Analysis was carried out using an ACCUCORE HPLC (C18, 150 × 2.1, 1.7 µm) system coupled to a triple quadrupole tandem mass spectrometer (ACQ-TQD-QBB1152, Waters acuity PDA detector, Waters Corporation, Milford, MA, USA) equipped with an orthogonal ESI source. The sample was analysed in both positive and negative modes in the range of m/z 150 – 2000 with the source temperature set to 120°C and desolvation at 350°C. The mobile phase A (water and 0.1 % formic acid) and mobile phase B (mixed acetonitrile and 0.1 % formic acid) were used as eluents in a linear gradient flow. The injection volume was 5 µl and the flow rate was maintained at 0.3 ml/min throughout the gradient. Data analysis was performed using OpenLynx™ and MassLynx™ Software (Waters Corporation, Milford, MA).

2.7. Cell morphology analysis using dual Acridine Orange/Ethidium Bromide staining

The apoptosis-inducing potential of the B2 fraction of *E. caudata* was studied using the dual fluorescent staining technique. 1×10^5 cells were seeded into each well of a 6 well plate and allowed to adapt for 24 h after which different doses (10, 25, and 50 µg/ml) of B2 fraction were added. Cells were harvested after 24 h of treatment by trypsinization. 25 µl of cells were suspended in 2.5 µl of ethidium bromide (100 µg/ml) and acridine orange (100 µg/ml) and incubated for 2 mins. The morphology of apoptotic cells was then investigated under a fluorescent microscope (Thermo Fisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615). At least 300 cells were scored, and the apoptotic index was determined as follows:

Apoptotic index (%) = Number of apoptotic cells scored × 100/Total number of cells counted.

2.8. Antioxidant Assays

The effect of the B2 fraction of *E. caudata* on the antioxidant-oxidant system was evaluated by measuring the levels of antioxidants and lipid peroxidation. For this, 1×10^6 cells were seeded into each well of a 6-well plate and treated with 25 µg/ml of B2 fraction. The cells were harvested, and 5% cell homogenate was prepared. Protein estimation was performed by standard protocol (Lowry *et al.*, 1951) and subjected to antioxidant assays as follows:

2.8.1. Glutathione (GSH)

The level of GSH was estimated by the standard method with slight modification (Moron *et al.*, 1979). Briefly, 80 µl of cell homogenate was incubated for 2 min at room temperature with 900 µl of 0.02 M sodium phosphate buffer and 20 µl of 10 mM DTNB followed by absorbance measurement at 412 nm in a UV-visible spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai). The level of GSH was calculated from a standard graph and expressed in µmol/mg protein.

2.8.2. Glutathione S-transferase (GST)

The level of GST was measured using the standard method (Beutler, 1984). Briefly, 50 µl of 5 mM CDNB was incubated with 850 µl of 0.1M phosphate buffer (pH 6.5) at 37°C for 10 min followed by the addition of 50 µl each of 20 mM GSH and cell homogenate. The absorbance was measured at 340 nm at 1 min intervals for 5 min. GST activity was expressed as unit/mg protein. GST activity was measured as:

$$\text{GST Activity} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / 9.6 \times \text{volume of test sample} \times 100$$

2.8.3. Superoxide dismutase (SOD)

The NBT reduction method was employed to estimate the level of SOD (Fried, 1975). Briefly, cell homogenate (100 µl), 186 µM of PMS (100 µl), 3 mM NBT (300 µl), and 780 µM NADH (200 µl) was incubated at 30°C for 90 sec and the reaction was stopped by adding 1 ml of acetic acid and 4 ml of n-butanol. The absorbance of the mixture was then recorded at 560 nm and the activity was expressed in unit/mg protein (1 unit = 50 % inhibition of NBT reduction).

$$\% \text{ inhibition} = (\text{OD}_{\text{blank}} - \text{OD}_{\text{sample}}) / \text{OD of blank} \times 100$$

2.8.4. Lipid peroxidation (LPO) assay

Malondialdehyde (MDA), which is generated by the breakdown of polyunsaturated fatty acids is a useful marker for evaluating the level of lipid peroxidation. The level of MDA was measured using the standard method with slight modifications (Beuege and Aust, 1978). In brief, 10% TCA, 0.8% TBA, and 0.02 N HCl were combined in a 1:2 ratio with cell homogenate and boiled for 10 min. The mixture was then cooled immediately and centrifuged at 12000 rpm for 10 min. The supernatant was collected, and absorbance was taken at 535 nm against blank. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$.

2.9. Single-Cell Gel Electrophoresis (Comet Assay)

The alkaline single-cell gel electrophoresis (Comet assay) was performed according to standard procedure with minor modifications (Singh *et al.*, 1988). Briefly, 1×10^5 cells were treated with different concentrations (10, 25, and 50 $\mu\text{g/ml}$) of B2 fraction. The cells were harvested and were suspended in 0.5% low-melting point agarose (75 μl) prepared in 1X PBS and spread onto a frosted slide precoated with 1% normal-melting point agarose. Slides were immersed for 2 h in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Trizma base, 1% Triton X-100, and 10% DMSO, pH 10.0). Following lysis, slides were placed in a horizontal electrophoresis tank containing freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13) for 20 min to allow unwinding of DNA. Electrophoresis was then carried out for 30 min at 24 V and 300 mA and the slides were then neutralized by washing with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min. Slides were washed with distilled water after neutralization and then stained with ethidium bromide solution (2 $\mu\text{g/ml}$) for 5 min. Each slide was prepared in triplicate and 100 randomly selected cells from each slide were examined using a fluorescence microscope with a magnification of 200x. The images captured were analysed with Image J software.

2.10. Caspase 3 and 6 activity assays

A quantitative enzymatic activity assay for caspases 3 and 6 was performed as per the manufacturer's protocols (BioVision Incorporated, USA). In brief, 2×10^6 cells were seeded in a T-25 flask with 5 ml of media followed by treatment with 25 µg/ml of B2 fraction. 5FU (100 µg/ml) was used as a positive control. The cells were harvested after 24 h and lysis was performed using 50 µl of chilled lysis solution and incubated for 10 min on ice after which the lysate was centrifuged at $15,000 \times g$ for 1 min at 4°C. The supernatant was collected, and protein estimation was performed and measured as per the Bradford method (Bradford, 1976). A specific colorimetric substrate, DEVD-pNA for caspase-3 and VEID-pNA for caspase-6, was used to measure the activity of 150 µg of protein from each sample. The assay was carried out in 96 well plates with a total volume of 100 µl. After incubating the mixture for 2 h at 37°C, the absorbance of free p-nitroanilide (pNA), which was generated when activated caspase 3 and caspase 6 cleaved from their respective substrates, was measured using a microplate reader at 405 nm.

2.11. *In silico* identification of molecular targets of B2 fraction

2.11.1. ADMET and druglikeness prediction

The Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) aspects of a drug's pharmacokinetic profile are necessary in determining how it is absorbed, distributed, metabolized, and excreted in the body. The canonical Simplified Molecular Input Line Entry Systems (SMILES) of the phytocompounds identified in the B2 fraction were retrieved from PubChem and utilized for further analysis. Identifying drug candidates with therapeutic importance that might function as a safe and effective medication requires an understanding of pharmacokinetics and physicochemical qualities. Therefore, the druglikeness and bioavailability of the compounds were then screened using Lipinski, Ghose, Veber, Egan, and Muegge's rules with swissADME software (<https://www.SwissADME.ch>). The phytocompounds that displayed no violation of the druglikeness were then further analysed for their pharmacokinetic properties using pkCSM software (<https://biosig.lab.uq.edu.au/pkcsml/prediction>) (Daina *et al.*, 2017).

2.11.2. Screening of potential targets

The target proteins of the selected phytocompound were obtained from the Swiss prediction database (<http://www.swisstargetprediction.ch/>) (Daina *et al.*, 2019). Proteins related to Non-Small Cell Lung Carcinoma (NSCLC) and Colorectal Carcinoma (CRC) were obtained from the GeneCard database (<https://www.genecards.org/>). Finally, Jvenn, a plug-in of the jQuery JavaScript library (<https://jvenn.toulouse.inrae.fr/>) was utilized to determine the shared targets of the selected phytocompound, NSCLC, and CRC (Bardou *et al.*, 2014).

2.11.3. Protein-Protein Interaction (PPI) network construction and core target screening

The protein-protein interaction network of shared targets was then generated using the STRING database (<https://string-db.org/>) (Szklarczyk *et al.*, 2023). The results were downloaded in tsv. format and then imported into Cytoscape 3.10.2 software for further analysis. The Cytohubba plug-in was used to rank the related nodes based on their degree value to screen for potential protein targets.

2.11.4. Analysis of expression pattern of target protein in non-small cell lung carcinoma (NSCLC) and colorectal cancer (CRC)

The expression pattern of the target protein in both NSCLC and CRC was analysed using the UALCAN database (<https://ualcan.path.uab.edu/analysis.html>) in a TCGA STAD cohort. Prognosis analysis was achieved using the Kaplan-Meier plotter database (<http://kmplot.com/analysis/>) (Uhlén *et al.*, 2015; Chandrashekar *et al.*, 2022).

2.11.5. Molecular docking

The three-dimensional (3D) structures of the selected protein from network pharmacology were obtained from the Protein Data Bank (PDB) (<https://www.rcsb.org/>). The chemical structure of the chosen bioactive was also obtained from the NCBI PubChem database (<http://pubchem.nlm.nih.gov/>). Molecular docking was done using the UCSF CHIMERA plugin by VINA, and the docked result was visualized using Discovery Studio Visualizer (Ralte *et al.*, 2023).

2.11.6. Molecular dynamic simulation

Molecular dynamics simulations were conducted to assess the interaction strength and stability of the receptor-ligand complex. Topology files for small molecules were generated via SOBTOP, whereas the protein topology was acquired through GROMACS commands. The Amber14sb force field and SPC water model were employed to simulate physiological conditions, maintaining a 20 buffer between protein atoms and the water box edge. The system was neutralized using 0.15 mol/l saline solution. Before the simulations, energy minimization was conducted utilizing 50,000 steepest-descent iterations. The system was equilibrated with NVT and NPT ensembles for 100 ps with a two fs timestep. A molecular dynamics simulation lasting 100 nanoseconds was subsequently performed at 310 Kelvin. Trajectory analysis was performed after the elimination of periodic boundary conditions, and the resulting structure was overlaid onto the initial complex to evaluate interactions. Key metrics, such as RMSD, RMSF, radius of gyration, etc, were evaluated, and the Gibbs free energy landscape was constructed. The total binding free energy was calculated using gmx_MMPBSA (Ralte *et al.*, 2023).

2.12. Statistical analysis

All data were expressed as mean $\pm$ standard error of the mean (SEM). One-way ANOVA followed by Tukey's test was employed to test the significant variations. SPSS ver.16.0 software (SPSS Inc, Chicago, Illinois, USA) and GraphPad Prism ver. 8.0 was used for statistical and graphical analysis. A p-value of less than 0.05 was the threshold considered to be statistically significant.

3. RESULTS

3.1. The cytotoxic effect of various fractions of *E. caudata* against neoplastic cells

Non-small cell lung carcinoma (A549) and human colorectal carcinoma cells (HCT116) were treated with different concentrations of the fractions of *E. caudata* for 24 h to screen their cytotoxicity using MTT assay. Out of the 17 fractions screened, four fractions – A2, B1, B2, and H2 displayed a dose-dependent increase in cytotoxicity against both cancer cells. The inhibition (%) of the cells by the fractions were plotted against their log doses for the determination of their IC_{50} (Figure 3.1A-D). Among the various fractions screened, the B2 fraction was found to be the most potent with an IC_{50} of $25.70 \pm 1.87 \mu\text{g/ml}$ for HCT116 and $26.78 \pm 2.32 \mu\text{g/ml}$ for A549 (Figure 3.2A & B). The most potent fraction i.e., B2 fraction was further assessed for its cytotoxicity against normal murine cell line L929. Even at a concentration of $200 \mu\text{g/ml}$, the IC_{50} of fraction B2 could not be determined, demonstrating the fraction's safety in a normal cell line. The vehicle control was also found to be non-toxic for both cancer cell lines.

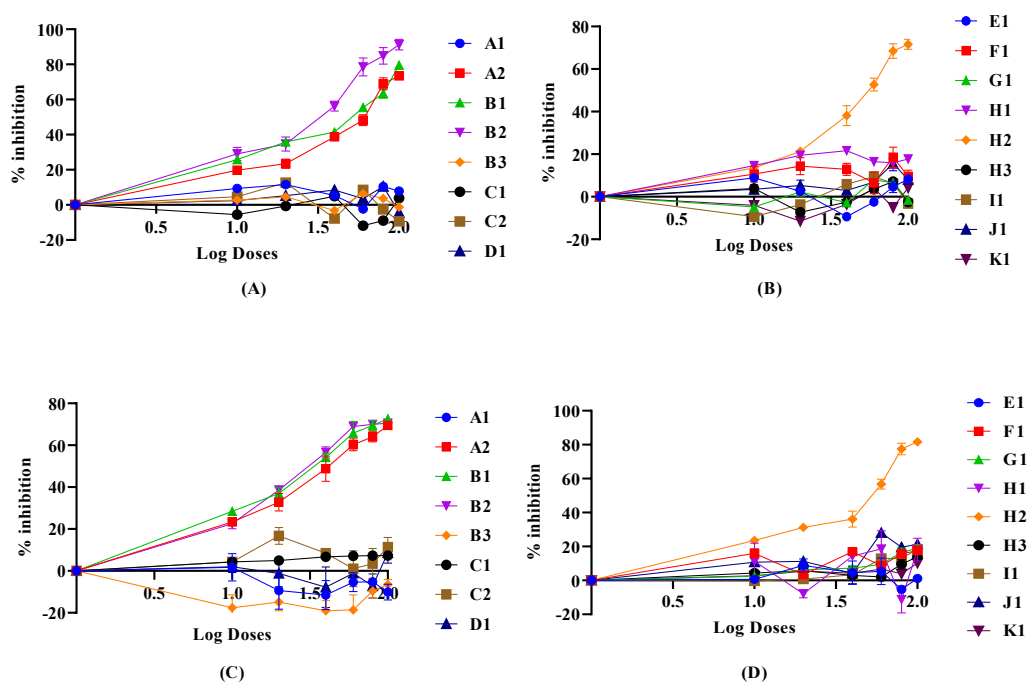
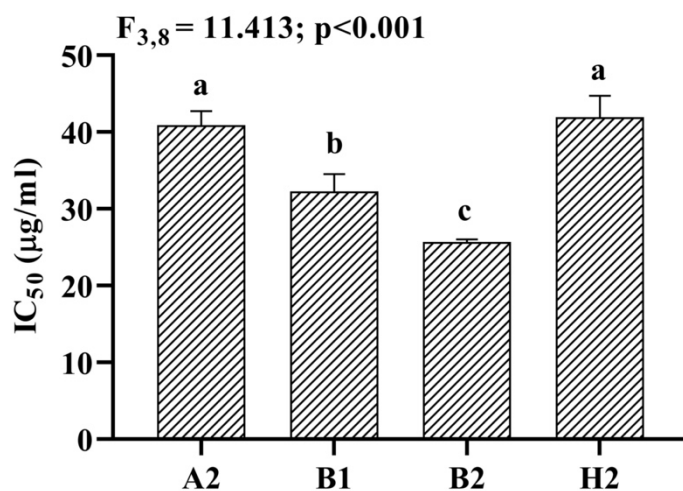
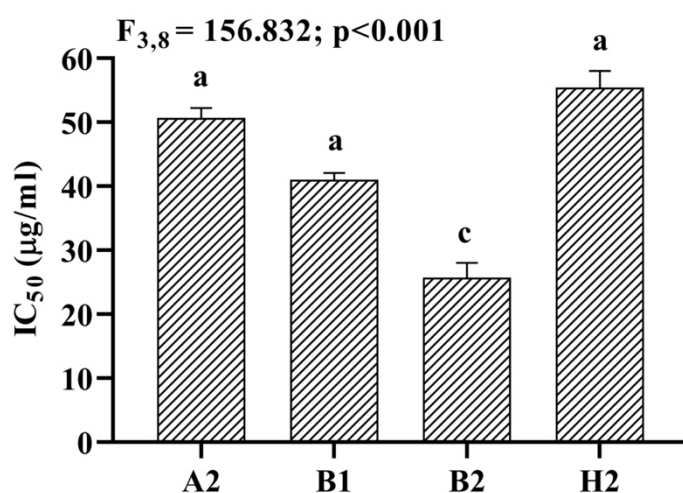


Figure 3.1. Log-dose plotted against % inhibition of different fractions of *E. caudata* for the calculation of IC_{50} against HCT116 cells (A & B) and A549 cells (C & D).



(A)



(B)

Figure 3.2. IC₅₀ of different fractions of *E. caudata* against (A) HCT116 cells and (B) A549 cells. Values are expressed as Mean $\pm$ SEM (n = 3). Different letters indicate significant variation.

3.2. Identification of phytochemicals in the B2 fraction using LC-HRMS

Liquid Chromatography coupled with High-Resolution Mass Spectroscopy (LC-HRMS) was employed to identify the compounds present in the most potent fraction of *E. caudata* i.e., the B2 fraction. LC-MS chromatogram analysis identified

the presence of 12 compounds belonging to flavonoid, alkaloid, and rotenoid, and the detail of compounds identified from the B2 fraction is given in Table 3.1.

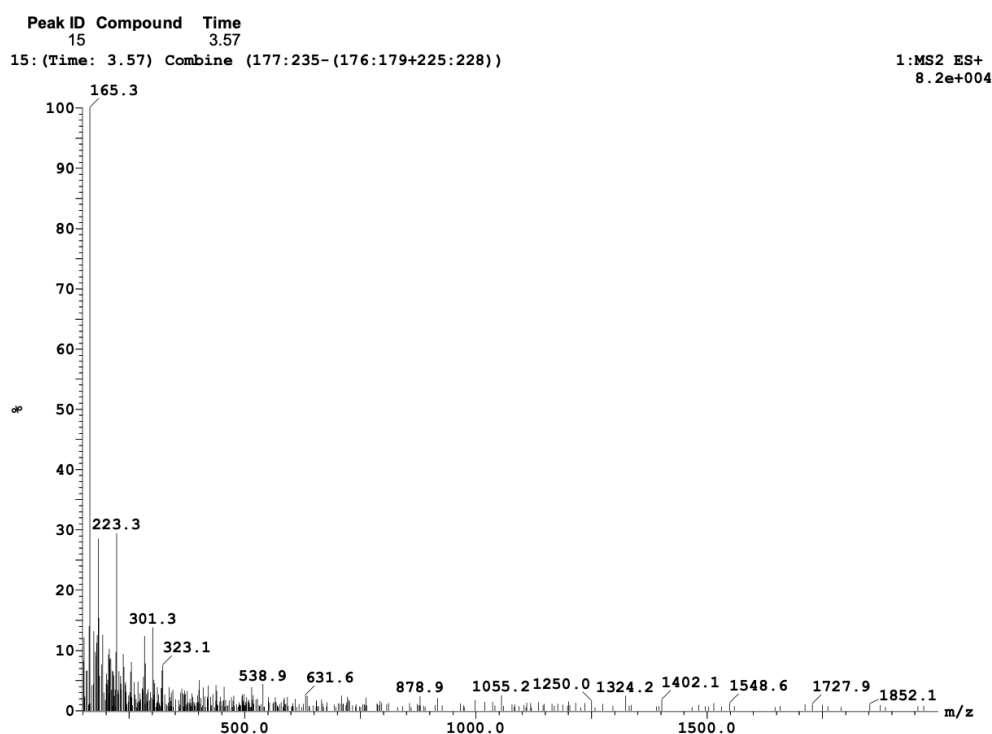


Figure 3.3. LC-HRMS chromatogram analysis of B2 fraction of *E. caudata*.

Table 3.1. Compounds identified in the B2 fraction of *E. caudata* using LC-HRMS.

Sl.	Name of Compound	m/z ratio	Class
1.	4' Hydroxyflavone	236.89	Flavonoid
2.	6,2'-Dihydroxyflavone	254.24	Flavonoid
3.	Aloe-emodin 8-glycoside	432.94	Dihydroxyanthraquinone
4.	Amorphigenin	432.94	Rotenoid
5.	Bergapten	216.89	Furanocoumarins
6.	Chrysoeriol-7-O Glucoside	605.92	Flavonoid
7.	Genipin	248.89	Iridoid glycoside
8.	Halocynthiaxanthin	158.21	Carotenoids
9.	Harmane	183.23	Indole Alkaloid
10.	Pinocembrin	256.89	Flavonoid
11.	Sinapic Acid	209.89	Phenylpropanoid
12.	Vanillic Acid	229.89	Phenolic acid

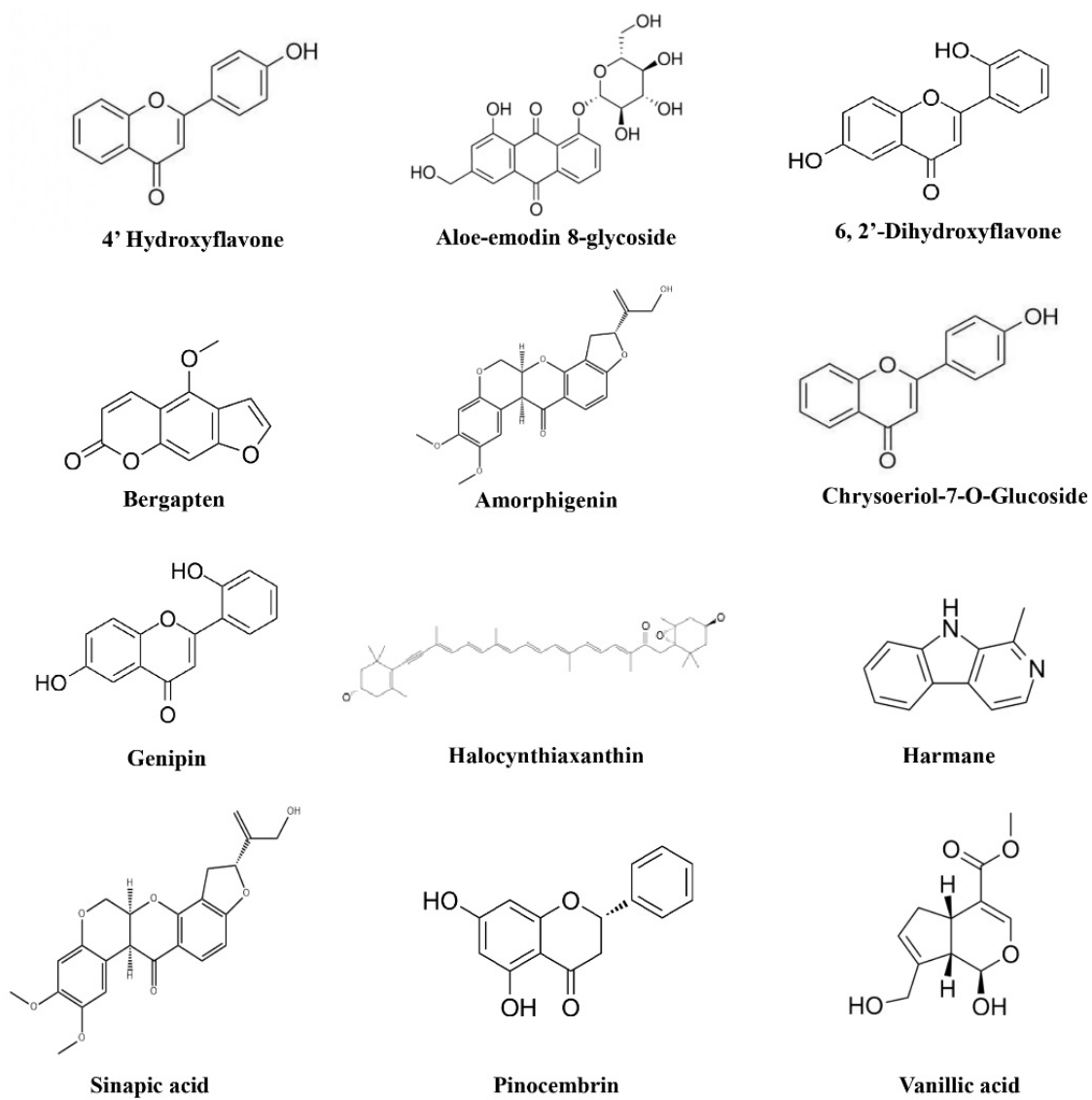


Figure 3.4. Molecular structures of compounds identified from the B2 fraction of *E. caudata*.

3.3. Morphological evidence of apoptosis induced by B2 fraction

Apoptosis is the optimal choice of cellular death without the generation of inflammation. Therefore, the acridine orange/ethidium bromide (AO/EtBr) dual staining was used to ascertain whether the B2-induced cellular death in the cancer cells was by apoptosis. HCT116 and A549 cells treated with different doses of B2 fraction of *E. caudata* showed a dose-dependent increase in the number of apoptotic cells. Fluorescence microscopy revealed apoptotic evidence such as membrane blebbing, chromatin condensation, and fragmentation in the B2-treated cancer cells (Figures 3.5 & 3.6). The apoptotic index % was also observed to increase in a dose-dependent manner for both cancer cells. Furthermore, the apoptotic-inducing activity of B2 fraction at the dose of 50 $\mu\text{g/ml}$ was found to be significantly higher than the apoptotic-inducing action of the standard drug 5FU (100 $\mu\text{g/ml}$) in both cancer cells (Figure 3.7A & B).

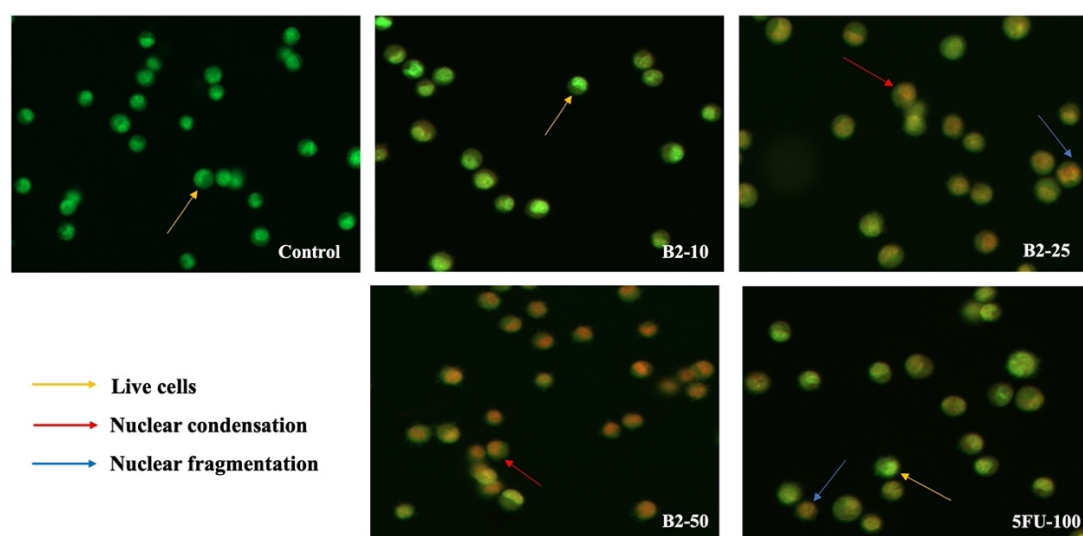


Figure 3.5. Acridine orange/Ethidium bromide dual staining of HCT116 cells after treatment with different doses of B2 fraction for 24 h. Control: Cancer cells without treatment; B2-10, B2-25, and B2-50: Cancer cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of *E. caudata* respectively; 5FU: Cancer cells treated with 100 $\mu\text{g/ml}$ of 5FU (positive control).

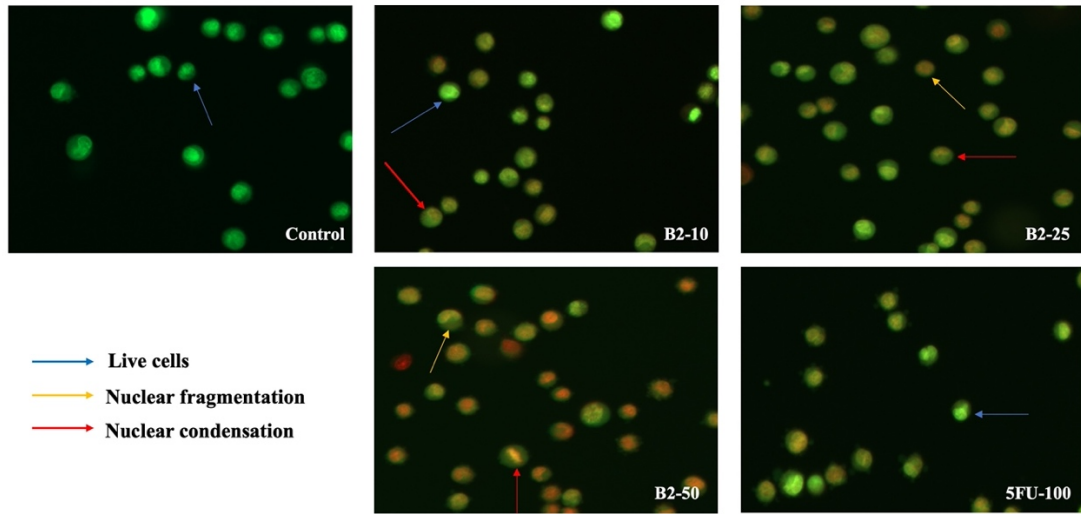


Figure 3.6. Acridine orange/Ethidium bromide dual staining of A549 cells after treatment with different doses of B2 fraction for 24 h. Control: Cancer cells without treatment; B2-10, B2-25, and B2-50: Cancer cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of *E. caudata* respectively; 5FU: Cancer cells treated with 100 $\mu\text{g/ml}$ of 5FU (positive control).

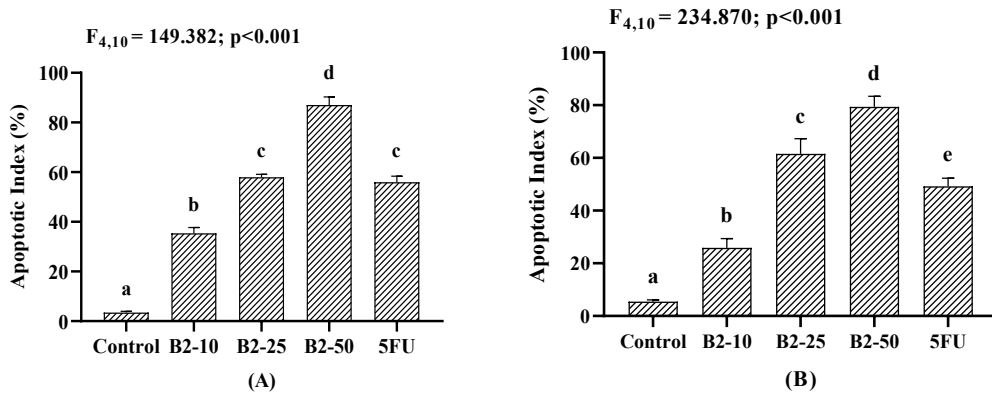


Figure 3.7. Apoptotic index of HCT116 cells (A) and A549 cells (B) after treatment with different doses of B2 fraction of *E. caudata*. Control: Cancer cells without treatment; B2-10, B2-25, and B2-50: Cancer cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of *E. caudata* respectively; 5FU: Cancer cells treated with 100 $\mu\text{g/ml}$ of 5FU (positive control). Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

3.4. Analysis of DNA damage in B2-treated neoplastic cells using Comet Assay

The activation of apoptosis induced by DNA damage in response to anticancer drug treatment is a crucial component of anticancer therapy. The alkaline single-cell gel electrophoresis or Comet Assay was performed to evaluate whether different doses of B2 fraction could induce DNA damage in the treated cancer cells (Figure 3.8 & Figure 3.10). Our result shows that treatment of both HCT116 cells and A549 cells with different doses of B2 fraction could significantly induce DNA damage as is evident from the dose-dependent increase in the tail length and olive tail moments when compared to the untreated cancer cells (Figure 3.9A & B & Figure 3.11A & B). Furthermore, the levels of DNA damage induced by 25 $\mu\text{g/ml}$ of B2 fraction were found to be similar to that of the standard drug 5FU in the A549 cells.

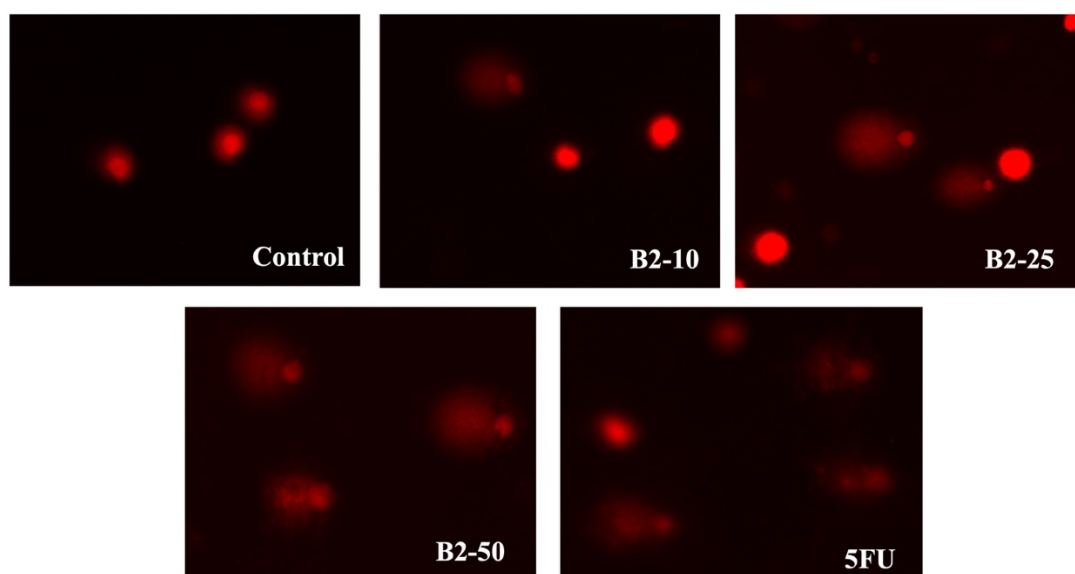


Figure 3.8. Fluorescent image of comet observed in HCT116 cells treated with different concentrations of B2 fraction of *E. caudata*. Control: HCT116 cells without treatment; B2-10, B2-25, and B2-50: HCT116 cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of *E. caudata* respectively; 5FU: HCT116 cells treated with 100 $\mu\text{g/ml}$ of 5FU.

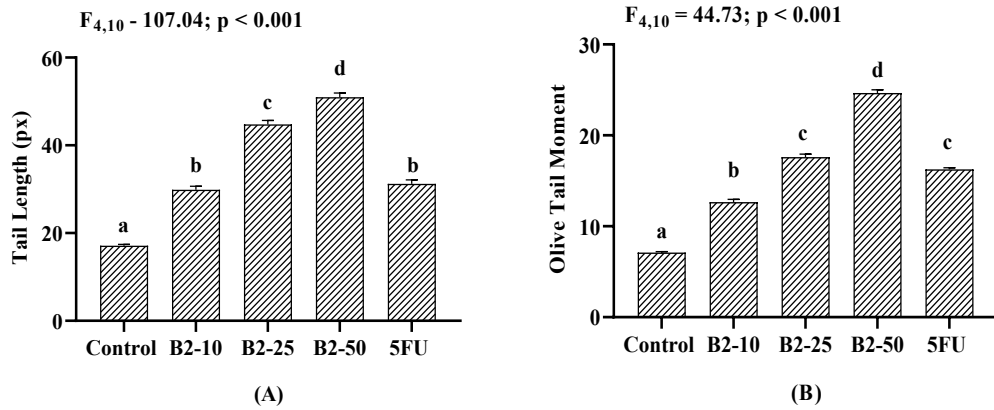


Figure 3.9. The extent of DNA damage induced by the B2 fraction of *E. caudata* in HCT116 cells expressed in terms of (A) tail length and (B) tail olive moment. Control: HCT116 cells without treatment; B2-10, B2-25, and B2-50: HCT116 cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of *E. caudata* respectively; 5FU: HCT116 cells treated with 100 $\mu\text{g/ml}$ of 5FU. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

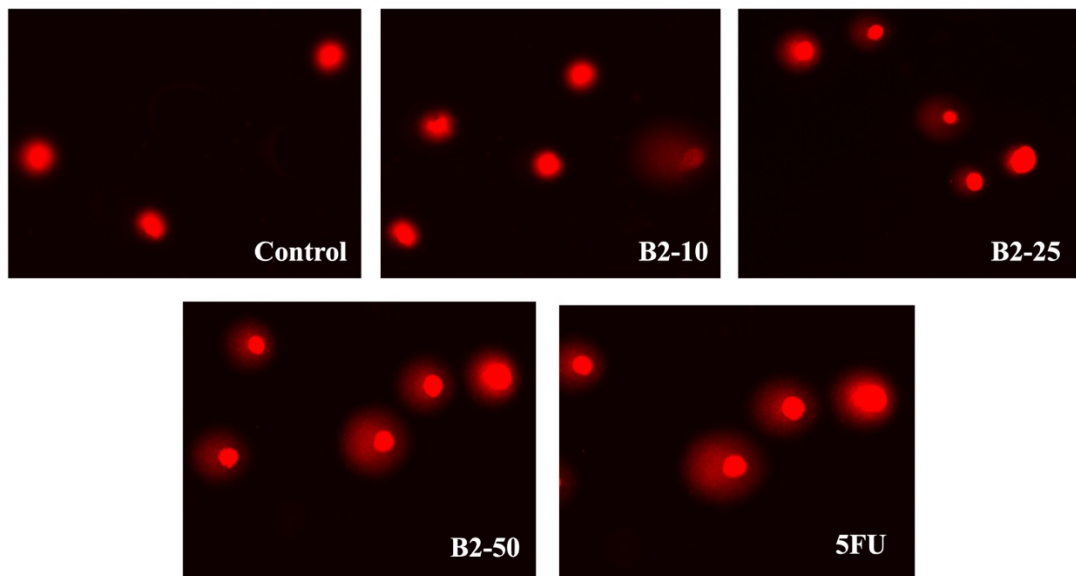


Figure 3.10. Fluorescent image of comet observed in A549 cells treated with different concentrations of B2 fraction of *E. caudata*. Control: A549 cells without treatment; B2-10, B2-25, and B2-50: A549 cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of *E. caudata* respectively; 5FU: A549 cells treated with 100 $\mu\text{g/ml}$ of 5FU.

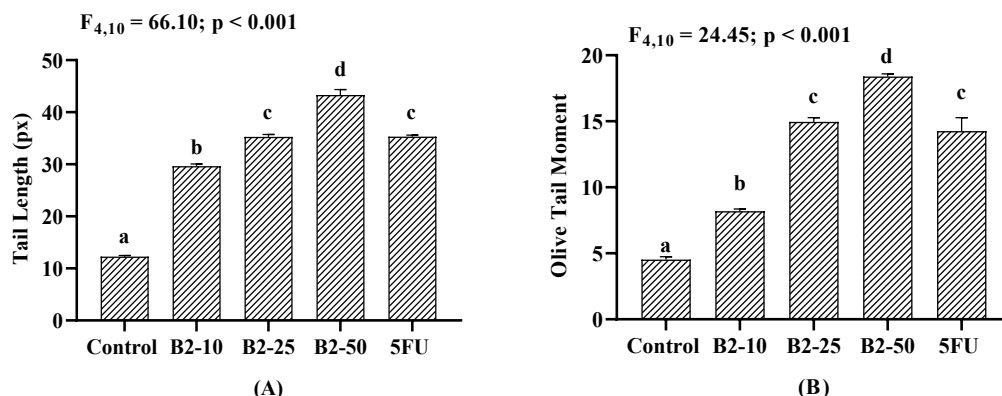


Figure 3.11. The extent of DNA damage induced by the B2 fraction of *E. caudata* in A549 cells expressed in terms of (A) tail length (B) tail olive moment. Control: A549 cells without treatment; B2-10, B2-25, and B2-50: A549 cells treated with 10, 25, and 50 $\mu\text{g/ml}$ of B2 fraction of *E. caudata* respectively; 5FU: A549 cells treated with 100 $\mu\text{g/ml}$ of 5FU. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

3.5. Effects of B2 fraction on the redox system of cancer cells

To assess whether B2 fraction alters the redox system of the cancer cells, the level and activities of antioxidants including glutathione (GSH), glutathione S-transferase (GST), and superoxide dismutase (SOD) were measured in both HCT116 and A549 cells after treated with 25 $\mu\text{g/ml}$ of B2 fraction. Treatment of cancer cells with B2 fraction of *E. caudata* significantly decreases the level of GSH as well as the activities of GST and SOD when compared to the untreated control (Figure 3.12A – C & Figure 3.13A – C). Furthermore, the level of malondialdehyde (MDA), a by-product of lipid peroxidation (LPO) was also assessed as a biomarker of oxidative stress in the cancer cells. Our study revealed that treatment of cancer cells with a B2 fraction of *E. caudata* could significantly alter the redox balance by elevating the levels of MDA when compared to their untreated counterparts (Figure 3.12D & Figure 3.13D). Furthermore, the effect of B2 fraction on the activities of GST and induction of LPO was observed to be similar to that of the standard drug in HCT116 cells.

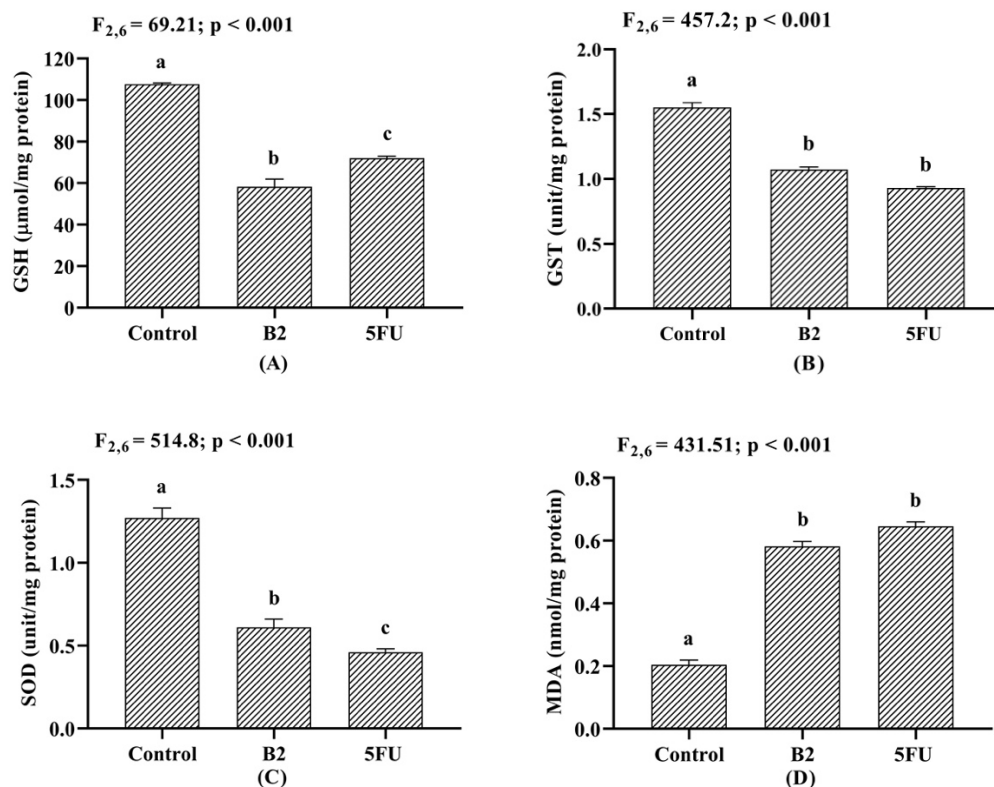


Figure 3.12. Effects of B2 fraction of *E. caudata* on the levels and activities of GSH (A), GST (B), SOD (C), and MDA (D) of HCT116 cells. Control: HCT116 cells without treatment; B2: HCT116 cells treated with 25 $\mu\text{g/ml}$ of B2 fraction of *E. caudata*; 5FU: HCT116 cells treated with 100 $\mu\text{g/ml}$ of 5FU (positive control). Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

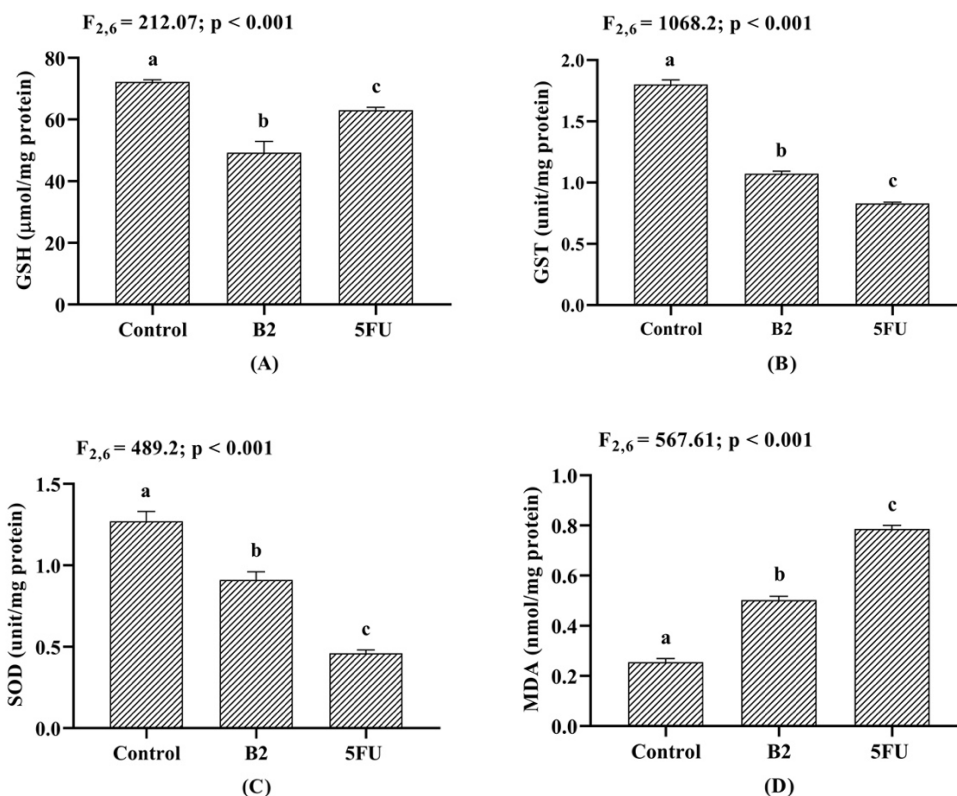


Figure 3.13. Effects of B2 fraction of *E. caudata* on the levels and activities of (A) GSH (B) GST (C) SOD and (D) MDA of A549 cells. Control: A549 cells without treatment; B2: A549 cells treated with 25 μg/ml of B2 fraction of *E. caudata*; 5FU: A549 cells treated with 100 μg/ml of 5FU (positive control). Values are expressed as Mean ± SEM. Different letters indicate significant variations.

3.6. B2 fraction induces apoptosis by activation of caspase 3/6

Effector caspases (cysteine-aspartic proteases) 3 and 6 are critical for triggering apoptosis in cells. Thus, the effect of fraction B2 on apoptosis in both the HCT116 cells and A549 cells was evaluated by assessing the activity of caspase 3 and 6. Treatment of both the cancer cells with 25 μg/ml of fraction B2 resulted in a significant increase in the activities of both caspase 3 and 6. Our results show that there was a 2.1- and 2.8-fold increase in the activities of caspase 3 and 6 respectively in B2 fraction-treated HCT116 cells when compared to the untreated control (Figure 3.14A & B). A similar result was also observed in A549 cells where the B2 fraction treatment induced a 1.7- and 2.0-fold increase in the activities of caspase 3 and 6 respectively when compared to the untreated cancer cells (Figure 3.14C & D). In

addition, HCT116 cells treated with 25 $\mu\text{g/ml}$ of B2 fraction exhibited the same effect on caspase 6 activity as the standard drug (Figure 3.14B).

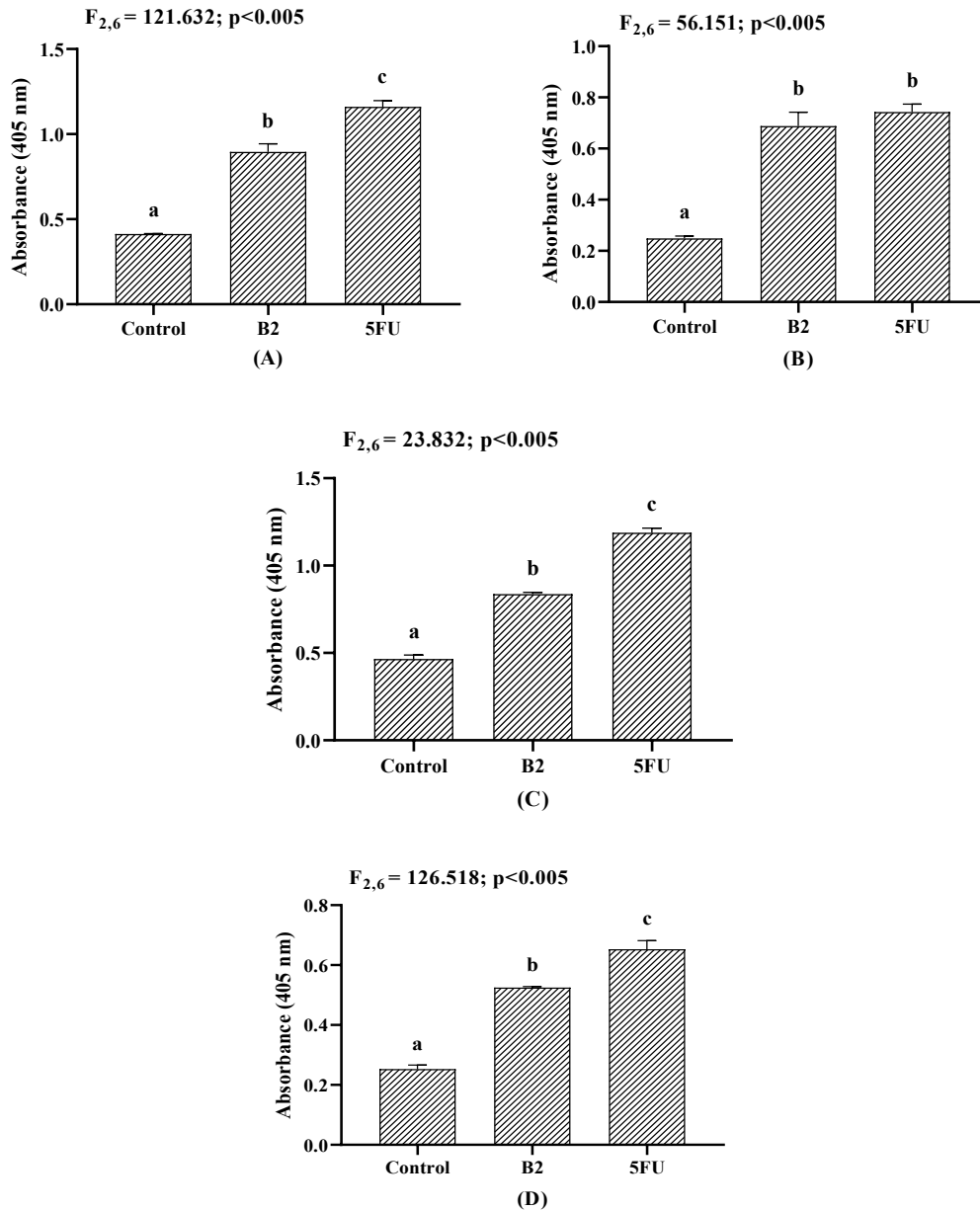


Figure 3.14. Effects of B2 fraction of *E. caudata* on activities of (A) Caspase-3 in HCT116 cells; (B) Caspase-6 in HCT116 cells; (C) Caspase-3 in A549 cells; and (D) Caspase-6 in A549 cells after 24 h treatment. Control: Cancer cells without treatment; B2: Cancer cells treated with 25 $\mu\text{g/ml}$ of B2 fraction of *E. caudata*; 5FU: Cells treated with 100 $\mu\text{g/ml}$ of 5FU. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

3.7. Druglikeness and physicochemical analysis of phytocompounds from B2 fraction

To determine the druglikeness and physicochemical properties of the phytocompounds from the B2 fraction, the SwissADME prediction tool was employed. The druglikeness was calculated based on the rule of Lipinski, Ghose, Veber, Egan, and Muegge. Of the 12 phytocompounds screened, seven compounds viz., 4'-Hydroxyflavone, 6,2'-Dihydroxyflavone, Amorphigenin, Bergapten, Genipin, Pinocembrin, and Sinapic acid displayed zero violation of all the druglikeness rules (Table 3.2). The physicochemical properties of the phytocompounds for druglikeness prediction are displayed in Table 3.3. The compounds, which displayed zero violation of all the druglikeness rules, were then further analysed for their Oral Bioavailability (OB), from which two compounds, Genipin and Amorphigenin, showed their OB parameters within the desired shaded area in the OB radar diagram (Figure 3.15).

Table 3.2. Drug-likeness analysis of drug candidates. ‘Yes’, indicate zero violation (A) 4'-Hydroxyflavone (B) 6,2'-Dihydroxyflavone (C) Aloe Emodin 8-Glucoside (D) Amorphigenin (E) Bergapten (F) Chrysoeriol-7-O-Glucoside (G) Genipin (H) Halocynthiaxanthin (I) Harman (J) Pinocembrin (K) Sinapic Acid (L) Vanillic Acid.

Rule	A	B	C	D	E	F	G	H	I	J	K	L
Lipinski	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No
Ghose	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Veber	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Egan	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes
Muegge	Yes	Yes	No	Yes	Yes	No	Yes	No	No	Yes	Yes	No
Bioavailability score	0.55	0.55	0.55	0.55	0.55	0.17	0.56	0.17	0.55	0.55	0.56	0.85

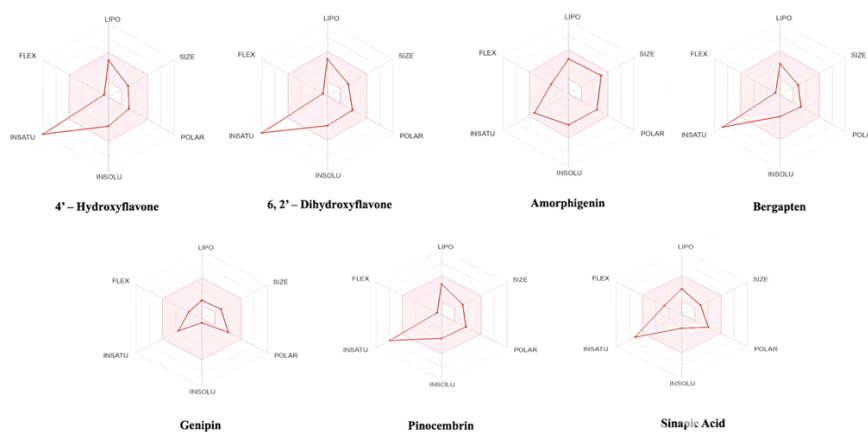


Figure 3.15. Oral Bioavailability chart of selected phytocompounds. The colored zone is the suitable physicochemical space for oral bioavailability. LIPO: Lipophilicity; SIZE: Molecular size; POLAR: Polarity; INSOLU: Insolubility; INSATU: Insaturation and FLEX: Flexibility

Table 3.3. Physicochemical properties of phytochemicals based on SwissADME prediction. (A) 4'- Hydroxyflavone (B) 6,2'- Dihydroxyflavone (C) Aloe Emodin 8-Glucoside (D) Amorphigenin (E) Bergapten (F) Chrysoeriol – 7 – O – Glucoside (G) Genipin (H) Halocynthiaxanthin (I) Harman (J) Pinocembrin (K) Sinapic Acid (L) Vanillic Acid.

Properties	A	B	C	D	E	F	G	H	I	J	K	L
Heavy atoms	18	19	31	30	16	33	16	44	14	19	16	12
Heavy aromatic atoms	16	16	12	12	13	16	0	0	13	12	6	6
Fraction Csp ³	0	0	0.33	0.35	0	0	0.55	0.53	0.08	0.13	0.18	0.12
Rotatable bonds	1	1	4	4	0	0	3	9	0	1	4	2
H-bond acceptors	3	4	10	7	4	11	0.08	0.32	1	4	5	4
H-bond donors	1	2	6	1	0	6	1	5	1	2	2	2
Molar refractivity	69.94	71.97	102.05	107.32	58.75	112.60	54.51	185.59	58.57	69.55	58.12	41.92
Topological Polar Surface Area (Å ²)	50.4	70.67	173.98	83.45	52.58	179.28	75.99	70.06	28.68	66.76	75.99	66.76

3.8. ADMET analysis

The two selected phytocompounds were then analysed for their ADMET properties (Table 3.4). Based on this analysis, the compound Amorphigenin was found to be more suitable for a drug candidate due to better absorption, distribution, and lesser toxicity.

Table 3.4. ADMET analysis of Genipin and Amorphigenin using PkCSM software

Pharmacokinetics Parameters	Unit	Amorphigenin	Genipin
Absorption			
Water solubility	Log mol/L	-1.313	-3.72
Caco2 permeability	Log Papp in 10 ⁻⁶ cm/s	1.041	0.221
Intestinal absorption	% Absorbed	94.001	59.431
Skin permeability	Log Kp	-2.758	-3.872
Distribution			
Vdss (human)	Log L/kg	-0.151	-1.313
Fraction unbound	Fu	0.15	0.221
BBB permeability	Log BB	-0.049	59.431
CNS permeability	Log PS	-1.982	-3.872
Metabolism			
CYP2D6 substrate	Yes/No	No	No
CYP3A4 substrate	Yes/No	Yes	No
CYP1A2 inhibitor	Yes/No	No	No
CYP2C19 inhibitor	Yes/No	Yes	No
CYP2D6 inhibitor	Yes/No	No	No
CYP3A4 inhibitor	Yes/No	Yes	No

Excretion			
Total clearance	Log(ml/min/kg)	0.226	0.448
Renal OCT2 substrate	Yes/No	No	No
Toxicity			
AMES toxicity	Yes/No	No	Yes
Max. tolerated dose (Human)	Log mg/kg/day	0.111	1.088
hERG I inhibitor	Yes/No	No	No
hERG II inhibitor	Yes/No	No	No
LD ₅₀ Rat	Mol/kg	2.663	2.094
Oral Rat Chronic Toxicity	Log mg/kg_bw/day	1.57	2.223
Hepatotoxicity	Yes/No	No	No
Skin sensitization	Yes/No	No	No
<i>T. pyriformis</i> toxicity	Log µg/ml	0.32	0.205
Minnow toxicity	Log mM	1.158	3.134

3.9. Common Targets of Amorphigenin, NSCLC and CRCC

Based on the druglikeness, oral bioavailability, and AMDET analysis, Amorphigenin was selected for further *in silico* analysis. Swiss target prediction revealed a total of 101 possible protein targets for Amorphigenin. A total of 10137 proteins and 17094 protein targets were also compiled for NSCLC and CRC respectively from the GeneCard database. Mutual matching performed using the Jvenn tool resulted in the identification of 73 proteins (Figure 3.16).

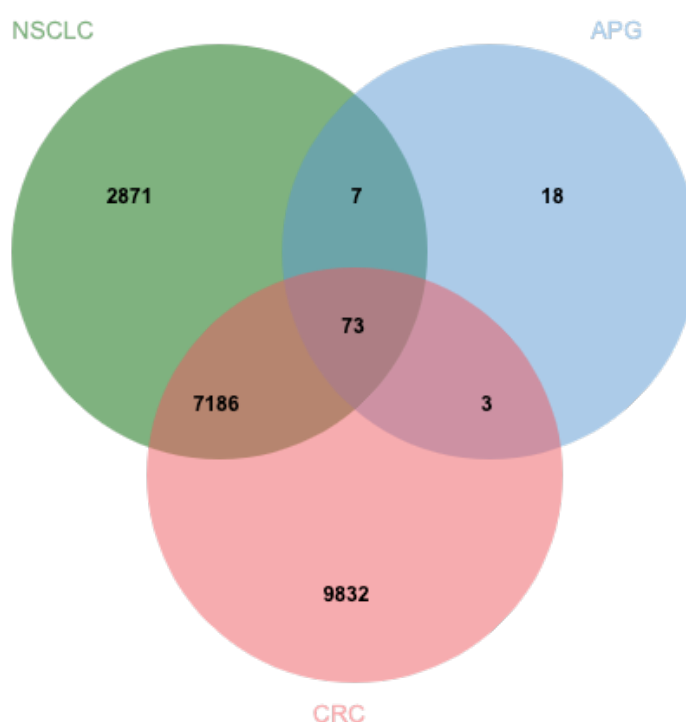


Figure 3.16. Venn diagram of proteins involved in NSCLC and CRC targeted by Amorphigenin. NSCLC: Non-Small Cell Lung Cancer; CRC: Colorectal Cancer; APG: Amorphigenin.

3.10. Analysis of Protein-Protein Interaction (PPI) Network

The 73 protein targets were loaded into the STRING database for PPI network analysis. The resulting network consists of 73 nodes and 409 edges with an enrichment p-value $< 1.0e-16$ (Figure 3.17). The network was then imported into Cytoscape software and analyzed by determining centrality and other metrics. The Cytohubba plug-in revealed the top ten genes ranked by degree as depicted in Figure 3.18. The

top 10 core gene targets were HSP90AA1, CASP3, PIK3CA, MTOR, GRB2, MDM2, MAPK3, JAK2, MAPK1 and MAPK8.

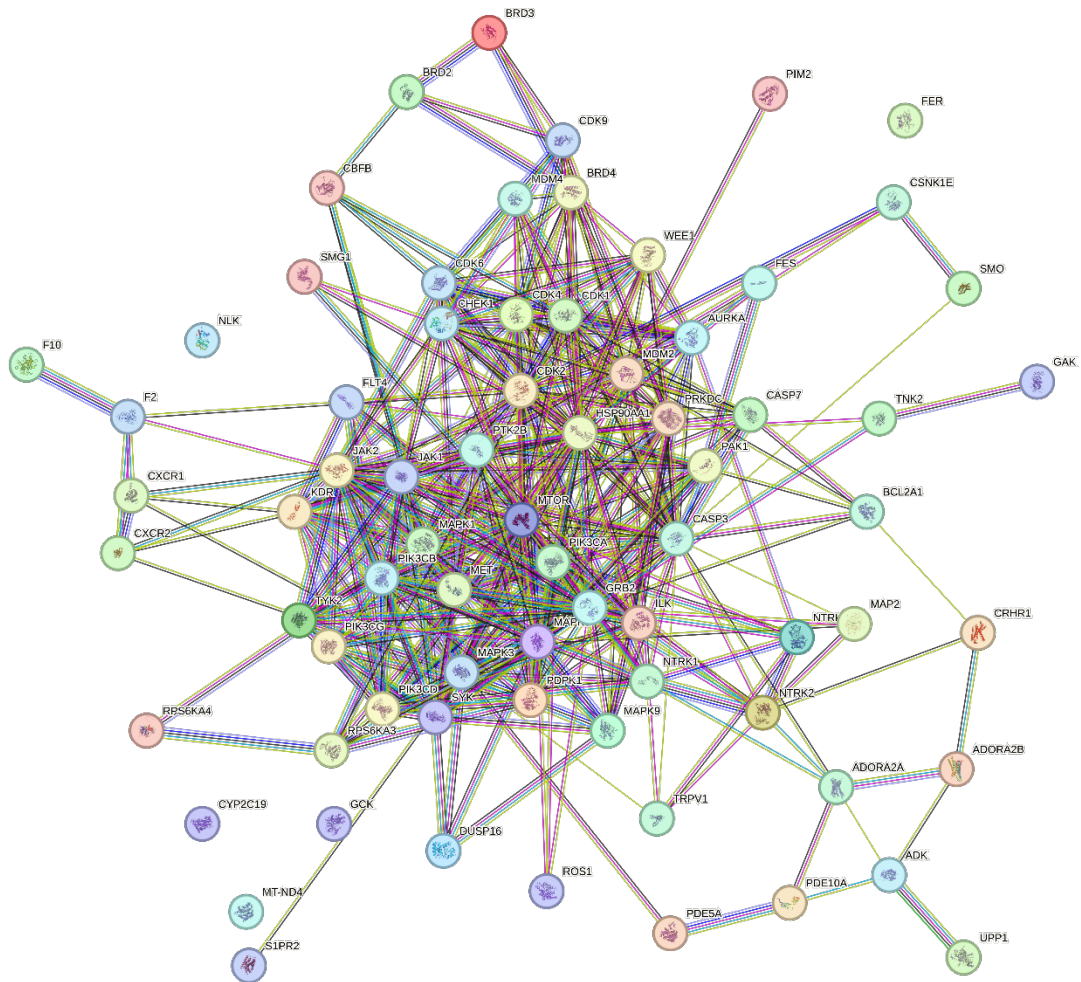


Figure 3.17. Protein-Protein Interaction (PPI) network of 73 protein targets.

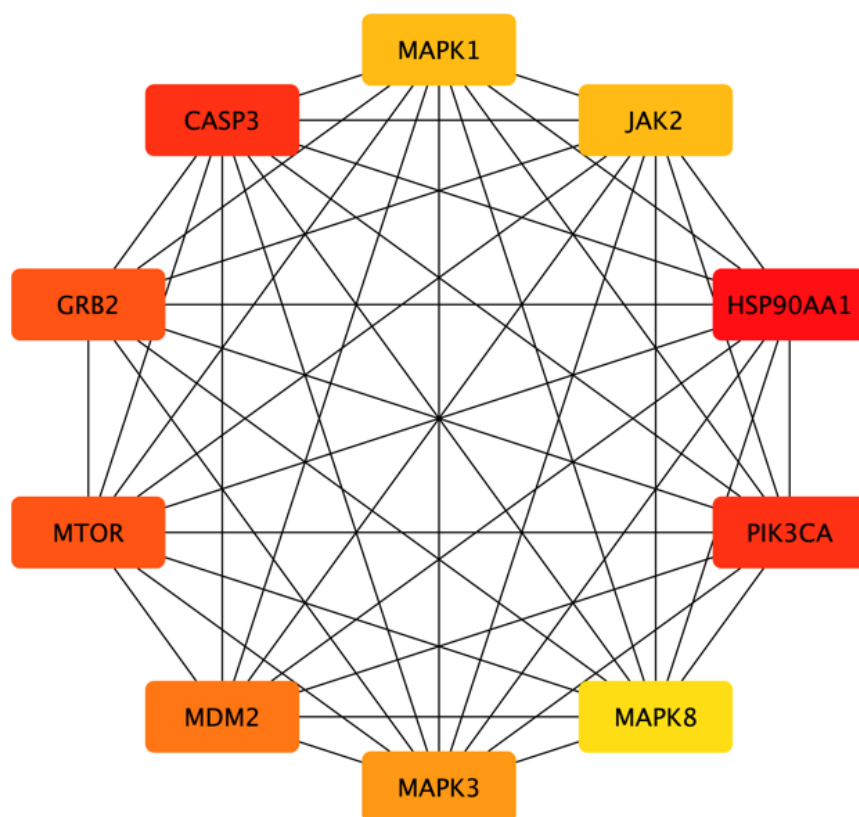


Figure 3.18. The top 10 potential target networks ranked by degree value as generated by Cytoscape 3.10.2.

3.11. Pattern and survival probability of HSP90AA1 expression in NSCLC and CRC

The UALCAN database showed that HSP90AA1 was expressed in all normal, lung, and colon carcinomas. Notably, the expression pattern of HSP90AA1 in both NSCLC and CRC was significantly higher than in their normal counterparts (Figure 3.19A & B). In addition, it was also observed that higher expression of HSP90AA1 was associated with tumor progression and metastasis (Figure 3.20 A & B; Figure 3.21A & B). Kaplan-Meier Plotter database showed that the high expression of HSP90AA1 was associated with an overall decreased survival rate suggesting that HSP90AA1 upregulation may be an indicator of poor prognosis (Figure 3.22).

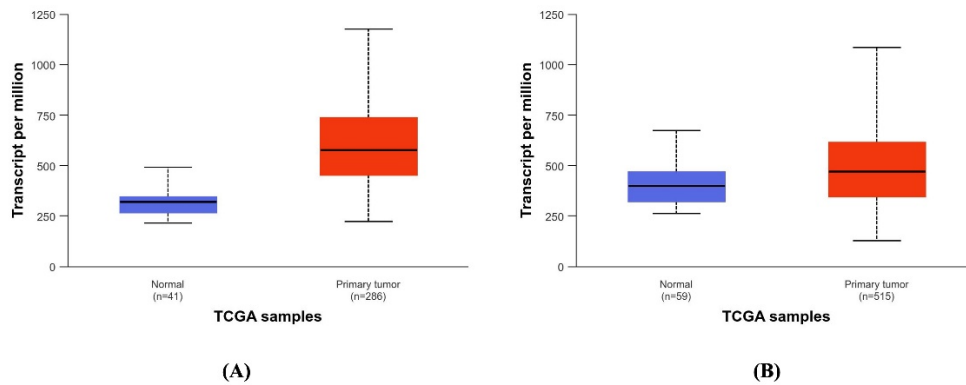


Figure 3.19. Expression of HSP90AA1 in (A) Colorectal Adenocarcinoma (B) Non-Small Cell Lung Cancer.

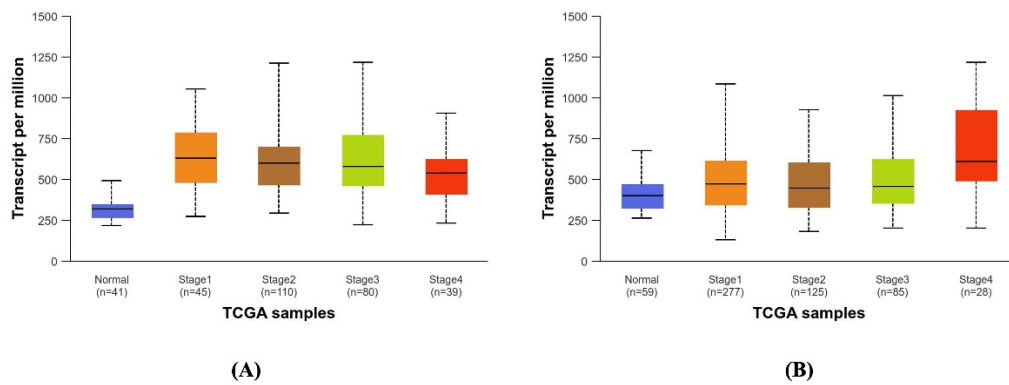


Figure 3.20. Expression patterns of HSP90AA1 based on individual cancer stages in (A) Colorectal cancer (B) Non-Small Cell Lung carcinoma.

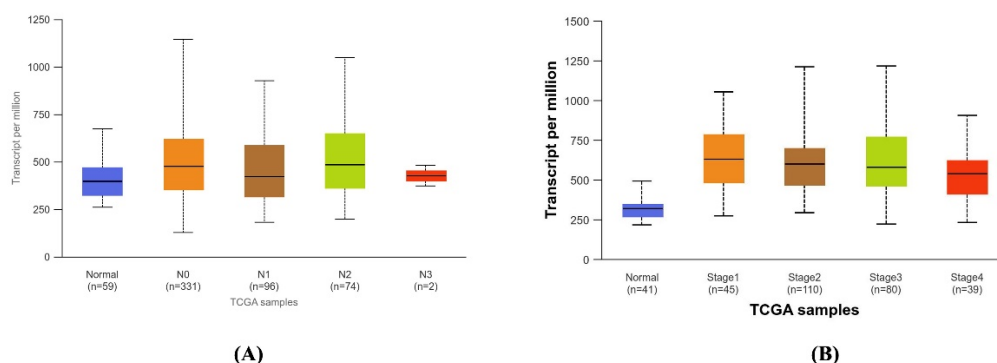


Figure 3.21. Expression patterns of HSP90AA1 based on nodal metastasis status in (A) Colorectal cancer (B) Non-Small Cell Lung carcinoma.

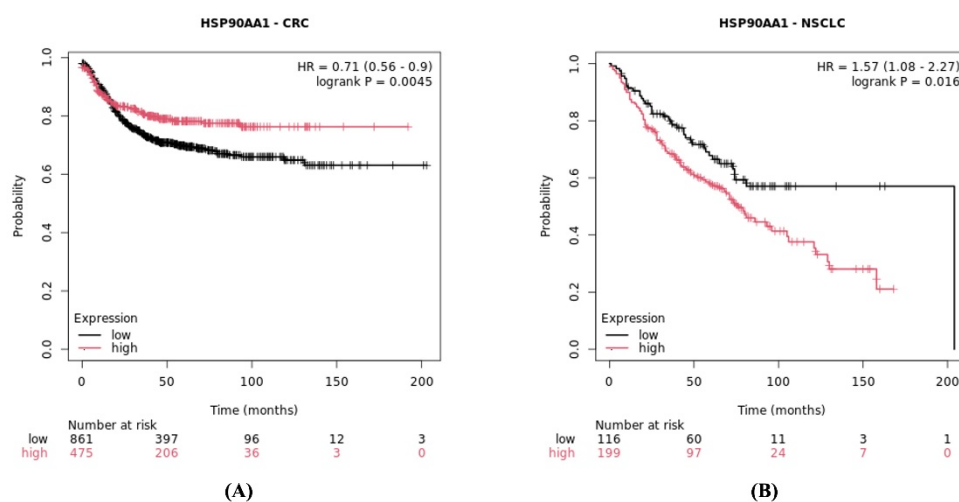
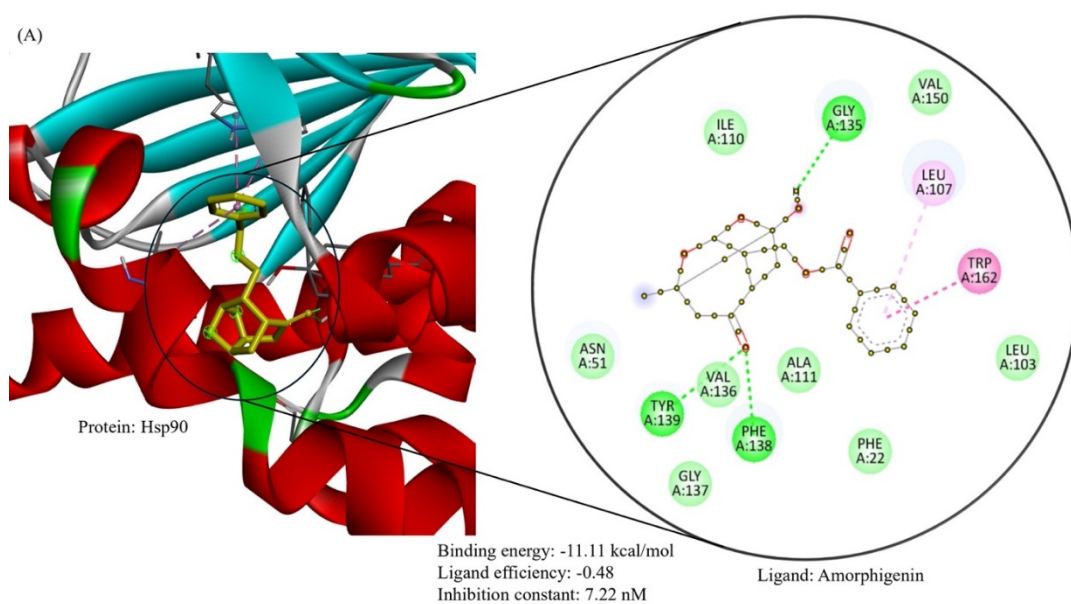


Figure 3.22. Kaplan-Meier survival plot for HSP90AA1 expression in (A) Colorectal Carcinoma and (B) Non-Small cell lung carcinoma.

3.12. Molecular Docking

Considering the elevated expression pattern of HSP90 in non-small cell lung carcinoma and colorectal carcinoma and its correlation with metastasis and poor prognosis, it appears that targeting HSP90 could be a promising therapeutic strategy for combating non-small cell lung carcinoma and colorectal carcinoma. HSP90 enhances and promotes the functions of its client proteins through its ATPase cycle. Therefore, the phytochemical Amorphigenin was selected and assessed for its inhibitory potential against the N-terminal domain of HSP90 by using the molecular

docking technique. UCSF CHIMERA plugin by VINA was employed to execute molecular docking to evaluate the potential interaction between Amorphigenin (CID 92207) and the primary target HSP90 (PBD ID 4BQG). The molecular docking investigation of HSP90 with Amorphigenin and the control ligand Geldanamycin for anticancer potential demonstrated that Amorphigenin displayed a superior binding affinity, with a binding energy of -11.11 kcal/mol, a ligand efficiency of -0.48, and an inhibitory constant (K_i) of 7.22 nM (Figure 3.23A). In contrast, Geldanamycin exhibited a marginally reduced binding energy of -10.11 kcal/mol, a ligand efficiency of -0.24, and an elevated inhibition constant of 70.51 nM (Figure 3.23B).



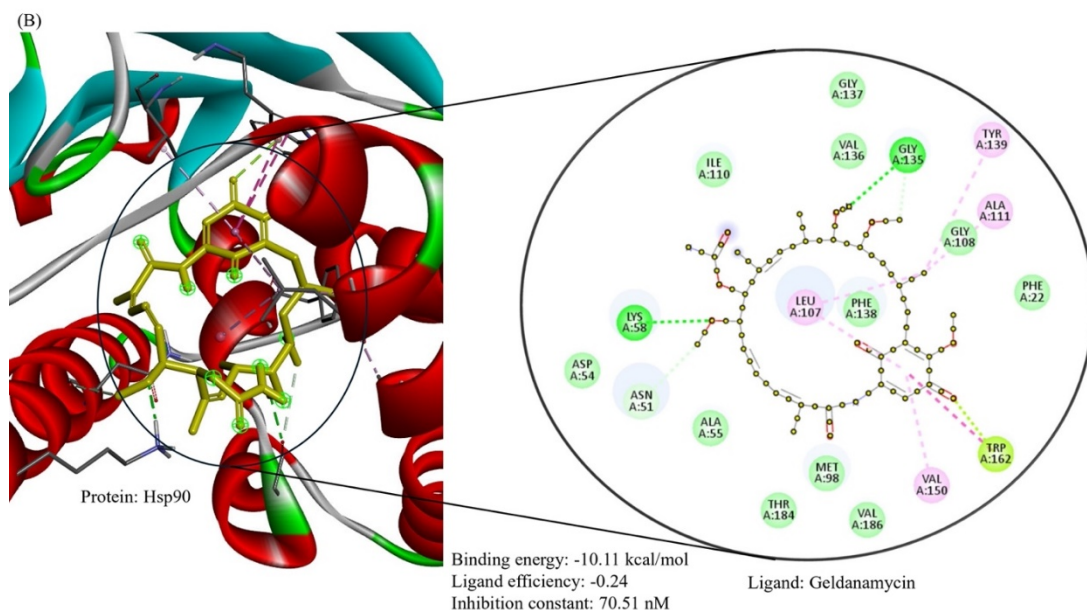


Figure 3.23. 3D structure of HSP90 showing binding site (left) and the main residues involved in the HSP90- (A) Amorphigenin and (B) Geldanamycin (control) interaction with bond type and length (right).

3.13. Molecular dynamic simulation

RMSD analysis was performed to assess the structural stability of the Amorphigenin-HSP90 complex in comparison to the Geldanamycin control during a 100 ns molecular dynamics simulation. The RMSD plot demonstrates that Geldanamycin displays markedly greater variations, with RMSD values increasing from around 1.5 nm at 10 ns to over 6.0 nm at approximately 90 ns. Conversely, the Amorphigenin-HSP90 complex exhibits a very constant RMSD, oscillating within a narrower range of 0.5 – 1.5 nm over the simulation. The average RMSD for Amorphigenin is approximately 1.0 nm, indicating a more stable binding conformation relative to the control (Figure 3.24). RMSF analysis was used to evaluate the flexibility of specific residues in the Amorphigenin-HSP90 complex relative to the control (Geldanamycin). The RMSF profile reveals that the majority of residues display changes between 0.05 and 0.25 nm, indicating significant flexibility in both systems. Nevertheless, several areas, especially near residue 3000, exhibit markedly greater fluctuations, attaining 0.45 nm, signifying enhanced mobility. The Amorphigenin-HSP90 bound complex exhibits marginally reduced fluctuations in

critical binding site residues relative to the control, indicating that Amorphigenin aids in structural stabilization in these areas (Figure 3.25). This decrease in flexibility may be due to the ligand binding affinity and association stability with HSP90. An investigation in the number of hydrogen bonds (H-bonds) was performed to evaluate the stability and interaction strength of Amorphigenin and Geldanamycin with HSP90 throughout the 100 ns simulation. The findings demonstrate that both ligands sustained hydrogen bonds with the protein at differing frequencies. Geldanamycin demonstrated a greater frequency of hydrogen bonding, peaking at 5 H-bonds at 20 ns, while Amorphigenin typically formed 1 – 2 H-bonds during the simulation, with peaks above 3 H-bonds (Figure 3.26). Although Amorphigenin forms fewer hydrogen bonds, the consistent presence of these interactions indicates a robust binding affinity, possibly reinforced by supplementary hydrophobic and van der Waals interactions inside the active region. An investigation of SASA then was conducted to assess the protein-ligand complex exposure to the surrounding solvent. The Amorphigenin and Geldanamycin-bound HSP90 systems demonstrated steady SASA values during the 100 ns simulation, oscillating between 110 and 115 nm². The Amorphigenin complex exhibited marginally elevated SASA values relative to the Geldanamycin control, signifying a modest increase in solvent exposure (Figure 3.27). This may indicate variations in ligand placement inside the binding pocket, potentially influencing interactions with adjacent residues. The consistency of the SASA values over time indicates that neither ligand substantially modifies the overall compactness of the protein structure during the experiment.

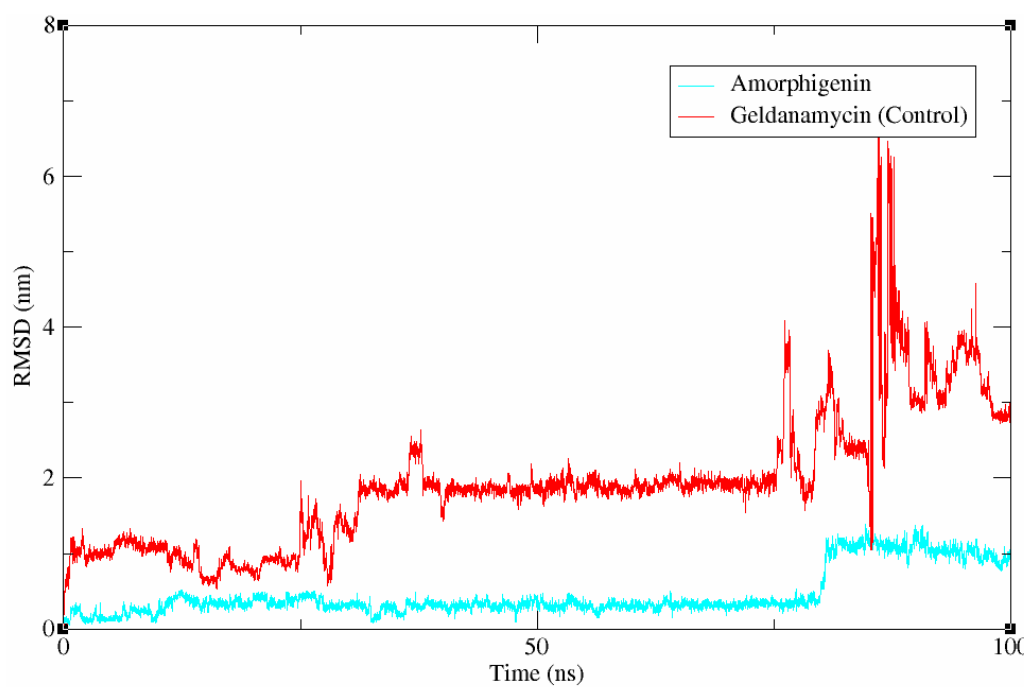


Figure 3.24. RMSD profile of HSP90-Amorphigenin complex compared to control HSP90-Geldanamycin complex for 100 ns of MD simulation.

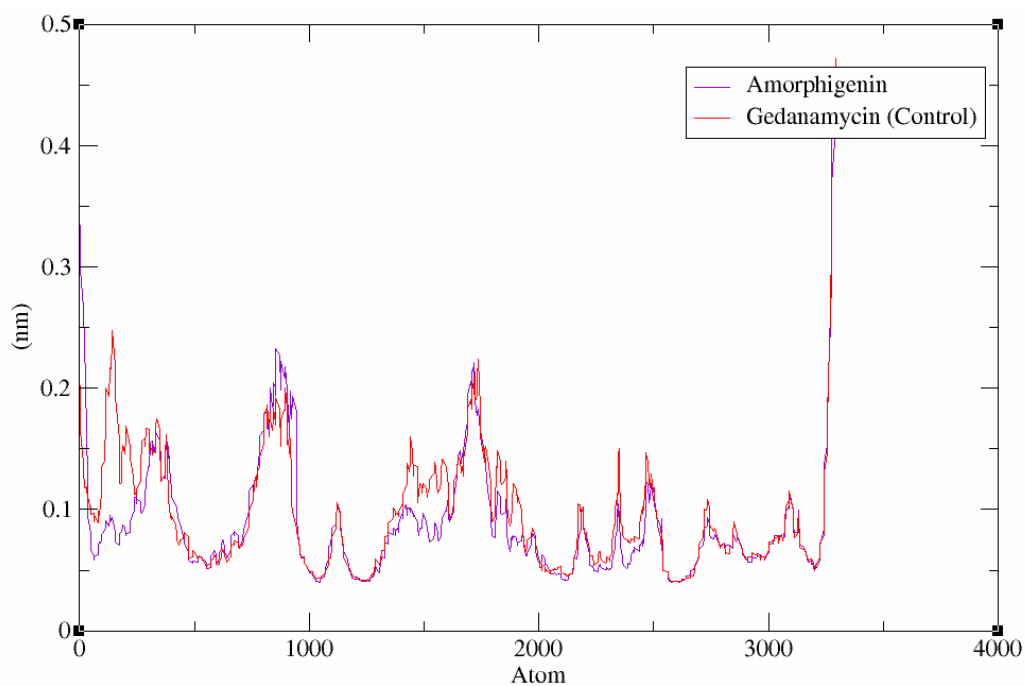


Figure 3.25. RMSF profile of HSP90 and Amorphigenin complex compared to control HSP90-Geldanamycin complex for 100 ns of MD simulation.

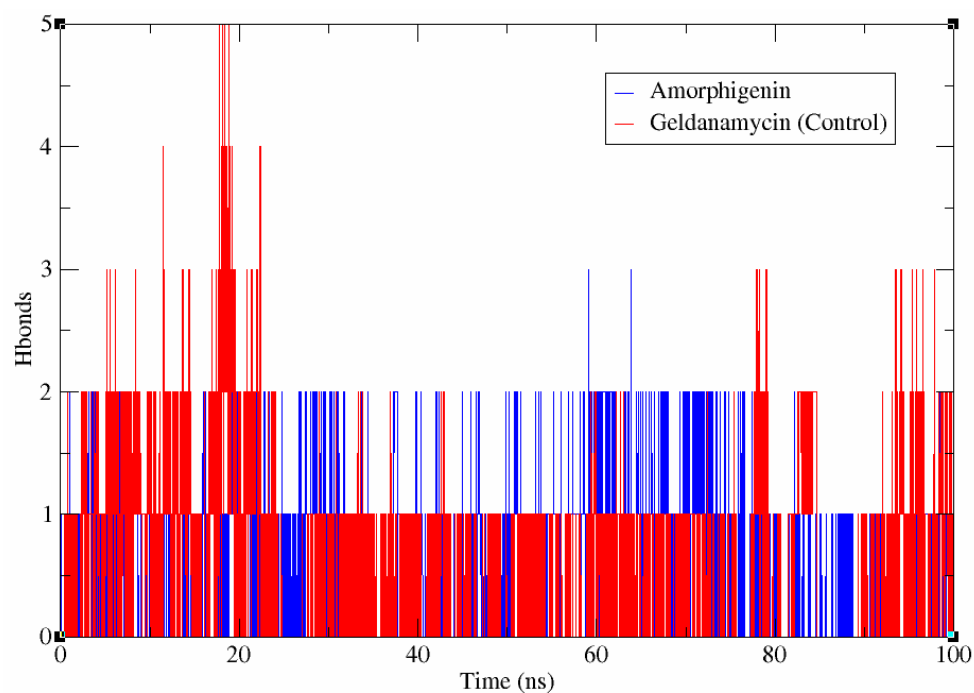


Figure 3.26. Number of hydrogen bond formation in HSP90-Amorphigenin complex compared to control HSP90-Geldanamycin complex for 100 ns of MD simulation.

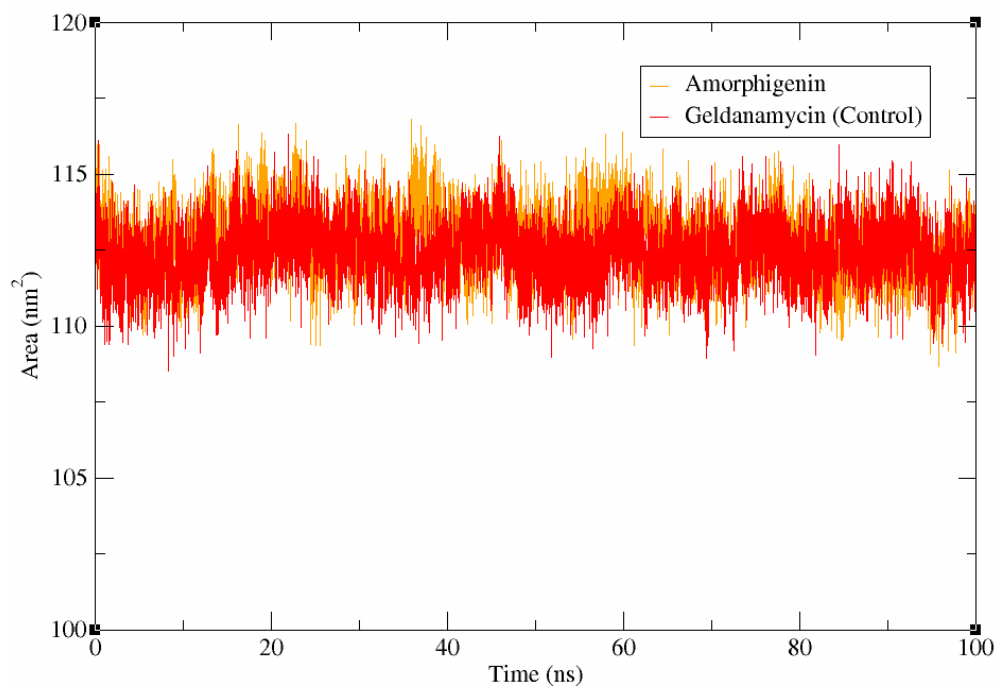


Figure 3.27. Solvent Accessible Surface Area between HSP90-Amorphigenin complex compared to control HSP90-Geldanamycin complex for 100 ns of MD simulation.

3.14. gmx_MMPBSA

The time series plots depicted as shown in Figure 3.28 displayed the fluctuation in total binding energy (black line) of the protein-ligand complex across about 1,000 (100 ns) simulation frames, with a moving average (red dashed line) indicating a smoothed trend. The binding energy varies between approximately -35 and -17 kcal/mol, with an average of about -25 kcal/mol. Periodic variations are evident, with certain regions exhibiting transient instability, probably resulting from structural rearrangements within the complex. Nonetheless, the overarching trend stays constant, indicating that the ligand sustains consistent binding throughout the simulation, despite transient energy fluctuations. The bar plot as shown in Figure 3.29 displayed the total binding energy of van der Waals (VDWAALS -40 kcal/mol), electrostatic interactions (EEL -5 kcal/mol), polar solvation (EGB +25 kcal/mol), and non-polar solvation (ESURF +2 kcal/mol). The total gas-phase energy (GGAS -45 kcal/mol) represents the cumulative electrostatic and van der Waals interactions that significantly promote binding. Nonetheless, the solvation energy (GSOLV +22 kcal/mol) hinders binding, chiefly because of the adverse polar solvation component (EGB). The conclusive total binding energy (-23 kcal/mol) signifies a positive binding relationship between the protein and ligand, indicating that non-covalent interactions, especially van der Waals forces, are the primary stabilizing mechanism.

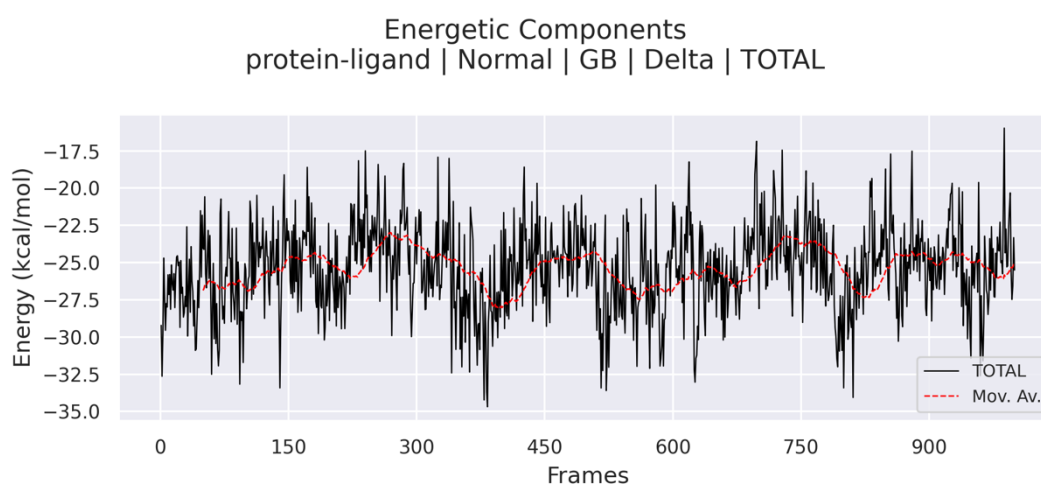


Figure 3.28. gmx_MMPBSA analysis showing time series plot.

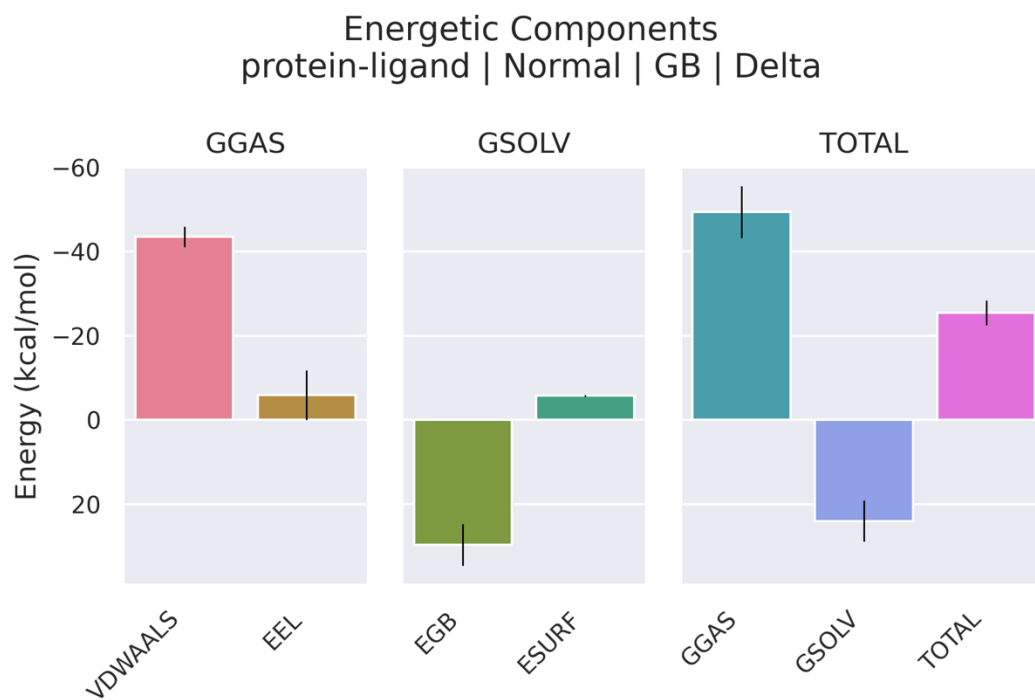


Figure 3.29. gmx_MMPBSA analysis showing energy decomposition bar plot.

4. DISCUSSION

Over the past decades, scientific progress in natural product research has accumulated substantial evidence to support the effectiveness of naturally occurring bioactive compounds for targeting various pathological conditions including malignancies. As a result, there has been a growing emphasis in the pharmaceutical sector on substituting synthetic drugs with these natural alternatives due to their comparatively higher efficacy and reduced toxic side effects. Consequently, the focus has been placed on screening plants and plant-based formulations utilized in traditional medicine to discover novel compounds that can be employed as an anticancer drug. Numerous studies have documented a diverse range of these pharmacologically active compounds in the genus *Elaeagnus* that can target cancers (Nazir *et al.*, 2020). Despite extensive research into this genus, information on the bioactive components and pharmacological activities of *E. caudata* remains limited. Therefore, using HCT116 and A549 cells as our model, we sought to elucidate the *E. caudata* anticancer activities using the bio-assay guided fractionation of methanolic extract.

Apoptosis is a crucial and highly controlled process by which cells undergo death without affecting healthy cells; thus, the dysregulation of such process can result in various chronic conditions including cancer (Elmore 2007). Therefore, the apoptosis-inducing activity of the B2 fraction was investigated using various assays. The present study demonstrated that the B2 fraction selectively triggered cellular death in the cancer cells through the apoptotic pathway, as evident from the Et/Br dual staining (Figure 3.7) and Comet assays (Figure 3.9; Figure 3.11). Our study observed a significant increase in caspase-3 and caspase-6 activities in the cancer cells after treatment with B2 fraction (Figure 3.14). This indicates that the B2 fraction-induced apoptotic cellular death occurs through a caspase-mediated pathway.

The level of reactive oxygen species (ROS) in the tumour microenvironment could influence cancer disease progression or induction of cell death. While moderate levels of ROS favour cell proliferation and survival, an excessive amount of ROS is cytotoxic (Fang *et al.*, 2009). Cancer cells have been reported to retain certain levels of ROS, which have been linked with their involvement in the initiation, progression, and metastasis, as well as in the development of chemoresistance (Colavitti and Pani,

2002). Subsequently, cancer cells strive to balance the levels of ROS in their system to a certain level that is enough to support their proliferation. However, to not succumb to their toxic effect, they upregulated their antioxidant transcription factors and reprogrammed their metabolism to increase the production of NADH and *de novo* synthesis of glutathione (Tang *et al.*, 2022). Several anticancer drugs, including doxorubicin, paclitaxel, and vinblastine, as well as certain drugs currently in clinical trials, are known to trigger apoptosis in cancer cells by inhibiting the antioxidant system (Lalsangpuii *et al.*, 2024). The B2 fraction in our study presents similar behaviour. Treatment of cells with B2 fraction exhibits a significant decrease in the levels of antioxidant activities (Figure 3.12; Figure 3.13). Subsequently, an increase in the levels of malondialdehyde was also observed. This effect may be attributed to a decrease in antioxidant activity, resulting in the accumulation of ROS within the cells, thereby inducing lipid peroxidation. Lipid peroxidation-induced DNA damage is an important regulator of apoptotic cell death. This is employed in many clinical settings by many chemotherapy drugs such as Olaparib (Tang *et al.*, 2022).

The alkaline comet assay revealed the DNA damaging potential of the B2 fraction in the cancer cells (Figure 3.9; Figure 3.11). The mechanism, however, remains unclear. The efficient DNA damaging effect of the fraction may be attributed to its ability to generate malondialdehyde, a by-product of lipid peroxidation, which can form mutagenic adducts that damage DNA. Furthermore, the induction of these DNA damages may also be initiated via a caspase-dependent pathway as is evident from the increased activity of caspase 3 in the treated cancer cells. Caspase 3, an executioner caspase, is the key role player in the activation of the endonuclease Caspase-activated DNase (CAD) by the proteolysis cleavage of inhibitor of CAD (ICAD) (Sakahira *et al.*, 1998).

Thus, the apoptosis-inducing activity of B2 fraction invites further investigation of the compound(s) involved that could contribute to such efficient bioactivity. To identify the compounds that may lead to the anticancer and apoptotic nature of B2 fraction of *E. caudata*, Liquid Chromatography coupled with High-Resolution Mass Spectroscopy (LC-HRMS) analysis was conducted. Several of these compounds identified in the B2 fraction of *E. caudata* have been reported to possess anticancer activity. Pinocembrin has been reported to be effective against malignant

cell lines such as B16F10, A375, CF-7, SKBR3, and MDA-MB-231 cells (Zheng *et al.*, 2018; Zhu *et al.*, 2021). Other compounds such as Harman, Bergapten, Pinocembrin, etc have also been shown to possess cytotoxic as well as pro-apoptotic activity against a variety of malignant cells including A549 and HCT116 cells as well (Zhong *et al.*, 2016; Zhang *et al.*, 2023). The presence of such a diversified spectrum of bioactive compounds may explain why the B2 fraction of *E. caudata* has considerable anticancer and apoptotic activities.

In recent years, the application of various *in silico* tools to drug discovery research has garnered considerable scientific interest as they minimize the expenses and duration of drug development. Due to the synergistic effects of plant extracts and their chemical constituents, it can be challenging to unravel the molecular mechanism by which they act on specific molecular targets. Network pharmacology research focuses on the screening of drug active ingredients, the network analysis of pharmaceutical components and disease-related gene links, and the prediction of drug target proteins (Zhang *et al.*, 2019). Therefore, network pharmacology has enhanced our comprehension of the intricate molecular pathways that drive diseases, aiding in the identification of possible protein targets of a particular drug. Among the phytochemicals identified in the B2 fraction of *E. caudata*, Amorphigenin was selected for *in silico* analysis based on druglikeness, oral bioavailability, and ADMET properties.

Network pharmacology has revealed HSP90 as the top target of Amorphigenin. HSP90 proteins are ATP-dependent, crucial molecular chaperones that regulate intracellular protein homeostasis by aiding their folding, heat stress stabilization, and degradation. Among various other cellular processes, HSP90 and their interaction with client proteins are highly involved in cell proliferation, differentiation, and survival (Schopf *et al.*, 2017). These interactions are especially essential for cancerous cells because they encounter a more severe disruption in proteostasis compared to normal cells. This is owing to their elevated levels of mutant oncoproteins which accelerates their proliferation (Niu *et al.*, 2022). Various research has linked HSP90 with many signaling pathways involved in malignancy such as RAF/ERK, PI3K/AKT/mTOR, etc (Basso *et al.*, 2002; Ohji *et al.*, 2006; Chatterjee and Burns, 2017). Thus, HSP90

has become a significant target in cancer treatment and has subsequently received substantial focus in targeted cancer drug development over the past few decades.

HSP90 enhances and promotes the functions of its client proteins through its ATPase cycle. Molecular docking analysis of the N-terminal domain of HSP90 and Amorphigenin revealed a strong binding affinity of -11.11 kcal/mol (Figure 3.23). The structural stability of the docked Amorphigenin-HSP90 bound complex was further analyzed using molecular dynamic simulation. Molecular dynamic simulation revealed that the ligand remains stably bound to the receptor throughout the 100 ns simulation (Figure 3.24 – 3.27). The RMSD and RMSF measurements indicated that the Amorphigenin-HSP90 combination exhibits greater conformational stability than the Geldanamycin-bound system. Although Amorphigenin forms fewer hydrogen bonds than Geldanamycin, it sustains stable connections, perhaps enhanced by hydrophobic and van der Waals forces. This indicates that hydrogen bonding alone may not entirely dictate binding stability. The SASA investigation reveals negligible variations in solvent exposure, with Amorphigenin marginally elevating SASA, potentially attributable to modified ligand orientation. Nonetheless, the overall structural compactness stays unchanged. These findings indicate that Amorphigenin demonstrates stable binding to HSP90, potentially providing an alternative to Geldanamycin with a unique interaction profile.

The gmx_MMPBSA study of the HSP90-Amorphigenin complex elucidates the binding stability and energetic contributions essential for its potential as an anticancer therapy (Figure 3.28 & 3.29). The time series study indicates that the overall binding energy stabilizes around -25 kcal/mol, with fluctuations reflecting modest conformational changes but no substantial loss of interaction. The energy decomposition reveals that van der Waals interactions (-40 kcal/mol) serve as the primary stabilizing factor, supported by advantageous electrostatics (-5 kcal/mol), but polar solvation (+25 kcal/mol) counteracts binding. Notwithstanding solvation penalties, the overall negative binding energy (-23 kcal/mol) substantiates a robust ligand affinity for HSP90, indicating that Amorphigenin may effectively impede its activity.

Both Amorphigenin and HSP90 were shown to be involved in apoptotic cell death pathways (Rodina *et al.*, 2007; Massey *et al.*, 2010; He *et al.*, 2013; Zhong

et al., 2016; Le *et al.*, 2022). Based on our *in-silico* analysis, Amorphigenin was found to inhibit HSP90 activity by binding to its N-terminal domain, which is crucial for its ATPase activity. Interference of the N-terminal ATPase site by Amorphigenin is a plausible explanation for why HSP90 fails to serve its numerous client proteins, many of which are important regulators of cell survival. Thus, the potential of Amorphigenin as a lead compound for HSP90-targeted chemotherapy has been endorsed by HSP90's essential function in the survival of cancer cells through the stabilization of oncogenic proteins, and the advantageous binding energetics of Amorphigenin. However, additional refinement via structural optimization or *in silico* alterations may improve its binding efficacy and pharmacological characteristics.

This study demonstrates the significant anticancer potential of *E. caudata*. The B2 fraction, isolated from the methanolic extract, exhibited potent cytotoxic activity against A549 and HCT116 cancer cells. This cytotoxic effect was primarily mediated through apoptosis, as evidenced by increased caspase 3/6 activity, DNA damage, and lipid peroxidation, while simultaneously decreasing antioxidant levels. LC-HRMS analysis identified several bioactive compounds within the B2 fraction, including Amorphigenin, which emerged as a promising drug candidate based on its drug-like properties and potent anticancer activity. Network pharmacology and molecular dynamic simulation studies revealed that Amorphigenin interacts with HSP90, a key molecular chaperone involved in cancer cell survival. These findings suggest that *E. caudata* and its bioactive compounds, particularly Amorphigenin with its favourable binding energetics for HSP90, hold significant promise for the development of novel HSP90-targeted therapies for treating non-small cell lung carcinoma and colorectal cancer. Further research is, however, warranted to fully elucidate the molecular mechanisms underlying the anticancer activity of *E. caudata* and to explore its potential as a therapeutic agent.

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CHAPTER 4

Isolation, identification, and anticancer activity of the lead compound of *Elaeagnus caudata*

ABSTRACT

Natural products have long been integral to the discovery of novel therapeutic agents, particularly in oncology, where plant-derived natural compounds contribute significantly to chemotherapeutic development. This study focuses on the isolation of a novel compound from *Elaeagnus caudata* which may hold promise for a new anticancer chemotherapy. A flavonoid, 6,2'-Dihydroxyflavone (MW 254.24 g/mol) was isolated from the methanolic extract of *E. caudata* guided by bioassay and confirmed using FTIR, CHNS(O) analysis, LC-HRMS, and ¹H and ¹³C NMR. MTT assay revealed that 6,2'-Dihydroxyflavone inhibited HCT116 cell proliferation in a dose- and time-dependent manner, significantly superior to 5-fluorouracil (5-FU) in efficacy at higher concentrations. The mode of cellular death via apoptosis was observed via increased apoptotic index as evident from the AO/EtBr dual staining, increased DNA damage and confirmed by the increased Caspase 3 and 6 activities, upregulation of pro-apoptotic genes and subsequent downregulation of anti-apoptotic genes. Oxidative stress markers were also evaluated, demonstrating a decrease in glutathione (GSH) levels and antioxidant enzyme activities, including glutathione S-transferase (GST) and superoxide dismutase (SOD). Conversely, lipid peroxidation (MDA levels) increased significantly, suggesting a redox imbalance contributing to apoptosis. These findings suggest that 6,2'-Dihydroxyflavone from *E. caudata* exhibits significant cytotoxic and pro-apoptotic properties against HCT116 cells via oxidative stress-mediated apoptosis and modulation of apoptotic gene expression. The compound's efficacy in comparison to 5-FU highlights its potential as a novel anticancer agent. Further studies, including *in vivo* evaluations and insight into its mechanism, are warranted to explore its therapeutic applications in cancer treatment.

1. INTRODUCTION

Natural products have been pivotal in the treatment of human diseases throughout history. They primarily refer to the secondary metabolites from plants, animals, and microorganisms which can be extracted and isolated using various methods (Zhongwen *et al.*, 2024). They represented a vast array of varied chemical compounds that exhibit a multitude of biological activities such as antimicrobial, anticancer, antioxidants, antihelminth, antitussive, antiangiogenic, anti-inflammatory, antipyretic, analgesic, etc. Therefore, natural compounds have laid a substantial foundation for traditional medicine across many cultures, functioning as primary healthcare remedies (Katz and Baltz, 2016). Approximately 80% of the global population still depends on these conventional medicines (Singh *et al.*, 2023). Consequently, the integration of modern pharmacological approaches with traditional medicinal knowledge has emerged as a highly productive area of scientific research. This convergence has significantly advanced the discovery and development of novel natural compounds, particularly in the fields of antibiotic and anticancer drug research.

Over time, cancer management has experienced significant advancements via the development of many treatment modalities, including chemotherapy, advanced surgery, radiation therapy, gene therapy, hormone therapy, laser therapy, combination therapy, immunotherapy, and pathway-targeted chemotherapy (Liu *et al.*, 2024). Chemotherapy has been the most conventional therapy for a diverse array of cancers, including metastatic and recurrent cancer (Anand *et al.*, 2022). Historically, medicinal plants have served as principal sources for anticancer drug discovery, where plant-derived compounds such as vinblastine, vincristine, etoposide, paclitaxel, docetaxel, topotecan, and irinotecan are among the most efficacious chemotherapy available for cancer treatment even in modern chemotherapeutic (Cragg *et al.*, 2015). Notably, more than 60% of currently approved anticancer chemotherapeutics are derived directly or indirectly from natural products, underscoring the enduring significance of natural compounds in the field of oncology (Newman and Cragg, 2020).

Nonetheless, the onset of both localized and systemic adverse effects, coupled with the increasing rate of chemoresistance has continuously necessitated

the need for the discovery of new chemotherapeutic drugs. The common side effects of chemotherapy include chemotherapy-induced alopecia (CIA), chemotherapy-induced peripheral neuropathy (CIPN), chemotherapy-induced nausea and vomiting (CINV), cardiotoxicity, diarrhea, infertility, and cognitive impairment often referred to as "chemo brain" (Brianna & Lee, 2023). Although numerous treatments exist to mitigate these adverse effects, they fail to reverse the toxicity of chemotherapy, prompting various researchers to explore alternative medicines aimed at relieving chemotherapy-induced side effects (Anand *et al.*, 2022). Consequently, the search for novel anticancer compounds derived from natural sources has gained considerable momentum in recent decades, leading to the extensive exploration of medicinal plants for their potential contributions to cancer therapy.

The members of the genus *Elaeagnus* have been widely used in the practice of traditional medicines, prompting scientific inquiry into the validation of their pharmacological activity, elucidation of their molecular mechanisms, and consequently the isolation of the bioactive compounds responsible for their significant medicinal properties. Different plants of this genus have been reported to possess diverse biological activities such as antioxidant, antidiabetic, antibacterial, muscle relaxant, anti-diarrheal, antinociceptive, anticancer, anti-inflammatory, hepatoprotective, neuroprotective, and anti-amnesic effects (Nazir *et al.* 2020). Bioactive groups including flavonoids with potent anticancer activity such as rutin, epigallocatechin gallate, phenolic compounds such as ferulic acid, triterpenoids, saponins such as terpengustifol, and alkaloids such as harmane and tetrahydroharmol, have also been isolated from various species of the *Elaeagnus* genus (Farzaei *et al.*, 2015; Zhu *et al.*, 2018).

Among the members of the *Elaeagnus* genus, *E. caudata* locally known as *Sarzuk* in Mizoram, has remained relatively unexplored in terms of its bioactivity and phytochemical constituents, despite its extensive utilization in the traditional medicine of several cultures. It has been traditionally used to treat various health problems such as stopping menses, induction of labor, expulsion of retained placenta, rheumatoid arthritis, and for treating various malignancies (Rai and Lalramnghinglova, 2010). In other parts of North-East India, *E. caudata* has been used by the Dimasa tribe of Assam for the prevention of miscarriage (Rout *et al.*,

2012). The crude extract of the root is also used for the treatment of hepatic jaundice and hepatomegaly (Sharma *et al.*, 2016; Das *et al.*, 2019). Therefore, for its extensive traditional use yet its underutilization, we sought to elucidate the pharmacological activity of *E. caudata* for its anticancer properties with the primary objective of isolating a lead compound which may hold promising prospects for developing a novel anticancer chemotherapy.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Minimum Essential Medium (MEM), Trypsin-EDTA, fetal bovine serum (FBS), bovine serum albumin (BSA), 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), sodium bicarbonate, glutathione (GSH) reduced, Folin-ciocalteu's reagent, nicotinamide adenosine dinucleotide (NADH), nitroblue tetrazolium (NBT), n-butanol, thiobarbituric acid (TBA), potassium chloride (KCl), 5, 5' dithio 2-nitrobenzoic acid (DTNB), sodium chloride (NaCl), phenazine methosulphate (PMS), ethidium bromide, dimethyl sulphoxide (DMSO), 1-chloro-2, 4 dinitrobenzene (CDNB), methanol, petroleum ether, chloroform, and Triton X-100 were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Ethylenediamine tetra-acetic acid (EDTA), L-glutamine, agarose (low gelling temperature), phenol red, trichloroacetic acid (TCA), silver nitrate, Trizma base, and Trizma hydrochloride were purchased from Sigma Chemical Co., Bangalore, India. 5-Fluorouracil (5FU) was purchased from GLS Pharma Ltd. (Hyderabad, India). The remaining chemicals were procured from Merck Specialties Pvt., Ltd (Mumbai, India).

2.2. Plant material and preparation of fraction

The plant material was collected, prepared, and extracted as described in the previous chapter (Chapter 1). The fraction B2 obtained from the fractionation of the methanolic extract of *E. caudata* was selected for compound isolation owing to its potent cytotoxicity and pro-apoptotic activity as described in Chapter 3.

2.3. Bioassay-guided sub-fractionation and isolation of lead compound

The B2 fraction was subjected to sub-fractionation with column chromatography using silica gel. The chromatographic parameters are as described in the previous chapter. Five sub-fractions were collected and labeled as B2.1, B2.2, B2.3, B2.4, and B2.5. Each sub-fraction was screened for its cytotoxicity after which the sub-fraction displaying the most cytotoxic potential compared to the other sub-fractions was further purified using silica gel 230-400 mesh size in a glass column. The sub-fractions were eluted with a mixture of Hexane/Ethyl Acetate from 77.8:22.2 to 77.2:22.8. The separation was monitored using Thin Layer

Chromatography. The sub-fraction with most potent cytotoxicity was then further purified on the Sephadex LH-20 column using MeOH as eluent. Finally, a pale-yellowish crystal was afforded from the sub-fraction which displayed a single spot in TLC analysis and was further subjected to structural elucidation and identification.

2.4. Structural elucidation and identification of isolated compound

2.4.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed to identify the functional groups present in the isolated compound. The analysis was recorded on the Jasco Model FT/IR – 4700 (United Kingdom).

2.4.2. ^1H and ^{13}C Nuclear Magnetic Resonance (NMR)

^1H NMR and ^{13}C NMR were employed to elucidate the structure of the isolated compound. The analysis was performed on Bruker Avance (Germany) at 400 MHz using DMSO- D_6 as solvent. TMS was used as an internal reference and the chemical shift values are expressed in δ , ppm units. MestReNova was used for spectral analysis.

2.4.3. Elemental analysis using CHNS(O) Analyzer

For CHNS(O) analysis, the sample was analyzed on Flash Smart V CHNS/O (Thermo Fisher Scientific) based on the Dumas Method. The combustion products are separated by a chromatographic column and detected by the thermal conductivity detector (TCD), which gives an output signal proportional to the concentration of the individual components of the mixture.

2.5. Cell line and cell culture

The human colorectal carcinoma cell line (HCT116) was employed for this study. It was procured from the National Centre for Cell Science (NCCS), Pune, India. All cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS and 1% L-Glutamine in a humidified incubator with 5% CO_2 at 37°C (Eppendorf, Hamburg, Germany).

2.6. Cytotoxicity analysis of sub-fractions and isolated compounds

The cytotoxicity of the different sub-fractions, and subsequently the isolated compound was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983). Briefly, 1×10^4 cells were seeded in a 96 well plate. The cells were treated with different concentrations of each sub-fraction/compound for the required treatment duration after which the drug-containing media was discarded and replaced with serum-free media. 10 μ l of MTT (5 mg/ml) was then added and incubated for another 2 h at 37°C in a CO₂ incubator. Following this, 100 μ l of DMSO was used to dissolve the insoluble purple formazan crystals. After 30 min of incubation, the absorbance of the solution was measured at 560 nm using a microplate reader (Spectramax m2e, Molecular Devices). For each treatment, three independent experiments with three replicates were conducted. Cytotoxicity was expressed as inhibition (%) which was calculated by the formula given below:

$$\% \text{ inhibition} = \text{Control} - \text{Treatment} / \text{Control} \times 100$$

2.7. Cell morphology analysis using dual Acridine Orange/Ethidium Bromide staining

The apoptosis-inducing potential of the isolated compound was studied using the dual fluorescent staining technique. 3×10^5 cells were seeded into each well of a 6 well plate and allowed to adapt for 24 h after which treatment was done using different doses of the isolated compound. Cells were harvested after 24 h of treatment by trypsinization. 25 μ l of cells were suspended in 2.5 μ l of ethidium bromide (100 μ g/ml) and acridine orange (100 μ g/ml) and incubated for 2 mins. The morphology of apoptotic cells was then investigated under a fluorescent microscope (ThermoFisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615). At least 300 cells were scored, and the apoptotic index was determined as follows:

$$\text{Apoptotic index (\%)} = \text{Number of apoptotic cells scored} \times 100 / \text{Total number of cells counted.}$$

2.8. Antioxidant Assay

The effect of the isolated compound on the antioxidant-oxidant system was evaluated by measuring the levels of antioxidants and lipid peroxidation. For this, 1×10^6 cells were seeded into each well of a 6 well plate and treated with the isolated compound for 24 h. The cells were harvested, and 5% cell homogenate was prepared. Protein estimation was performed by standard protocol (Lowry *et al.*, 1951) and subjected to antioxidant assays as follows:

2.8.1. Glutathione (GSH)

The level of GSH was estimated by the standard method with slight modification (Moron *et al.*, 1979). Briefly, 80 μ l of cell homogenate was incubated for 2 min at room temperature with 900 μ l of 0.02 M sodium phosphate buffer and 20 μ l of 10 mM DTNB followed by absorbance measurement at 412 nm in a UV-visible spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai). The level of GSH was calculated from a standard graph and expressed in μ mol/mg protein.

2.8.2. Glutathione S-transferase (GST)

The level of GST was measured using the standard method (Beutler, 1984). Briefly, 50 μ l of 5 mM CDNB was incubated with 850 μ l of 0.1 M phosphate buffer (pH 6.5) at 37°C for 10 min followed by the addition of 50 μ l each of 20 mM GSH and cell homogenate. The absorbance was measured at 340 nm at 1 min intervals for 5 min. GST activity was expressed as unit/mg protein. GST activity was measured as:

$$\text{GST Activity} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / 9.6 \times \text{volume of the test sample} \times 100$$

2.8.3. Superoxide Dismutase (SOD)

The NBT reduction method was employed to estimate the level of SOD (Fried, 1975). Briefly, cell homogenate (100 μ l), 186 μ M of PMS (100 μ l), 3 mM NBT (300 μ l), and 780 μ M NADH (200 μ l) was incubated at 30°C for 90 sec and the reaction was stopped by adding 1 ml of acetic acid and 4 ml of n-butanol. The absorbance of the mixture was then recorded at 560 nm and the activity was expressed in unit/mg protein (1 unit = 50 % inhibition of NBT reduction).

$$\% \text{ inhibition} = (\text{OD}_{\text{blank}} - \text{OD}_{\text{sample}} / \text{OD of blank}) \times 100$$

2.8.4. Lipid Peroxidation (LPO)

The level of lipid peroxidation was measured using the standard method with slight modifications (Beuege and Aust, 1978). In brief, 10% TCA, 0.8% TBA, and 0.02 N HCl were combined in a 1:2 ratio with cell homogenate and boiled for 10 min. The mixture was then cooled immediately and centrifuged at 12000 rpm for 10 min. The supernatant was collected, and absorbance was taken at 535 nm against the blank. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$.

2.9. Comet Assay

The alkaline single-cell gel electrophoresis (Comet assay) was performed according to standard procedure with minor modifications (Singh *et al.*, 1988). Briefly, 3×10^5 cells were treated with the isolated compound. The cells were harvested and were suspended in 0.5% low-melting point agarose (75 μl) prepared in 1X PBS and spread onto a frosted slide precoated with 1% normal-melting point agarose. Slides were immersed for 2 h in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Trizma base, 1% Triton X-100, and 10% DMSO, pH 10.0). Following lysis, slides were placed in a horizontal electrophoresis tank containing freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13) for 20 min to allow unwinding of DNA. Electrophoresis was then carried out for 30 min at 24 V and 300 mA and the slides were then neutralized by washing with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min. Slides were washed with distilled water after neutralization and then stained with ethidium bromide solution (2 $\mu\text{g}/\text{ml}$) for 5 min. Each slide was prepared in triplicate and 100 randomly selected cells from each slide were examined using a fluorescence microscope with a magnification of 200x. The images captured were analyzed with Image J software.

2.10. Quantitative Real-Time – Polymerase Chain reaction (qRT-PCR) analysis of pro-apoptotic and anti-apoptotic gene expression

The relative expression of pro-apoptotic and anti-apoptotic genes in the cancer cells treated with the isolated compound was analyzed using the qRT-PCR technique and compared with their untreated counterparts. Briefly, 3×10^6 cells were seeded in a T-25 flask with 5 ml media and allowed to adhere for 24 h after which cells were treated with the isolated compound for 24 h. 5FU was used as a positive control. Cells were then harvested by trypsinization post-treatment. Total RNA was extracted using Tri-reagent (BR Biochem, Life Science Pvt. Ltd, R1022) followed by quantification using a Nanodrop Spectrophotometer (Eppendorf Biophotometer Plus, Hamburg, Germany). DNA contamination was eliminated using the RQ1 DNase Kit (Promega, M198A, Madison, WI, USA). cDNA was synthesized from 3 μ g of total RNA using a cDNA kit (Thermo Scientific, K1621; Lithuania, Europe). Primer 3, Boston, MA, USA, was employed for designing gene-specific primers. Quant-Studio 5 (ThermoFisher Scientific, Foster City, CA, USA) was used for qPCR. A 7 μ l PCR reaction Mastermix was prepared for each gene by mixing 1 μ l of cDNA, 1 μ l each of gene-specific forward and reverse primer, 1 μ l of nuclease-free water (ThermoFisher Scientific, A199338, Bangalore, India) and 3 μ l of PowerUpTM SYBRTM Green Master Mix (ThermoFisher Scientific, A25742, Lithuania, Europe). One cycle at 95°C (20 sec), 35 cycles at 95°C (01 sec), 60°C (20 sec), and 95°C (01 sec) were the cycling conditions for the qPCR. An additional melt curve plot stage included one cycle at 60°C (20 sec) and one cycle at 95°C (01 sec). Melting curves were then generated for the confirmation of the presence of a single uniform peak. The GAPDH gene was chosen as a reference gene to ascertain the relative expression levels of target genes. Each sample was run in duplicate along with non-template and negative RT controls. The relative expression of genes was determined using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). The list and sequence of primers used are displayed in Table 4.1.

Table 4.1. Primer sequences used for qRT-PCR.

Genes	Forward (5'→3')	Reverse (5'→3')
<i>Bcl-X_L</i>	GGCCACTTACCTGAATGACC	AAGAGTGAGCCCAGCAGAAC
<i>Bcl-2</i>	GGATGCCTTTGTGGAAGTGT	AGCCTGCAGCTTTGTTTCAT
<i>Bax</i>	TCCCCCGAGAGGTCTTTT	CGGCCCCAGTTGAAGTTG
<i>Bid</i>	CCTTGCTCCGTGATGTCTTTC	GTAGGTGCGTAGGTTCTGGT
<i>Survivin</i>	AGAACTGGCCCTTCTTGAGAG	CTTTTTATGTTTCCTCTATGGGGTC
<i>p53</i>	GTTCCGAGAGCTGAATGAGG	TCTGAGTCAGGCCCTTCTGT
<i>PARP</i>	CCAGATGCTTGTCTTCCTGAGAG	AGTGACAGCAGGGTTGGCATGA
<i>Apaf-1</i>	AAGGTGGAGTACCACAGAGG	TCCATGTATGGTGACCCATCC
<i>GAPDH</i>	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCC GTTCTCAG

2.11. Caspase 3 and 6 activity assays

A quantitative enzymatic activity assay for caspases 3 and 6 was performed as per the manufacturer's protocols (BioVision Incorporated, USA). In brief, 2×10^6 cells were seeded in a T-25 flask with 5 ml of media followed by treatment with 25 µg/ml of B2 fraction. 5FU (100 µg/ml) was used as a positive control. The cells were harvested after 24 h and lysis was performed using 50 µl of chilled lysis solution and incubated for 10 min on ice after which the lysate was centrifuged at $15,000 \times g$ for 1 min at 4°C. The supernatant was collected, and protein estimation was assayed and measured as per the Bradford method (Bradford, 1976). A specific colorimetric substrate, DEVD-pNA for caspase 3 and VEID-pNA for caspase 6, was used to measure the activity of 150 µg of protein from each sample. The assay was carried out in 96 well plates with a total volume of 100 µl. After incubating the mixture for 2 h at 37°C, the absorbance of free p-nitroanilide (pNA), which was generated when activated caspase 3 and caspase 6 cleaved from their respective substrates, was measured using a microplate reader at 405 nm.

2.12. Statistical analysis

All data were expressed as mean $\pm$ standard error of the mean (SEM). One-way ANOVA followed by Tukey's test was employed to test the significant variations. SPSS ver.16.0 software (SPSS Inc, Chicago, Illinois, USA) and GraphPad Prism ver. 8.0 was used for statistical and graphical analysis. A p-value of less than 0.05 was the threshold considered to be statistically significant.

3. RESULTS

3.1. Bioassay-guided isolation of lead compound

The B2 fraction was sub-fractionated using column chromatography yielding five sub-fractions B2.1, B2.2, B2.3, B2.4, and B2.5. Each sub-fraction was subjected to cytotoxicity screening using MTT assay and their inhibition (%) was plotted against log doses for determining their IC_{50} (Figure 4.1). Among the five sub-fractions, the sub-fraction B2.5 was observed to possess the most efficient cytotoxicity with an IC_{50} of $10.54 \mu\text{g/ml} \pm 0.978$ (Figure 4.2). Further purification of the B2.5 sub-fraction afforded a pale-yellow crystal weighing 18.2 mg and was proceeded for further analysis.

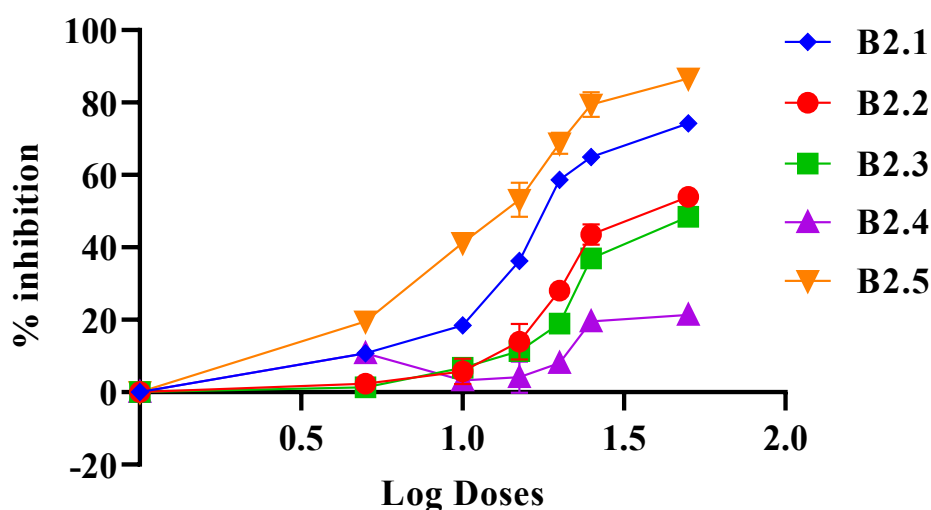


Figure 4.1. Log-dose plotted against % inhibition of different sub-fractions of B2 fraction for determination of IC_{50} against HCT116 cells.

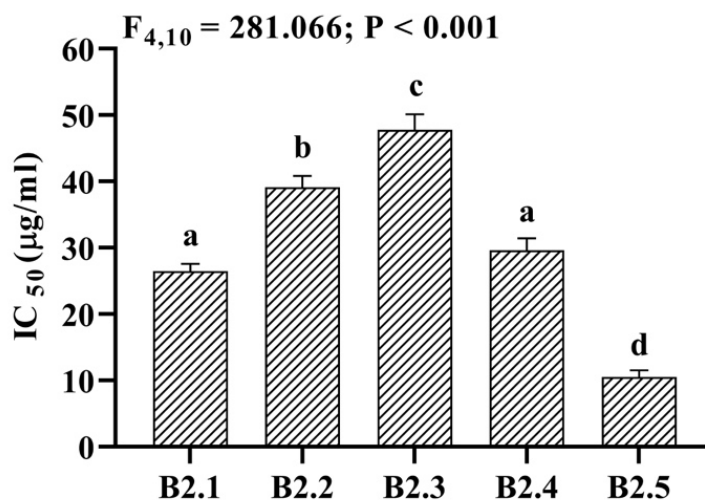


Figure 4.2. IC₅₀ of sub-fractions of B2 fraction against HCT116 cells. Values are expressed as Mean $\pm$ SEM. Different letters indicates significant variation.

3.2. Structural elucidation, elemental analysis, and identification of isolated compound

The structural elucidation, elemental analysis, and identification of the isolated compound were done using ^1H NMR (Figure 4.4), ^{13}C NMR (Figure 4.5), CHNS(O) analysis (Figure 4.6 & 4.7), FTIR (Figure 4.8). Based on combined analysis of CHNS(O), LC-HRMS, and ^{13}C NMR data, the molecular formula of the isolated compound was determined to be $\text{C}_{15}\text{H}_{10}\text{O}_4$ (observed $[\text{M}]^+$ peak at m/z 254.24), which corresponds to 11 degrees of unsaturation. This high degree of unsaturation suggests the presence of multiple rings and/or double bonds. The ^1H NMR spectrum exhibited aromatic and olefinic proton signals in the downfield region δ_{H} 7.01-7.89 (Table 4.2) indicating that the structure of the isolated compound is composed of conjugated π -systems. Further, structural elucidation using ^{13}C NMR and ^1H NMR spectra revealed characteristic signals consistent with a flavone scaffold, notably an olefinic proton (H-3) at δ_{H} 7.07 (s, 1H), which correlated with the corresponding C-3 carbon signal at δ_{C} 110.57. Moreover, the ^1H NMR spectrum showed no signals indicative of alkyl substituents and methylene linkages. Additionally, the appearance of two distinct downfield singlets at δ_{H} 10.67 (1H, s) and δ_{H} 9.99 (1H, s) indicated the presence of two hydroxyl protons. The spectrum

also displayed two additional singlets, two doublets, two doublet of doublets, one doublet of triplet and one triplet, with no overlapping or coupled peaks. This pattern of signals suggests that each of the two aromatic rings is monosubstituted with a hydroxyl group. The FTIR spectrum further supported the structure characteristic of flavones. The spectrum exhibited a broad characteristic peak for O–H stretching at 3015–3300 cm^{-1} , which can be attributed to the presence of hydroxyl groups. Additionally, weak C–H stretching vibrations were observed at 2721 cm^{-1} . A strong band observed at 1617 cm^{-1} can be attributed to the carbonyl (C=O) stretching of the 4-pyrone ring, while C=C stretching appeared at 1560 cm^{-1} , and C–O stretching was observed at 1240 cm^{-1} . The ^1H NMR, ^{13}C NMR, CHNS(O), LC-HRMS and FTIR spectral data confirm that the isolated compound possesses a flavone core, and the structure is consistent with 6-hydroxy-2-(2-hydroxyphenyl)-4H-chromen-4-one, commonly known as 6,2'-Dihydroxyflavone (Figure 4.3).

Table 4.2. ^{13}C NMR and ^1H spectral data of isolated compound.

Position	δ_{C} (ppm)	δ_{H} (ppm) (Multiplicity, J in Hz)
5	107.86	7.06 (s, 1H)
3	110.57	7.07 (s, 1H)
1'	117.48	--
3'	118.49	7.34 (d, J = 3.0 Hz, 1H)
8	119.90	7.89 (dd, J = 8.0, 1.7 Hz, 1H)
5'	120.22	7.01 (t, J=7.6 Hz, 1H)
7	123.41	7.62 (d, J = 9.0 Hz, 1H)
9	124.49	--
6'	129.02	7.25 (dd, J=9.0, 3.0 Hz, 1H)
4'	132.84	7.40 (ddd, J = 8.5, 7.3, 1.7 Hz, 1H)
10	150.03	--
6	155.15	10.67 (s, 1H)
2'	156.91	9.99 (s, 1H)
2	160.88	--
4	177.60	--

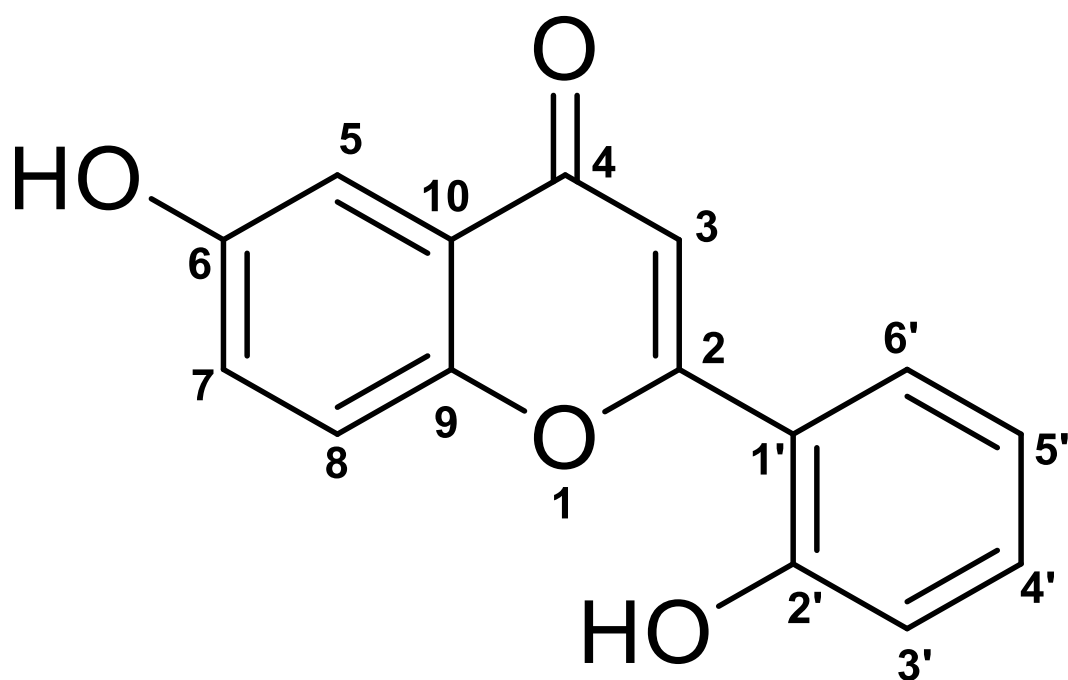
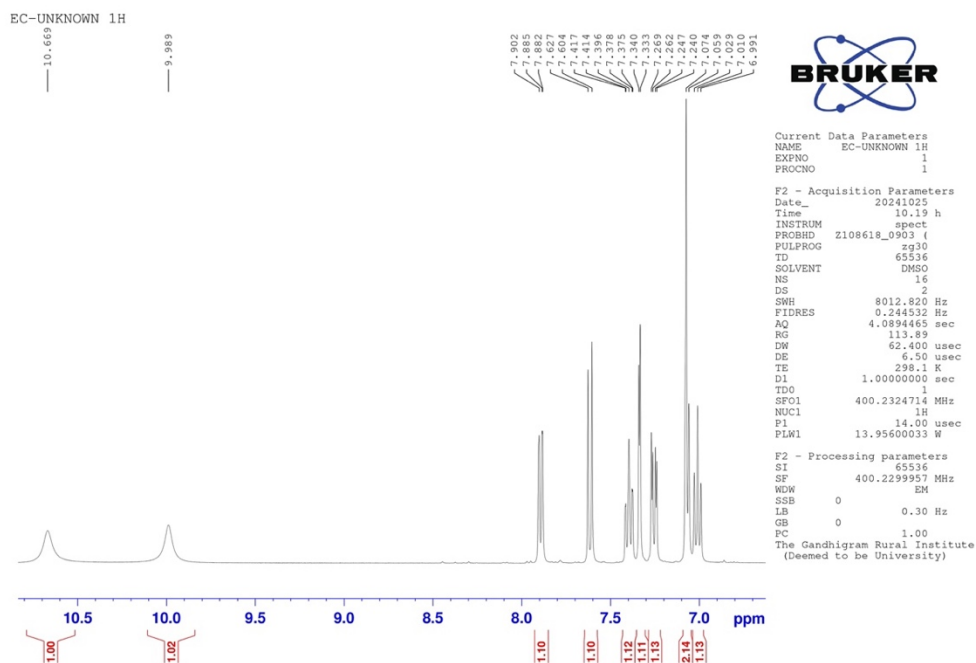
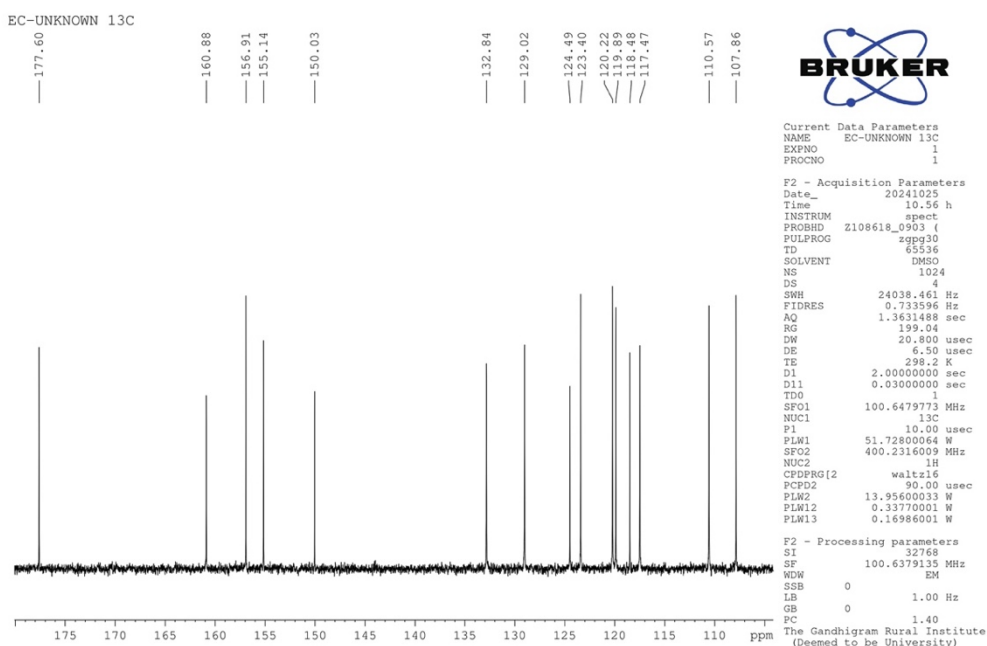


Figure 4.3. Chemical structure of the isolated compound.

Figure 4.4. ^1H NMR spectra of the compound isolated.Figure 4.5. ^{13}C NMR spectra of the compound isolated.

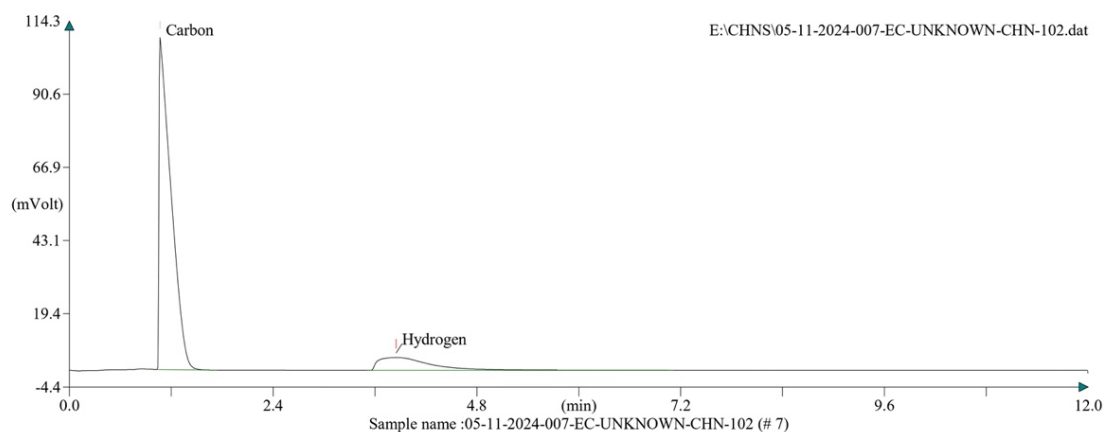


Figure 4.6. Carbon and hydrogen peaks of the isolated compound based on CHNS(O) analysis.

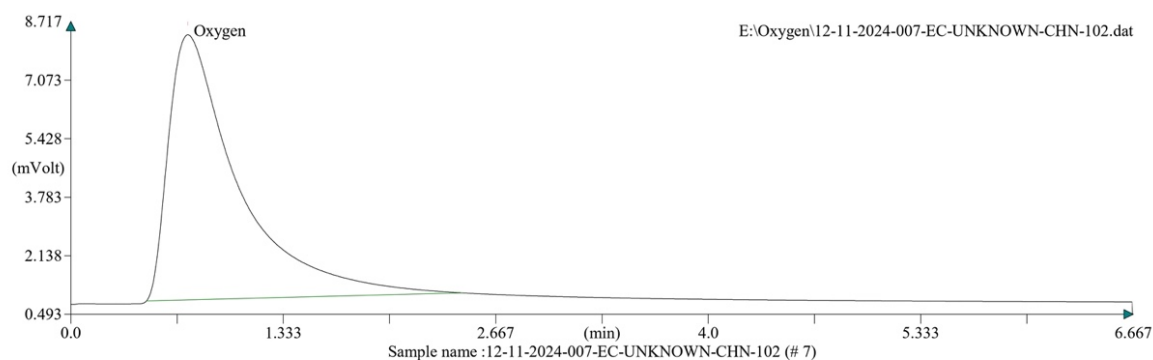


Figure 4.7. Oxygen peak of the isolated compound based on CHNS(O) analysis.

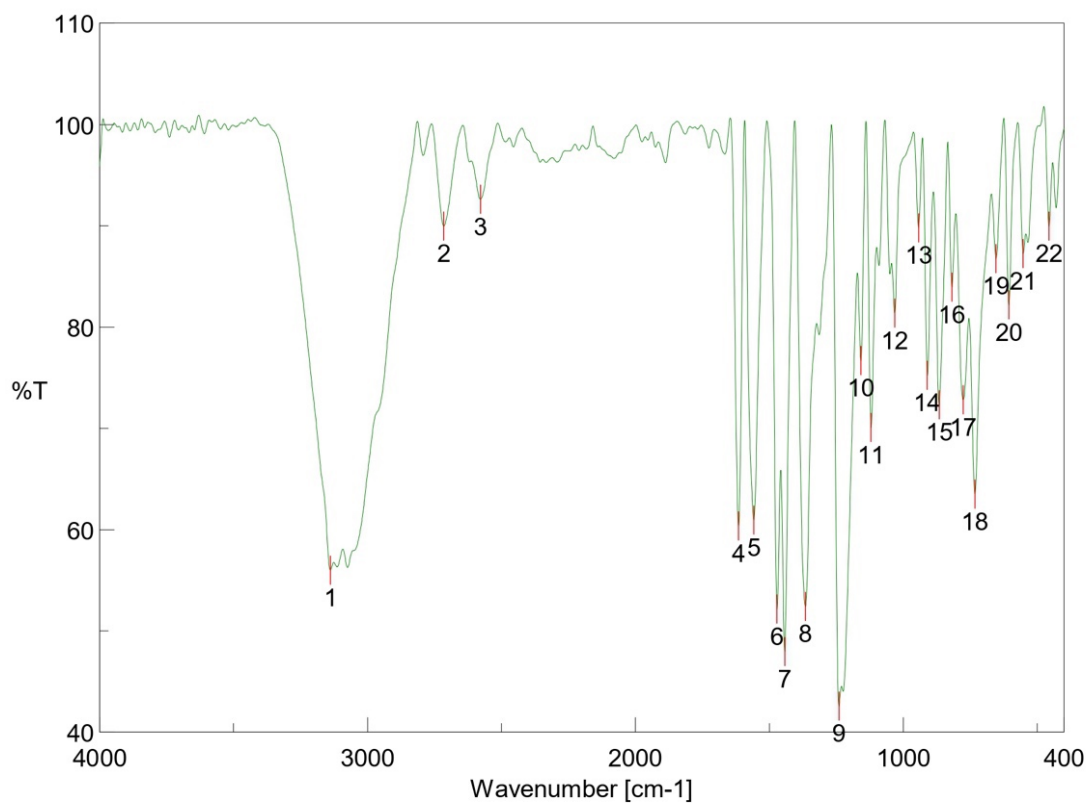


Figure 4.8. FTIR spectra of the isolated compound.

3.3. 6,2'-Dihydroxyflavone possesses cytotoxicity and anti-proliferative effect against human colorectal carcinoma cell line HCT116 cells

The cytotoxicity of the isolated compound 6,2'-Dihydroxyflavone was determined using the MTT Assay. HCT116 cells were treated with different concentrations (0.5 mM, 1.0 mM, 2.5 mM, 5 mM, and 10 mM) of 6,2'-Dihydroxyflavone for 6, 12 and 24 h. The inhibition (%) of the cells by the compound was plotted against log doses for the determination of their IC_{50} (Figure 4.9). The cytotoxicity of 6,2'-Dihydroxyflavone was found to increase in a dose and time-dependent manner. Notably, the treatment of HCT116 cells with 6,2'-Dihydroxyflavone for 12 h and 24 h displayed more efficacy than that of the standard drug 5-Fluorouracil treatment for 24 h (Figure 4.10).

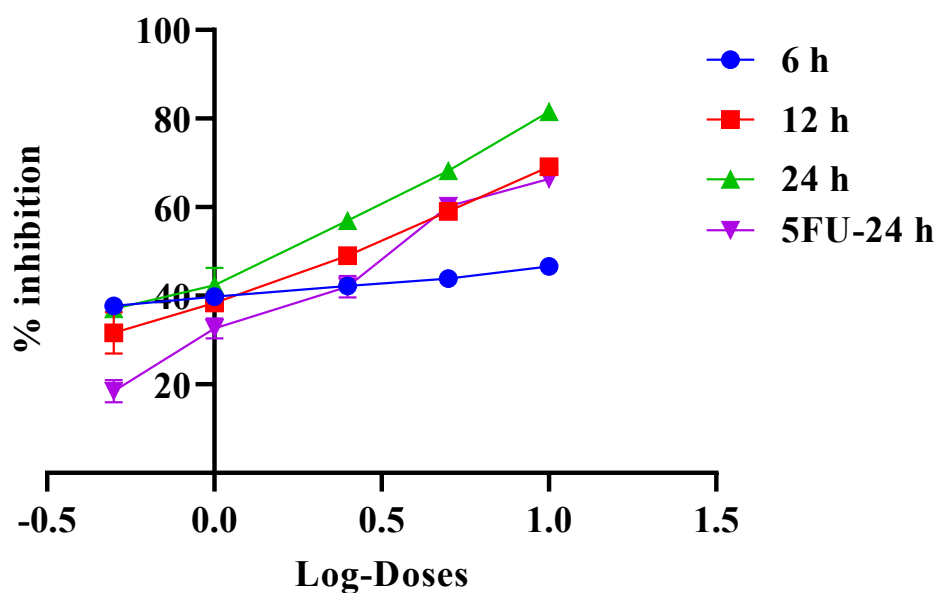


Figure 4.9. Log-dose plotted against % inhibition of 6,2'-Dihydroxyflavone for the calculation of IC_{50} against HCT116 cells.

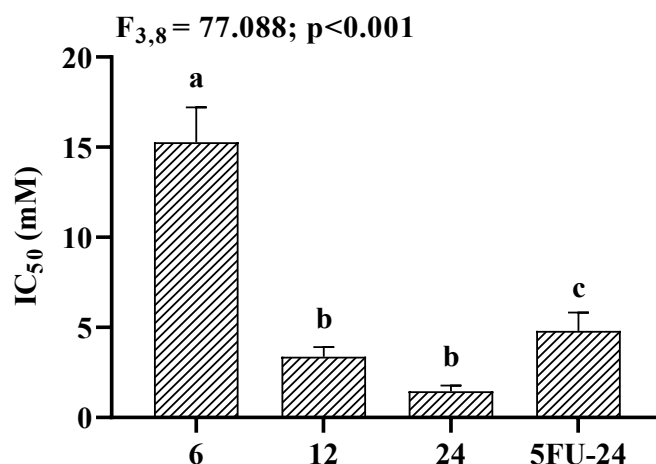


Figure 4.10. IC₅₀ of 6,2'-Dihydroxyflavone against HCT116 cells after 6, 12, and 24 h treatment. 6, 12, 24: HCT116 cells treated with different doses of 6,2'-Dihydroxyflavone for 6, 12 and 24 hours respectively; 5FU-24: HCT116 cells treated with different concentration of 5FU (positive control) for 24 hours. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

3.4. 6,2'-Dihydroxyflavone induces apoptotic morphological changes in HCT116 cells

The Acridine orange/Ethidium bromide (AO/EtBr) dual staining was employed to determine if the 6,2'-Dihydroxyflavone-induced cellular death in the HCT116 cells occurred via apoptosis. Characteristic morphological changes indicative of apoptosis were analysed under a fluorescent microscope. HCT116 cells treated with varying dosages of 6,2'-Dihydroxyflavone exhibited a dose-dependent increase in the number of apoptotic cells. Several apoptotic morphological markers such as nuclear condensation, membrane blebbing, chromatin fragmentation, etc., were observed in the treated HCT116 cells under a fluorescent microscope (Figure 4.11). Notably, the treatment of HCT116 cells with 1.5 mM of 6,2'-Dihydroxyflavone was statistically comparable to that of the standard drug 5FU (Figure 4.12).

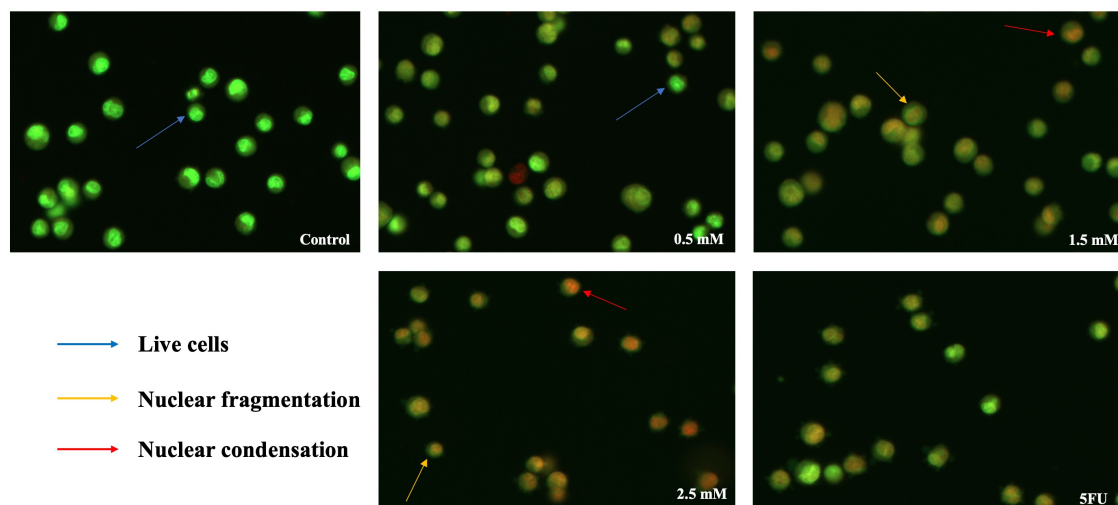


Figure 4.11. Acridine orange/Ethidium bromide (AO/EtBr) dual staining of HCT116 cells after treatment with different doses of 6,2'-Dihydroxyflavone for 24 h. Control: HCT116 cells without treatment; 0.5 mM, 1.5 mM, and 2.5 mM: HCT116 cells treated with 0.5, 1.5, and 2.5 mM of 6,2'-Dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5FU (positive control) for 24 h.

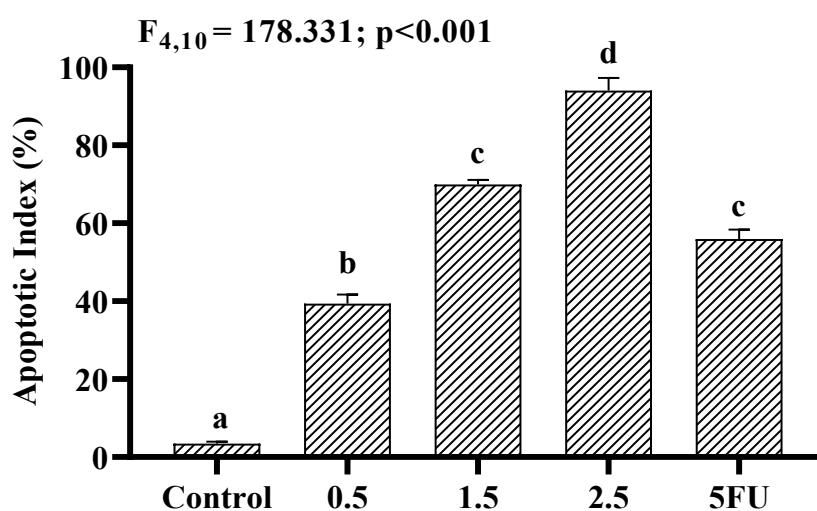


Figure 4.12. Apoptotic index of HCT116 cells after treatment with different doses of 6,2'-Dihydroxyflavone. Control: HCT116 cells without treatment; 0.5, 1.5, and 2.5: HCT116 cells treated with 0.5 mM, 1.5 mM, and 2.5 mM of 6,2'-Dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil

(positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

3.5. 6,2'-Dihydroxyflavone induces DNA damage in HCT116 cells

The ability of 6,2'-Dihydroxyflavone to induce DNA damage was assayed using the Comet Assay as DNA damage is often a target of cancer chemotherapy. Our results showed that treatment of HCT116 cells with different concentrations of 6,2'-Dihydroxyflavone effectively induces DNA strand breaks as is evident from the comet tails observed under a fluorescent microscope (Figure 4.13) as well as the increase in the amount of tail length (Figure 4.14A) and olive tail moment (Figure 4.14B). It was also observed that treatment of HCT116 cells with 2.5 mM of 6,2'-Dihydroxyflavone resulted in higher DNA damage as compared to the standard 5FU (Figure 4.14A & B).

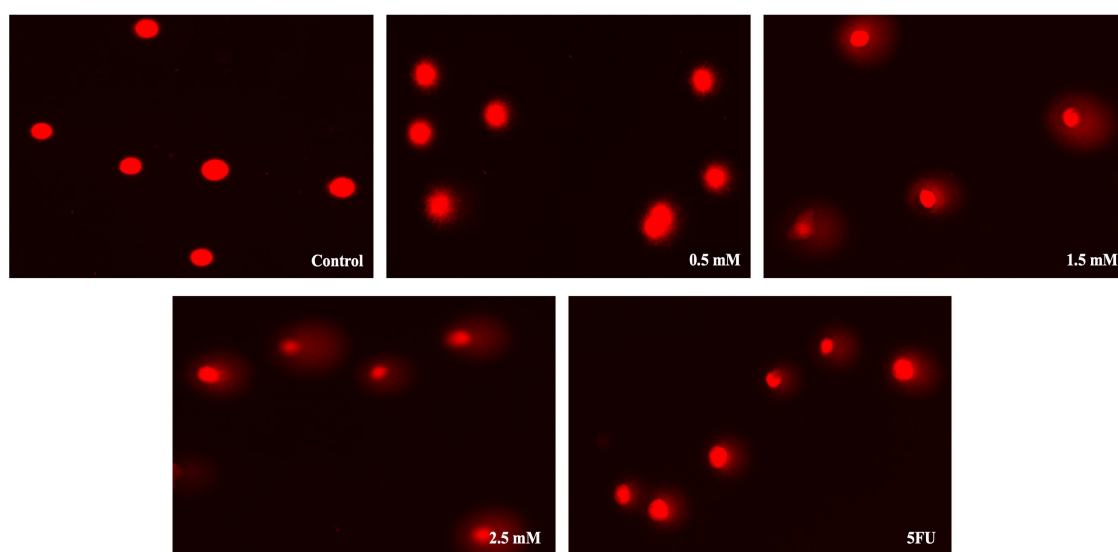


Figure 4.13. Fluorescent images of comets observed in HCT116 cells treated with different concentrations of 6,2'-Dihydroxyflavone. Control: HCT116 cells without treatment; 0.5 mM, 1.5 mM, and 2.5 mM: HCT116 cells treated with 0.5, 1.5 and 2.5 mM of 6,2'-Dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h.

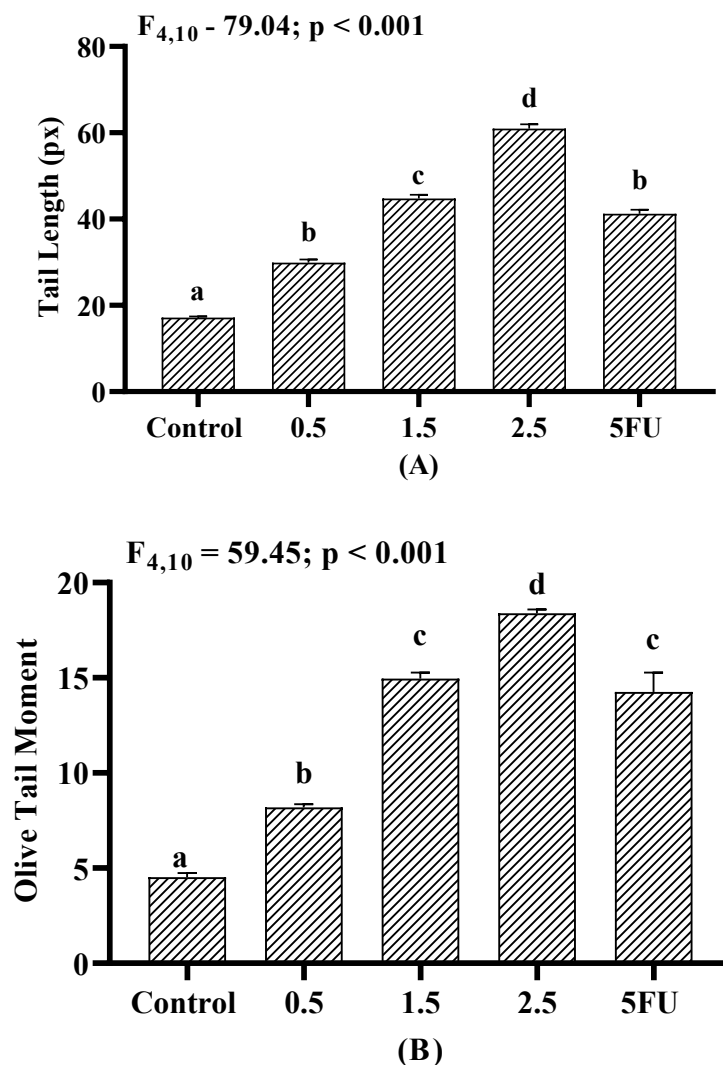
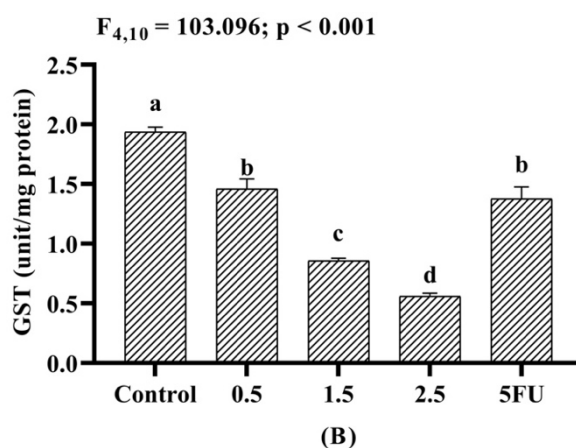
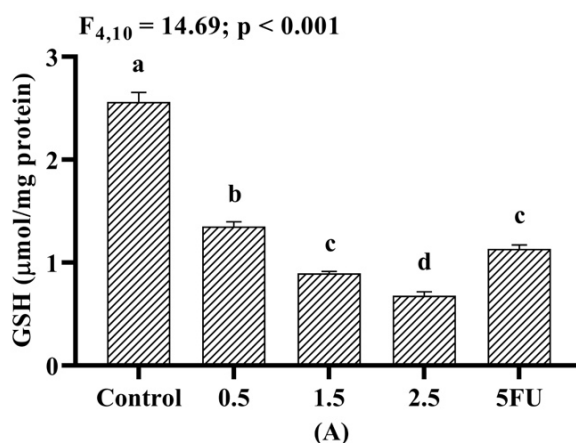


Figure 4.14. The extent of DNA damage induced by 6,2'-Dihydroxyflavone in HCT116 cells expressed in terms of (A) tail length and (B) olive tail moment. Control: HCT116 cells without treatment; 0.5, 1.5, and 2.5: HCT116 cells treated with 0.5 mM, 1.5 mM, and 2.5 mM of 6,2'-Dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

3.6. 6,2'-Dihydroxyflavone alters redox homeostasis in HCT116 cells

The level of the antioxidant glutathione (GSH) and activities of antioxidant enzymes glutathione S-transferase (GST) and superoxide dismutase (SOD) were measured in the HCT116 cells treated with 6, 2'-Dihydroxyflavone. The level of lipid

peroxidation as a marker of oxidative stress was also estimated in the treated cells and was compared with the untreated control HCT116 cells. A significant decrease in the level of GSH and the activities of GST and SOD was observed upon treatment of HCT116 cells with 6,2'-Dihydroxyflavone (Figure 4.15A, B & C). Consequently, the level of malondialdehyde (MDA) was observed to be significantly elevated in the compound-treated HCT116 cells when compared with the untreated control, indicative of increased lipid peroxidation (Figure 4.15D). Consistently, treatment of HCT116 cells with 1.5 mM of 6,2'-Dihydroxyflavone exhibits a comparable effect to that of the standard 5FU drug.



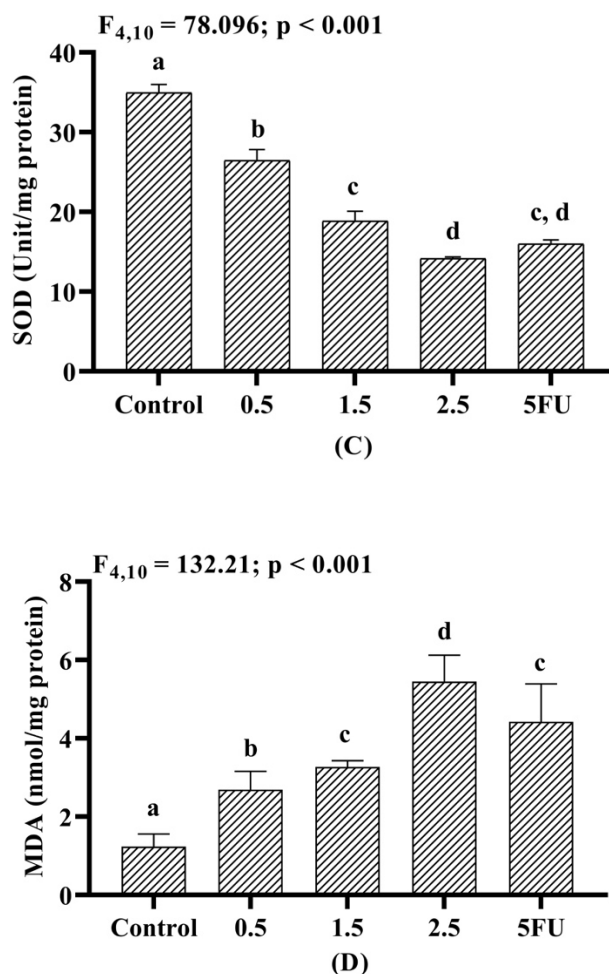


Figure 4.15. Effects of 6,2'-Dihydroxyflavone on the levels and activities of (A) GSH, (B) GST, (C) SOD, and (D) MDA in HCT116 cells. Control: HCT116 cells without treatment; 0.5, 1.5, and 2.5: HCT116 cells treated with 0.5 mM, 1.5 mM, and 2.5 mM of 6,2'-Dihydroxyflavone respectively for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

3.7. 6,2'-Dihydroxyflavone upregulates pro-apoptotic genes while downregulating anti-apoptotic gene expression in cancer cells

The relative expression of anti-apoptotic genes (*Bcl-X_L*, *Bcl-2*, *PARP*), pro-apoptotic genes (*Bax*, *Bid*, *Apaf-1*), and other genes involved in cellular survival such as *p53* and *Survivin* were analyzed in the 6,2'-Dihydroxyflavone-treated HCT116 cells using qRT-PCR. The treatment of HCT116 with 1.5 mM of 6, 2'-

Dihydroxyflavone resulted in the downregulation of the anti-apoptotic genes and the consequent upregulation of the pro-apoptotic genes. A significant increase was observed in the relative expression of pro-apoptotic genes *Bax*, *Bid*, and *Apaf-1* by 7.3, 5.7, and 6.8 folds in treated HCT116 cells (Figure 4.16 A, B & D). Meanwhile, the relative expression of anti-apoptotic genes *Bcl-X_L*, *Bcl-2*, and *PARP* was found to decrease by 3.0, 1.2, and 1.6 folds (Figure 4.17 (A, B & D)). Furthermore, an increase was also observed in the expression of the *p53* gene by 4.1 folds (Figure 4.16C) while the expression of the *Survivin* gene was decreased by 4.5 folds (Figure 4.17C) in HCT116 cells treated with 6,2'-Dihydroxyflavone. The effect of the 6,2'-Dihydroxyflavone on the relative expressions of *Bcl-2*, *Bax*, *Bid*, and *Apaf-1* was found to be statistically similar to that of the standard drug.

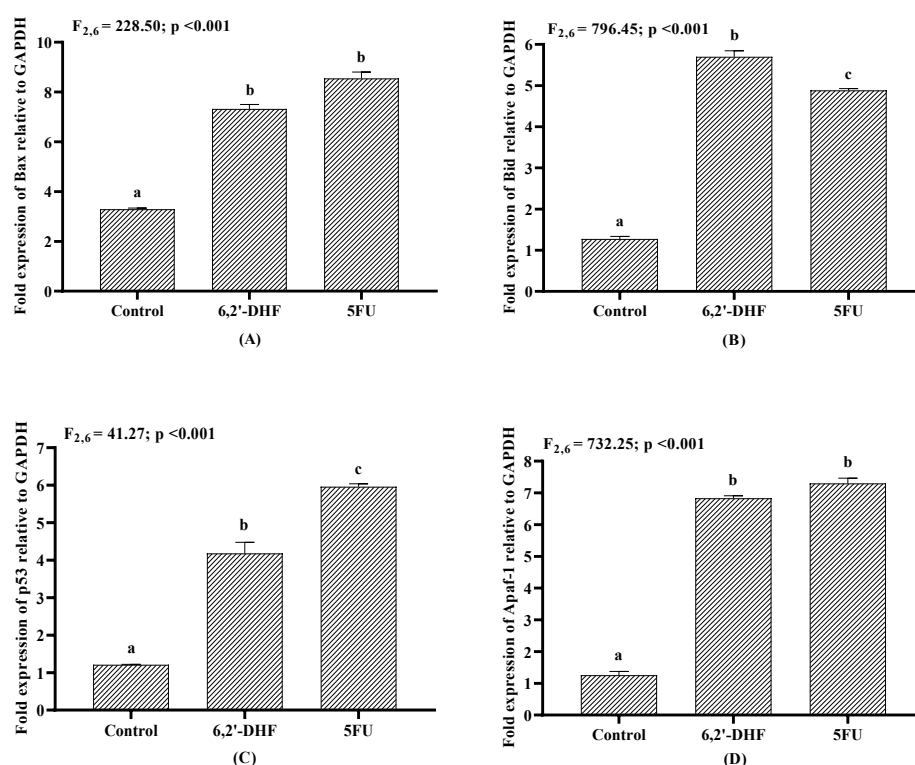


Figure 4.16. Quantification of the expression levels of pro-apoptotic genes (A) *Bax*, (B) *Bid*, (C) *p53*, and (D) *Apaf-1* relative to GAPDH in HCT116 cells. Control: HCT116 cells without treatment; 6,2'-DHF: HCT116 cells treated with 1.5 mM of 6,2'-Dihydroxyflavone for 24 h; 5FU: HCT116 cells treated with 5 mM of

5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

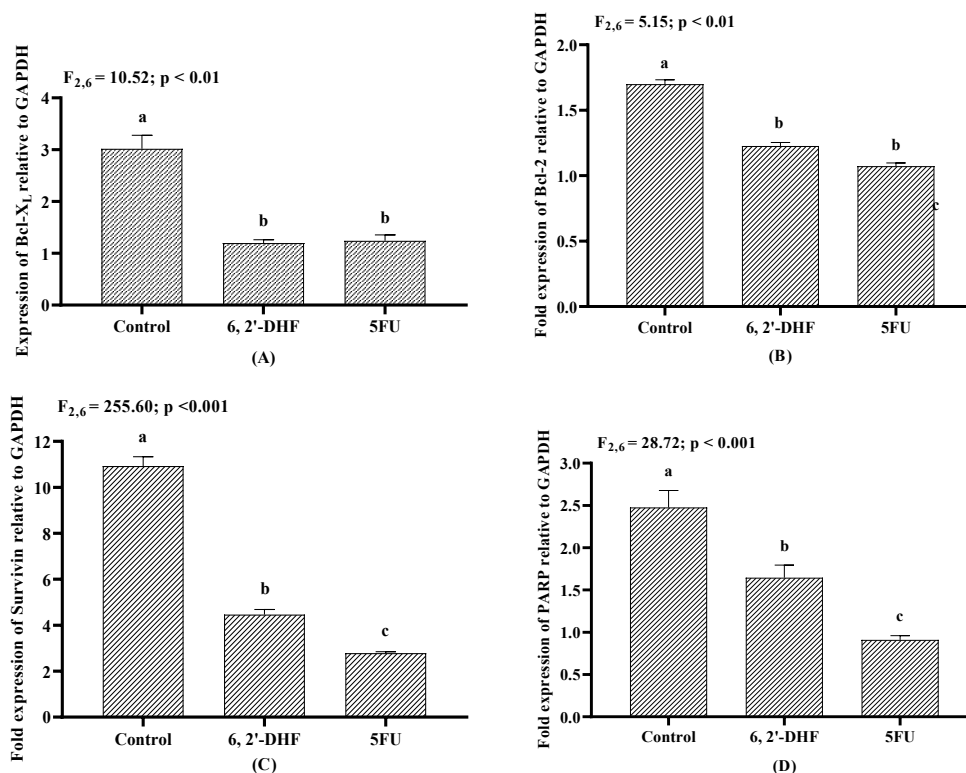


Figure 4.17. Quantification of the expression levels of anti-apoptotic genes (A) *Bcl-X_L*, (B) *Bcl-2*, (C) *Survivin*, and (D) *PARP* relative to GAPDH in HCT116 cells. Control: HCT116 cells without treatment; 6,2'-DHF: HCT116 cells treated with 1.5 mM of 6,2'-Dihydroxyflavone for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

3.8. 6,2'-Dihydroxyflavone increases activities of Caspase 3 and 6 in HCT116 cells

The induction of apoptosis in the treated HCT116 cells was confirmed by assessing the activities of Caspase 3 and 6. HCT116 cells were treated with 1.5 mM of 6,2'-Dihydroxyflavone for 24 h and the activities of Caspases 3 and 6 were analysed and compared with the untreated HCT116 cells. A 2.7-fold significant increase in the activity of caspase 3 was observed upon the treatment of HCT116 cells with 1.5 mM of 6,2'-Dihydroxyflavone (Figure 4.18). Treatment of HCT116 cells with 1.5 mM of 6,2'-Dihydroxyflavone also resulted in an 80.45 % increase in the activity of Caspase 6 which was significantly comparable to that of the standard control 5FU (76.26%) (Figure 4.19).

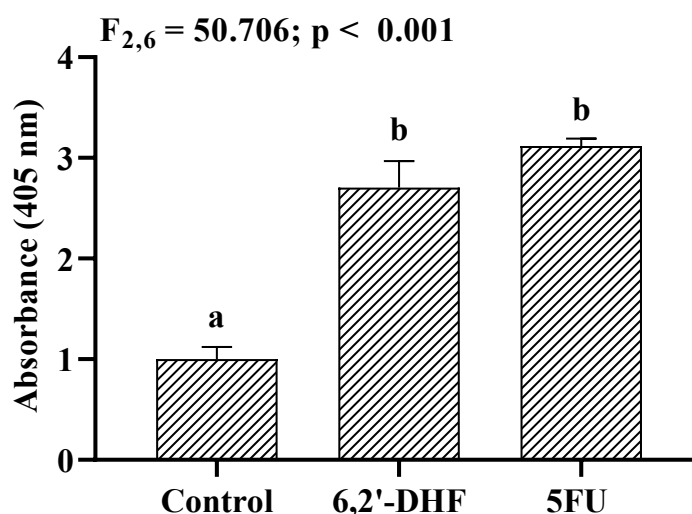


Figure 4.18. Effect of 6,2'-Dihydroxyflavone on the activity of Caspase 3 in HCT116 cells. Control: HCT116 cells without treatment; 6,2'-DHF: HCT116 cells treated with 1.5 mM of 6,2'-Dihydroxyflavone for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. Different letters indicate significant variation.

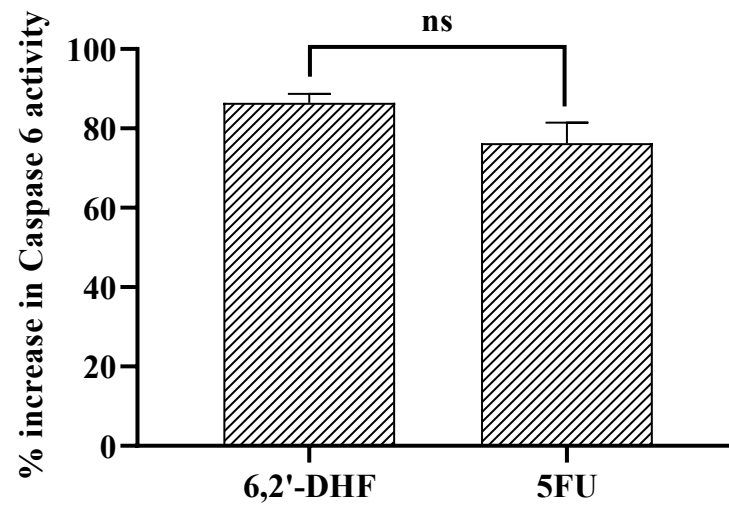


Figure 4.19. Effect of 6,2'-Dihydroxyflavone on the activity of Caspase 6 in HCT116 cells. 6,2'-DHF: HCT116 cells treated with 1.5 mM of 6,2'-Dihydroxyflavone for 24 h; 5FU: HCT116 cells treated with 5 mM of 5-Fluorouracil (positive control) for 24 h. Values are expressed as Mean $\pm$ SEM. ns: statistically not significant.

4. DISCUSSION

Despite the discovery and synthesis of numerous chemotherapeutic drugs for targeting cancer, the pursuit of new drugs is still very much high in demand due to the escalating concerns regarding the side effects linked to existing chemotherapy. Also, resistance to chemotherapy regimens has been documented for nearly all drugs employed in the treatment of malignancies (Ramos *et al.*, 2021). Resistance to doxorubicin (Smith *et al.*, 2006), paclitaxel (Harris *et al.*, 2006; Murray *et al.*, 2012), 5-Fluorouracil (Vulsteke *et al.*, 2014), cyclophosphamide (Sládek *et al.*, 2002), and carboplatin (Galluzzi *et al.*, 2014) have been demonstrated to lead to cancer recurrence in breast cancer, correlating with a poor prognosis and reduced survival rates. Resistance to 5-Fluorouracil (Zhang *et al.*, 2008), cisplatin (Mora-Lagos *et al.*, 2020), docetaxel (Li *et al.*, 2014), and oxaliplatin (Hu *et al.*, 2020) yields analogous outcomes in gastric cancer patients. Furthermore, the resistance to 5-Fluorouracil (Wang *et al.*, 2020), irinotecan (Nielsen *et al.*, 2017), and oxaliplatin (Lin *et al.*, 2021) in colorectal cancer, as well as cisplatin (Huang *et al.*, 2019), carboplatin (Liu *et al.*, 2019), and docetaxel (Cosaert and Quoix, 2002) in lung cancer, strongly suggests that the phenomenon of chemoresistance poses a significant threat to the health and survival of cancer patients. Therefore, the identification of a novel and more effective treatment that minimizes side effects and is resilient to chemoresistance is the need of the hour.

Since ancient times, medicinal plants have served as a promising source of natural products. The ever-growing interest in the quest for new anticancer therapies has driven the focus of chemotherapy research to look for new novel sources of anticancer drugs from natural sources such as plants due to their efficiency and reduced adverse effects. Thus, in this study, the plant *E. caudata*, with its extensive traditional use against cancer was identified as a candidate for bioassay-guided isolation of a novel anticancer compound.

The bioassay-guided fractionation and purification of the Hexane:Ethyl acetate extract of *E. caudata* resulted in the isolation of pale-yellow crystalline material. High-Resolution Mass Spectrometry (HRMS) analysis of the compound revealed a molecular ion peak at m/z 254.24, consistent with the molecular formula $C_{15}H_{10}O_4$, indicating 11 degrees of unsaturation. Comprehensive structural

characterization using ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy, CHNS(O) elemental analysis, and Fourier Transform Infrared Spectroscopy (FTIR) confirmed the compound as the flavonoid flavone, 6,2'-Dihydroxyflavone. Notably, this represents the first naturally occurring flavone reported with oxygenation exclusively at the C-6 position of ring A. Although structurally similar Dihydroxyflavones, such as 5,7'-Dihydroxyflavone, have been previously identified in other species within the *Elaeagnus* genus, such as *E. angustifolia* (Akbarzadeh *et al.*, 2014), this specific flavone derivative is novel. Flavonoids represent a diverse group of plant-derived polyphenolic compounds known for their potent anticancer activity. They have been reported to interfere with multiple signaling pathways involved in carcinogenesis such as AKT/PI3K/mTOR pathway, EGFR pathway, NF- κ B pathway, JAK/STAT pathway, etc., thereby limiting cellular proliferation, angiogenesis, and metastasis and ultimately inducing apoptosis (Ravishankar *et al.*, 2013; Hosseinzade *et al.*, 2019; Fattahi *et al.*, 2020; Zughaibi *et al.*, 2021). Currently, flavonoids such as Cyanidin, Catechins, Hesperidin, Apigenin, Luteolin, Quercetin 3-O glucoside, Genistein, and Daidzein are under clinical trials for targeting a variety of cancers including but not limited to breast cancer, colorectal adenocarcinoma, oral carcinoma, lung cancer, etc (Ponte *et al.*, 2021).

Induction of cellular death by various mechanisms is a prime target for all cytotoxic chemotherapy (Lopez and Tait, 2015). The isolated compound 6,2'-Dihydroxyflavone was revealed to possess potent cytotoxicity against HCT116 cells displaying a relatively low IC_{50} value of 1.45 mM based on the MTT assay which was more efficient than the standard 5-FU drug (Figure 4.10). However, the mode of cellular death was ascertained, thereby inviting further investigations. A comprehensive understanding of the specific mechanisms underlying cell death induction has become critical in anticancer drug development, as distinct pathways of cell death can yield varying therapeutic outcomes. Many conventional anticancer treatments induce cellular death by apoptosis, albeit in indirect ways. Apoptosis is a critical and highly controlled mechanism by which cells undergo death without damaging neighbouring healthy cells and therefore is the preferred mode of cellular death in a chemotherapy-induced cell death (Xu *et al.*, 2024). Thus, several functional assays were carried out to determine if the 6,2'-Dihydroxyflavone-induced

cell death in HCT116 cells was by apoptosis, and the differential expression of genes involved in apoptosis was also examined. Consequently, selective triggering of apoptosis was observed in the 6,2'-Dihydroxyflavone-treated HCT116 cells as evident by increased apoptotic morphological markers observed under EtBr/AO fluorescent dual staining (Figure 4.12), upregulation of pro-apoptotic genes (Figure 4.16) and the subsequent downregulation of the antiapoptotic genes (Figure 4.17). Further validation of apoptosis was displayed by a significant increase in the enzymatic activities of Caspase 3 (Figure 4.18) and Caspase 6 (Figure 4.19), collectively confirming the selective activation of the apoptotic pathway by 6,2'-Dihydroxyflavone in HCT116 cells.

The determination of whether the induced apoptosis follows the intrinsic or extrinsic pathway has become our limitation. However, based on our findings, it seems probable that the mitochondrial pathway was involved as indicated by the change in the relative expression of mitochondrial-pathway-associated genes such as *Bax* and their subsequent activation of effector caspases. The susceptibility of cells to apoptosis may be affected by the dysregulation of the balance of pro-apoptotic and anti-apoptotic proteins. An abundance of pro-apoptotic *Bcl-2* proteins and *Bax* on the surface of mitochondria leads to the formation of the permeability transition (PT) pore which then triggers the subsequent release of pro-apoptotic proteins including cytochrome *c*, *Smac/Diablo*, and apoptosis-inducing factor (AIF), ultimately resulting in the activation of caspase 3 and caspase 6 (Utpal *et al.*, 2025).

The effect of certain anticancer treatments, such as ionizing radiation and other chemotherapeutic drugs such as cisplatin that inflict DNA damage has been projected to propel the cancer cell, either directly or indirectly, towards apoptosis (Jurkovicova *et al.*, 2022). Increased DNA damage supported by increased tail length and olive tail moment was observed in the compound treated HCT116 cells using Comet assay (Figure 4.14) thereby signifying the involvement of DNA damage in the induction of apoptosis in the treated HCT116 cells.

Reactive oxygen species (ROS) are pivotal signaling molecules that significantly contribute to various diseases, including cancer onset, progression, and metastasis (Slika *et al.*, 2022). Although oxidative stress triggers various carcinogenic pathways, the excessive accumulation of ROS in the cancer cells can

counterintuitively precipitate anticancer effects, particularly cell cycle arrest, and apoptosis (Moloney and Cotter, 2018). Therefore, modulating ROS levels seems to be a feasible treatment strategy for combating cancer (Zhou *et al.*, 2016). Numerous anticancer agents, such as doxorubicin, paclitaxel, and vinblastine, along with certain compounds undergoing clinical trials, are recognized for inducing apoptosis in cancer cells through the generation of oxidative stress via inhibition of the antioxidant system. The compound 6,2'-Dihydroxyflavone was also found to inhibit the antioxidant system as the level of GSH and activities of GST and SOD were found to decrease while the level of oxidative stress marked by the increased lipid peroxidation was consequently elevated (Figure 4.15), signifying the involvement of redox imbalance in the induction of apoptosis in the treated HCT116 cells.

Furthermore, malondialdehyde (MDA), the by-product of lipid peroxidation has been reported to react with DNA to form adducts to deoxyguanosine and deoxyadenosine thereby inducing DNA damage (Marnett, 1999). Therefore, the increased generation of MDA coupled with the increased activity of caspase 3 may also contribute to the efficient DNA-damaging activity of 6,2'-Dihydroxyflavone. Caspase 3, an executioner caspase, plays a pivotal function in activating the endonuclease Caspase-activated DNase (CAD) through the proteolytic cleavage of its inhibitor, inhibitor of CAD (ICAD) (Sakahira *et al.*, 1998).

In this chapter, we report the isolation of a novel flavone, 6,2'-Dihydroxyflavone from the underutilized medicinal plant *Elaeagnus caudata*, which exhibits potent anticancer activity. The anticancer effect of the compound was primarily mediated through the induction of apoptosis, as evidenced by increased caspase 3 and caspase 6 activity, significant DNA damage, upregulation of pro-apoptotic gene expression, enhanced lipid peroxidation, and a concurrent reduction in antioxidant levels and anti-apoptotic gene expression. These findings suggest that 6,2'-Dihydroxyflavone holds considerable potential as a lead candidate for the development of a novel anticancer chemotherapeutic agent. However, further investigation is warranted to comprehensively elucidate the underlying molecular mechanisms and signaling pathways involved, alongside *in vivo* validation of these *in vitro* findings, to fully establish its therapeutic efficacy and safety profile.

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CHAPTER 5

Identification of molecular targets of lead compound in colorectal carcinoma using integrated network pharmacology and molecular dynamic simulation

ABSTRACT

Colorectal carcinoma remains a leading cause of cancer-related mortality worldwide, often compounded by chemoresistance and adverse effects from conventional therapies. This chapter employed an integrated *in silico* approach combining network pharmacology, molecular docking, and molecular dynamics (MD) simulations to investigate the anticancer potential of 6,2'-Dihydroxyflavone, a flavonoid isolated from *E. caudata*, against colorectal carcinoma. Target prediction and gene-disease association studies identified 98 common targets between 6,2'-Dihydroxyflavone and colorectal carcinoma. Protein-protein interaction (PPI) network analysis revealed critical hub genes, including EGFR, ESR1, PTGS2, and SRC, with EGFR emerging as the central target. Gene Ontology and KEGG pathway enrichment analyses indicated that 6,2'-Dihydroxyflavone potentially modulates several cancer-related pathways, particularly the ErbB, Estrogen, Prolactin, and Relaxin signalling pathways, which play vital roles in tumour progression, metastasis, and therapeutic resistance. Molecular docking analysis confirmed a strong interaction between 6,2'-Dihydroxyflavone and EGFR, with a binding energy of -9.21 kcal/mol, supporting its potential as an EGFR inhibitor. Comparative docking with Afatinib, a known EGFR inhibitor, revealed a slightly higher binding affinity for Afatinib. Subsequent 100 nanosecond MD simulations validated the stability of the 6,2'-Dihydroxyflavone-EGFR complex, with root mean square deviation (RMSD) and hydrogen bonding analysis confirming sustained interactions. Although 6,2'-Dihydroxyflavone exhibited greater flexibility and solvent exposure than Afatinib, binding free energy calculations (gmx_MMPBSA) demonstrated favourable energetics, supporting stable complex formation. Overall, this study suggests that 6,2'-Dihydroxyflavone exerts anticancer effects by targeting EGFR and disrupting critical oncogenic signalling pathways in colorectal carcinoma. These findings highlight the therapeutic potential of 6,2'-Dihydroxyflavone as a promising candidate for colorectal cancer treatment.

targeting the EGFR pathway, meriting further experimental validation through *in vitro*, *in vivo*, and clinical studies.

1. INTRODUCTION

Colorectal cancer remains a significant global health burden, ranking among the leading causes of cancer-related morbidity and mortality. In 2020, colorectal cancer accounted for 9.3% of all cancer deaths, second only to lung cancer in mortality rates (Bray *et al.*, 2024). Despite advancements in screening modalities and therapeutic interventions, both the incidence and mortality associated with colorectal cancer are projected to escalate, particularly in low- and middle-income countries. Epidemiological forecasts estimate a 60% increase in global colorectal cancer cases by 2030, reaching approximately 2.2 million new diagnoses and 1.1 million deaths annually (Arnold *et al.*, 2017).

The current standard of care for colorectal cancer typically involves multimodal treatment strategies, with surgical resection often complemented by radiotherapy and chemotherapy. In developed nations, the integration of advanced therapeutic approaches such as immunotherapy and targeted therapies—tailored to the molecular and genetic profiles of individual tumours—has further refined treatment paradigms (Faugeras *et al.*, 2017; McQuade *et al.*, 2017; Vassos and Piso, 2018). Nevertheless, colorectal carcinoma continues to be associated with high mortality rates, largely attributable to late-stage diagnoses, metastatic progression, and disease recurrence. Notably, the five-year survival rate for advanced-stage colorectal carcinoma remains below 14%, with more than 50% of patients receiving a diagnosis at a late stage (Chetroui *et al.*, 2021).

Furthermore, conventional chemotherapeutic regimens are frequently constrained by significant adverse effects, including gastrointestinal toxicity, myelosuppression, neurotoxicity, and hepatic or renal impairment. Escalating these challenges is the emergence of chemoresistance, which poses a formidable barrier to effective treatment and long-term disease control (Negarandeh *et al.*, 2020). Chemoresistance mechanisms—both intrinsic and acquired—reduce the responsiveness of tumour cells to standard cytotoxic agents, ultimately leading to therapeutic failure and disease progression.

Given these challenges, there is an urgent need to identify and develop novel therapeutic strategies that are both efficacious and associated with reduced toxicity. Alternative approaches capable of circumventing chemoresistance and improving

clinical outcomes are of paramount importance in advancing colorectal treatment and reducing its global disease burden.

In recent years, the utilization of *in silico* tools in drug discovery research has gained substantial scientific attention due to their potential to significantly reduce both the cost and time associated with drug development processes (Shaker *et al.*, 2021). The inherent complexity of plant extracts, resulting from the synergistic interactions among their diverse chemical constituents, presents considerable challenges in elucidating their precise molecular mechanisms of action on specific biological targets. Thus, the integration of bioinformatics and pharmacology has facilitated the emergence of network pharmacology as a robust analytical approach, enabling the systematic investigation of the biological mechanisms underlying complex diseases and drug-target interactions at the molecular level (Zhao and Iyengar, 2012). Network pharmacology primarily focuses on the identification of bioactive compounds, the construction and analysis of interaction networks linking pharmaceutical components to disease-associated genes, and the prediction of potential drug target proteins (Zhang *et al.*, 2019). This approach has significantly advanced our understanding of the intricate molecular pathways involved in disease pathogenesis and therapeutic interventions, thereby aiding in the identification of novel protein targets for drug development.

Additionally, molecular dynamic simulation has emerged as a powerful *in silico* technique, leveraging Newtonian mechanics to model the trajectories and dynamic behavior of molecular systems over time. By computing the configuration integral through sampling various molecular states, molecular dynamic simulation provides detailed insights into the temporal evolution of atomic interactions within biomolecular complexes (Yu *et al.*, 2024). This method enables the prediction of atomic-level movements within proteins and other molecular systems, offering a comprehensive understanding of the physicochemical principles governing interatomic forces and interactions.

In the previous scopes of this study, a novel flavonoid flavone 6,2'-Dihydroxyflavone was isolated from an underutilized medicinal plant called *E. caudata*. This compound exhibited significant cytotoxicity and potent pro-apoptotic activity against the human colorectal carcinoma cell line HCT116, indicating its

potential as a promising therapeutic candidate for colorectal cancer treatment. Therefore, with the reasoning above, we are desirous of dissecting the molecular mechanism of such bioactivity by identifying its prime molecular targets and pathways involved in inducing apoptosis in the colorectal cancer cell by employing *in silico* tools using integrating network pharmacology validated with molecular docking and molecular dynamic simulation.

2. MATERIALS AND METHODS

2.1. Screening of potential targets for 6,2'-Dihydroxyflavone

The canonical smile of 6,2'-Dihydroxyflavone was retrieved from the PubChem Database (<https://pubchem.ncbi.nlm.nih.gov/>) with CID number 688661. The target genes of the compound were then obtained from the Swiss prediction database (<http://www.swisstargetprediction.ch/>) (Daina *et al.*, 2019).

2.2. Potential target selection for Colorectal Cancer (CC)

The GeneCards database (<https://www.genecards.org/>) offers extensive information on all annotated and predicted human genes sourced from 150 databases. Thus, the genes related to colorectal carcinoma were retrieved from GeneCards (Stelzer *et al.*, 2016).

2.3. Drug-disease common target screening

The determination of the intersection of the targets of both 6,2'-Dihydroxyflavone and colorectal carcinoma were performed using Jvenn, a plug-in of the jQuery JavaScript library (<https://jvenn.toulouse.inrae.fr/>) (Bardou *et al.*, 2014). The intersecting genes represented the potential targets of 6,2'-Dihydroxyflavone for the treatment of colorectal cancer.

2.4. Protein-Protein interaction (PPI) network construction

The protein-protein interaction network of the shared targets was then generated using the STRING database (<https://string-db.org/>) (Szkarczyk *et al.*, 2023). The resultant network was downloaded as tsv. format and then imported into Cytoscape 3.10.2 software for further analysis. The Cytohubba plug-in was utilized to rank the related nodes based on their degree value to screen for the top ten core gene targets.

2.5. Gene Ontology and KEGG Pathway Enrichment Analysis

To further elucidate the relationship between target proteins and different cellular pathways, the functional annotation of Gene Ontology and KEGG pathway enrichment analysis of the top ten core genes was performed using the ShinyGO 0.80 tool (<http://bioinformatics.sdstate.edu/go/>). The species was set to human, pathway

minimum and maximum size were set to 2 and 5000 respectively while FDR cut off was set at 0.05 (Kanehisa *et al.*, 2021)

2.6. Molecular docking

Molecular docking was employed to determine the possibility of binding between the 6,2'-Dihydroxyflavone and the prime molecular target. The three-dimensional (3D) structure of the selected protein was obtained from the Protein Data Bank (PDB) (<https://www.rcsb.org/>). The 3D chemical structure of 6,2'-Dihydroxyflavone was also obtained from the NCBI PubChem database (<http://pubchem.nlm.nih.gov/>). Molecular docking was done using the UCSF CHIMERA plugin by VINA, and the docked result was visualized using Discovery Studio Visualizer (Ralte *et al.*, 2023).

2.7. Molecular dynamic simulation

Molecular dynamics simulations were conducted to assess the interaction strength and stability of the receptor-ligand complex. Topology files for small molecules were generated via SOBTOP, whereas the protein topology was acquired through GROMACS commands. The Amber14sb force field and SPC water model were employed to simulate physiological conditions, maintaining a 20 buffer between protein atoms and the water box edge. The system was neutralized using 0.15 mol/L saline solution. Before the simulations, energy minimization was conducted utilizing 50,000 steepest-descent iterations. The system was equilibrated with NVT and NPT ensembles for 100 ps with a two fs timestep. A molecular dynamics simulation lasting 100 nanoseconds was subsequently performed at 310 Kelvin. Trajectory analysis was performed after the elimination of periodic boundary conditions, and the resulting structure was overlaid onto the initial complex to evaluate interactions. Key metrics, such as RMSD, RMSF, radius of gyration, etc, were evaluated, and the Gibbs free energy landscape was constructed. The total binding free energy was calculated using gmx_MMPBSA (Ralte *et al.*, 2023).

3. RESULTS

3.1. Common targets of 6,2'-Dihydroxyflavone and colorectal carcinoma

Swiss target prediction revealed a total of 100 protein targets of 6,2'-Dihydroxyflavone belonging to different classes predominated by enzymes, lyase, kinases, receptors, cytochrome P450 family, etc., (Figure 5.1). A total of 23,714 genes involved in colorectal carcinoma were also compiled from the GeneCard database. Mutual gene matching performed between the compound and colorectal cancer using the Jvenn tool resulted in the identification of 98 genes (Figure 5.2) in total as represented in Table 5.1.

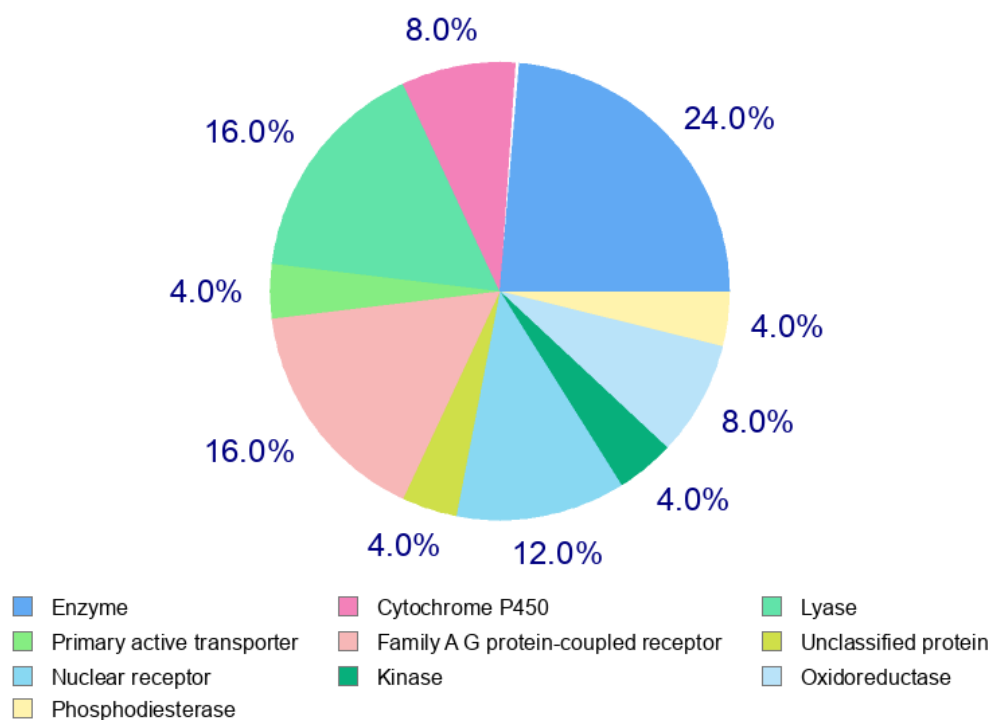


Figure 5.1. Pie-chart representing different classes of proteins that could be targeted by 6,2'-Dihydroxyflavone

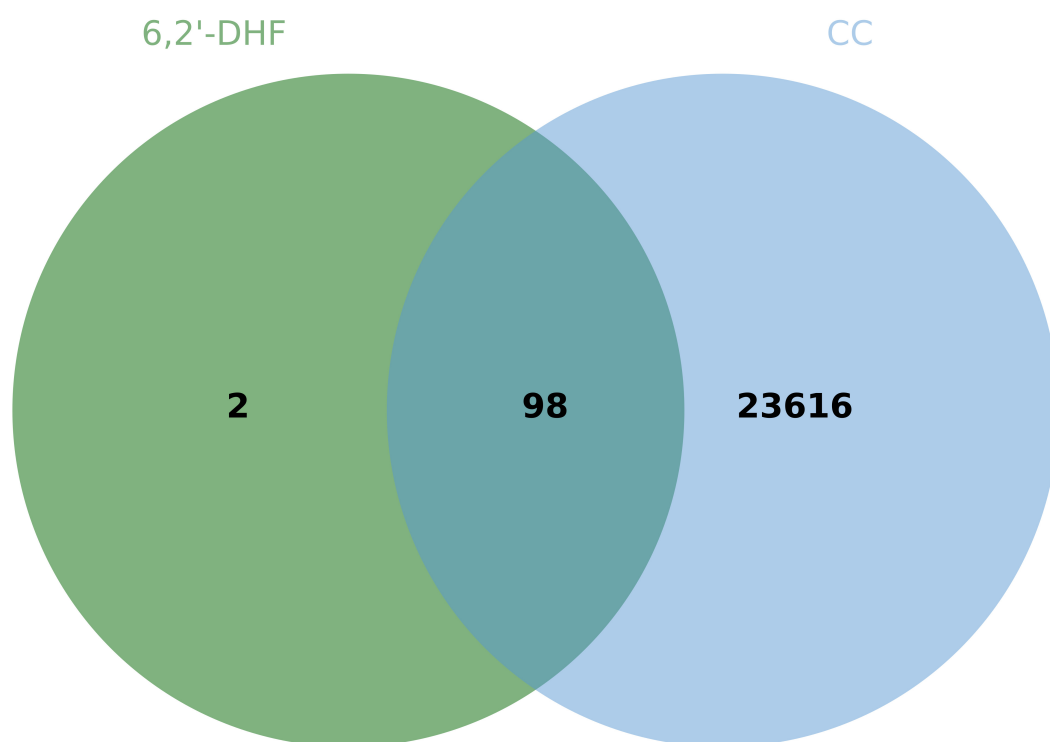


Figure 5.2. Venn Diagram of potential colorectal carcinoma genes targeted by 6,2'-Dihydroxyflavone. 6,2'-HDF-6,2'-Dihydroxyflavone, CC-Colorectal Carcinoma.

Table 5.1. Mutual genes matched between 6,2'-Dihydroxyflavone and Colorectal cancer in alphabetical order.

Sl.	GENE ID	GENE NAME
1.	ABCB1	ATP binding cassette subfamily B member 1
2.	ABCC1	Adenosine triphosphate binding cassette subfamily C member 1
3.	ABCG2	ATP-binding cassette subfamily G member 2
4.	ACHE	Acetylcholinesterase
5.	ADORA1	Adenosine A1 receptor
6.	ADORA2A	Adenosine A2A receptor
7.	ADORA3	Adenosine A3 receptor
8.	AHR	Aryl Hydrocarbon Receptor
9.	AKR1B1	Aldo-keto reductase family
10.	AKR1B10	Aldo-keto reductase family 1 member B10
11.	AKR1C1	Aldo-Keto Reductase 1C1
12.	ALK	Anaplastic lymphoma kinase
13.	ALOX12	Arachidonic acid 12-lipoxygenase
14.	ALOX15	Arachidonic acid 15-Lipoxygenase
15.	ALOX5	Arachidonic acid 5-Lipoxygenase
16.	AMY1A	AMY1A amylase alpha 1A
17.	APEX1	Apurinic/Apyrimidinic Endodeoxyribonuclease 1
18.	APP	Amyloid beta precursor
19.	AR	Androgen receptor
20.	ARG1	Arginase 1
21.	AURKB	Aurora kinase B
22.	BCHE	Butyrylcholinesterase
23.	CA1	Carbonic anhydrase 1
24.	CA12	Carbonic anhydrase 12
25.	CA2	Carbonic anhydrase 12
26.	CA3	Carbonic anhydrase 3
27.	CA4	Carbonic anhydrase 4
28.	CA7	Carbonic anhydrase 7
29.	CA9	Carbonic anhydrase 9
30.	CALM1	Calmodulin I
31.	CAMK2B	Calcium/calmodulin dependent protein kinase II beta
32.	CDK1	Cyclin-dependent kinase 1
33.	CDK2	Cyclin Dependent Kinase 2
34.	CHRNA7	Cholinergic Receptor Nicotinic Alpha 7 Subunit
35.	CSNK2A1	Casein kinase 2 alpha 1
36.	CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1

Sl.	GENE ID	GENE NAME
37.	CYP1A1	Cytochrome P450 Family 1 Subfamily A Member 1
38.	CYP1A2	Cytochrome P450 1A2
39.	CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1
40.	DAPK1	Death-Associated Protein Kinase 1
41.	DRD2	Dopamine Receptor D2
42.	EGFR	Epidermal growth factor receptor
43.	ESR1	Estrogen receptor 1
44.	ESR2	Estrogen receptor 2
45.	FLT3	FMS-like tyrosine kinase 3
46.	FYN	Proto-oncogene tyrosine-protein kinase Fyn
47.	GLO1	Glyoxalase 1
48.	GRK6	G protein-coupled receptor kinase 6
49.	GSK3B	Glycogen Synthase Kinase-3 β
50.	HSD17B1	Hydroxysteroid 17-Beta Dehydrogenase 1
51.	HSD17B2	Hydroxysteroid 17-Beta Dehydrogenase 2
52.	IGF1R	Insulin-like growth factor 1 receptor
53.	KDM4E	Lysine Demethylase 4E
54.	LCK	Lymphocyte-specific protein tyrosine kinase
55.	MAOA	Monoamine Oxidase A
56.	MAOB	Monoamine Oxidase B
57.	MAPK3	Mitogen-activated protein kinase 3
58.	MET	Mesenchymal-epithelial transition factor
59.	MMP12	Matrix metalloproteinase 12
60.	MMP13	Matrix metalloproteinase 13
61.	MMP2	Matrix metalloproteinase 2
62.	MMP3	Matrix metalloproteinase 3
63.	MMP9	Matrix metalloproteinase 9
64.	MPG	N-methylpurine DNA glycosylase
65.	NAE1	NEDD-8 activating enzyme 1
66.	NEK6	NIMA-related kinase 6
67.	NOX4	NADPH oxidase 4
68.	NTRK2	Neurotrophic Receptor Tyrosine Kinase 2
69.	NUAK1	NUAK family kinase 1
70.	ODC1	Ornithine decarboxylase
71.	PARP1	Poly (ADP-ribose) polymerase 1
72.	PDE5A	Phosphodiesterase Type 5
73.	PIK3CG	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma
74.	PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
75.	PiM1	Protease Inhibitor of alpha-1 antitrypsin

Sl.	GENE ID	GENE NAME
76.	PKN1	Protein Kinase N1
77.	PLA2G1B	Phospholipase A ₂ Group IB
78.	PLA2G2A	Phospholipase A ₂ group IIA
79.	PLA2G4A	Phospholipase A ₂ Group IVA
80.	PLK1	Polo-like kinase 1
81.	PRKDC	DNA-dependent protein kinase catalytic subunit
82.	PTGS2	Prostaglandin-Endoperoxide Synthase 2
83.	PTPRS	Protein Tyrosine Phosphatase Receptor Type S
84.	SIGMAR1	Sigma Non-Opioid Intracellular Receptor 1)
85.	SLC22A12	Solute carrier family 22 (organic anion/cation transporter), member 12
86.	SRC	Proto-oncogene tyrosine-protein kinase Src
87.	ST6GAL1	Beta-galactoside alpha-2,6-sialyltransferase 1
88.	SYK	Spleen tyrosine kinase
89.	TACR2	Tachykinin receptor 2
90.	TBXAS1	Thromboxane A synthase 1
91.	TERT	Telomerase Reverse Transcriptase
92.	TNKS	TRF1-interacting ankyrin-related ADP-ribose polymerase
93.	TNKS2	TRF1-interacting ankyrin-related ADP-ribose polymerase 2
94.	TOP1	Topoisomerase I
95.	TOP2A	Topoisomerase IIA
96.	TTR	Transthyretin
97.	TYR	Tyrosinase
98.	XDH	Xanthine dehydrogenase/oxidase

3.2. Analysis of Protein-Protein Interaction Network

For the PPI network analysis, the 98 gene targets were loaded into the STRING database. The resulting network is composed of 98 nodes and 559 edges. The PPI enrichment p-value was $< 1.0\text{e-}16$ (Figure 5.4). The network was then imported into the Cytoscape software and analysed by determining centrality and other metrics. The compound-target-network pathway (CTP) was also constructed (Figure 5.3). The Cytohubba plug-in revealed the top ten genes ranked by degree as depicted in Figure 5.5. The top 10 core gene targets were EGFR, ESR1, PTGS2, SRC, GSK3 β , MMP9, PARP1, MAPK3, ESR2, and MMP2 (Table 5.2).

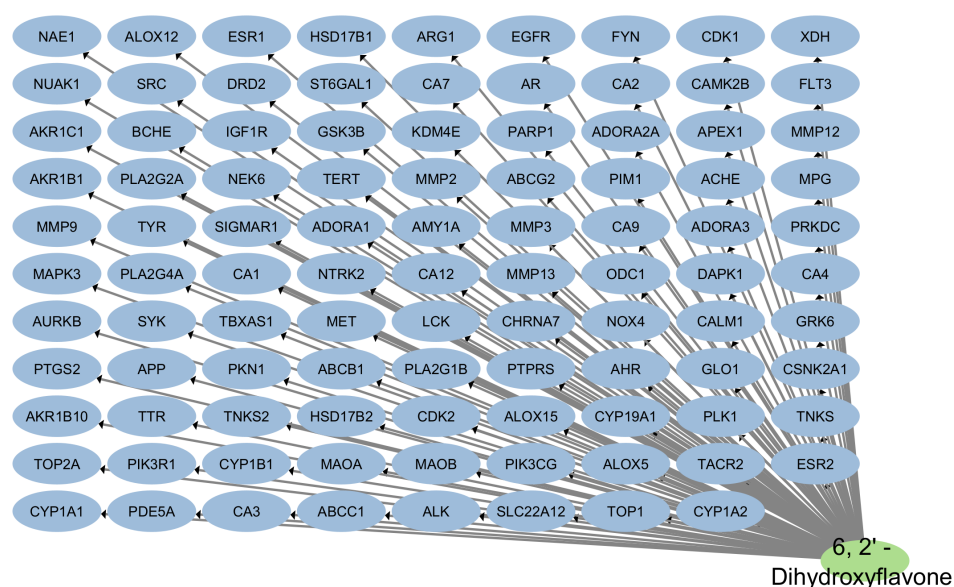


Figure 5.3. Compound-target-network Pathway (CTP) of 6,2'-Dihydroxyflavone in targeting colorectal carcinoma.

Table 5.2. Top 10 potential target rank and degree values as generated by Cytoscape 3.10.2.

SL	GENE ID	DEGREE VALUE
1.	EGFR	46
2.	ESR1	45
3.	PTGS2	40
4.	SRC	40
5.	GSK3B	35
6.	MMP9	30
7.	PARP1	29
8.	MAPK3	29
9.	ESR2	22
10.	MMP2	21

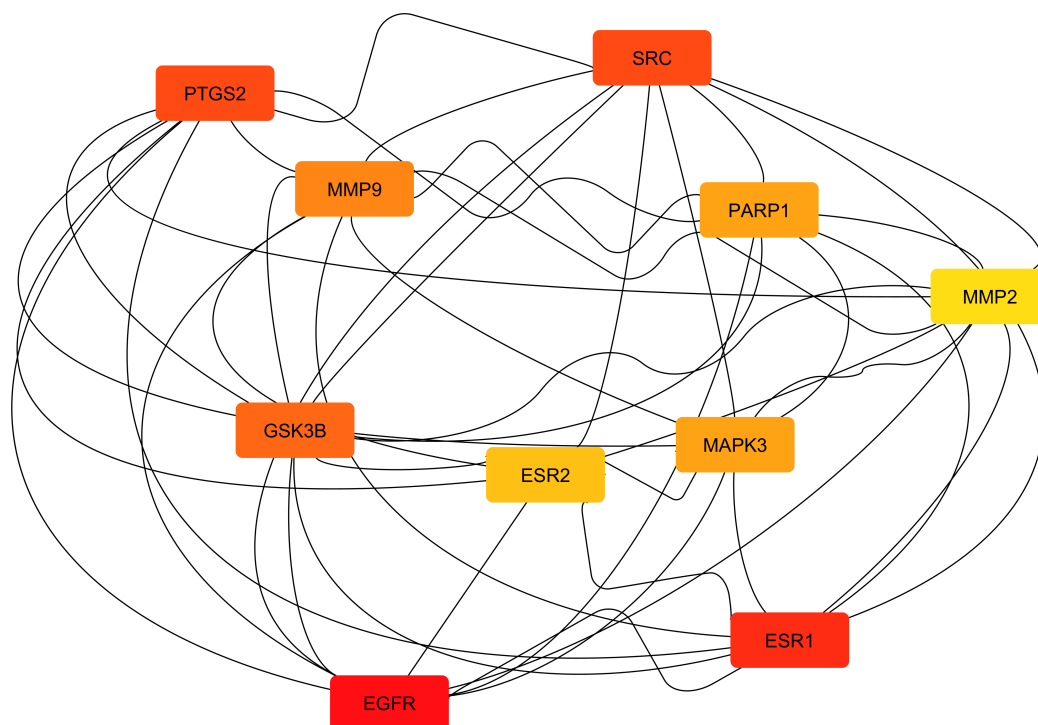


Figure 5.5. The top 10 potential target networks ranked by degree value as generated by Cytoscape 3.10.2 with interconnections.

3.3. Gene Ontology (GO) function Analysis

A total of 1268 GO items, comprising 1001 biological process (BP), 109 cellular components (CC), and 158 molecular functions (MF) were acquired from ShinyGO database ($p < 0.01$) and the top 10 BP, CC, and MF were chosen for visualization. The BP results show that the activity of 6,2'-Dihydroxyflavone in colorectal carcinoma was mostly focused on the regulation of cellular response to stress and oxidative stress. (Figure 5.6). The CC analysis includes caveola, membrane raft, membrane microdomain, membrane region, and plasma membrane raft (Figure 5.7). The MF analysis mainly includes Estrogen receptor binding, RNA polymerase II-specific DNA binding transcription factor binding, steroid hormone receptor binding, ATPase binding, and nuclear receptor binding (Figure 5.8).

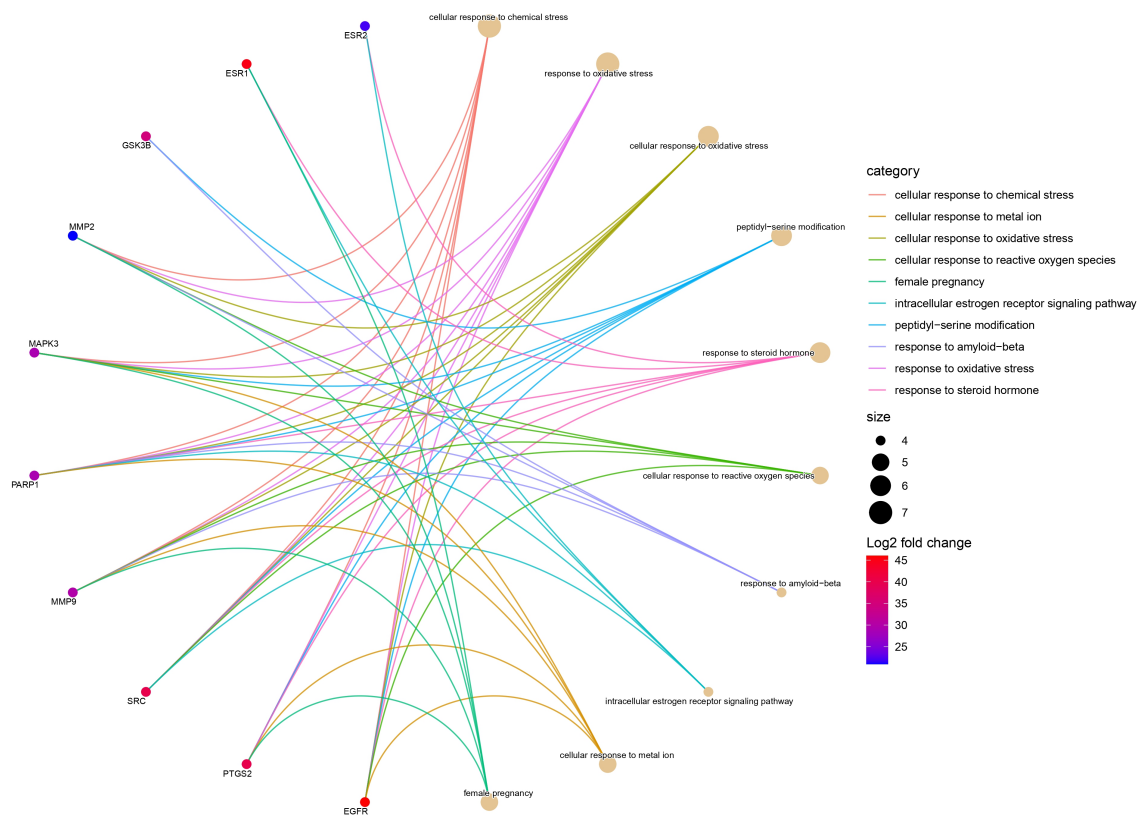


Figure 5.6. Net plot of Gene Ontology (Biological Processes) analysis of core genes.

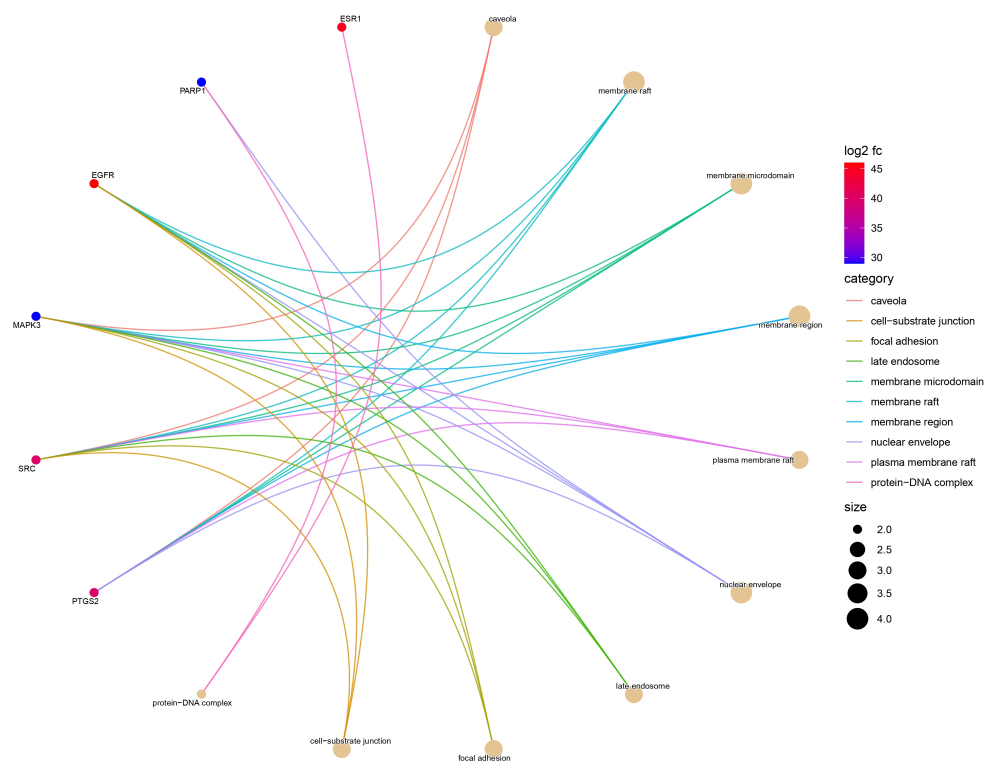


Figure 5.7. Net plot of Gene Ontology (Cellular Components) analysis of core genes.

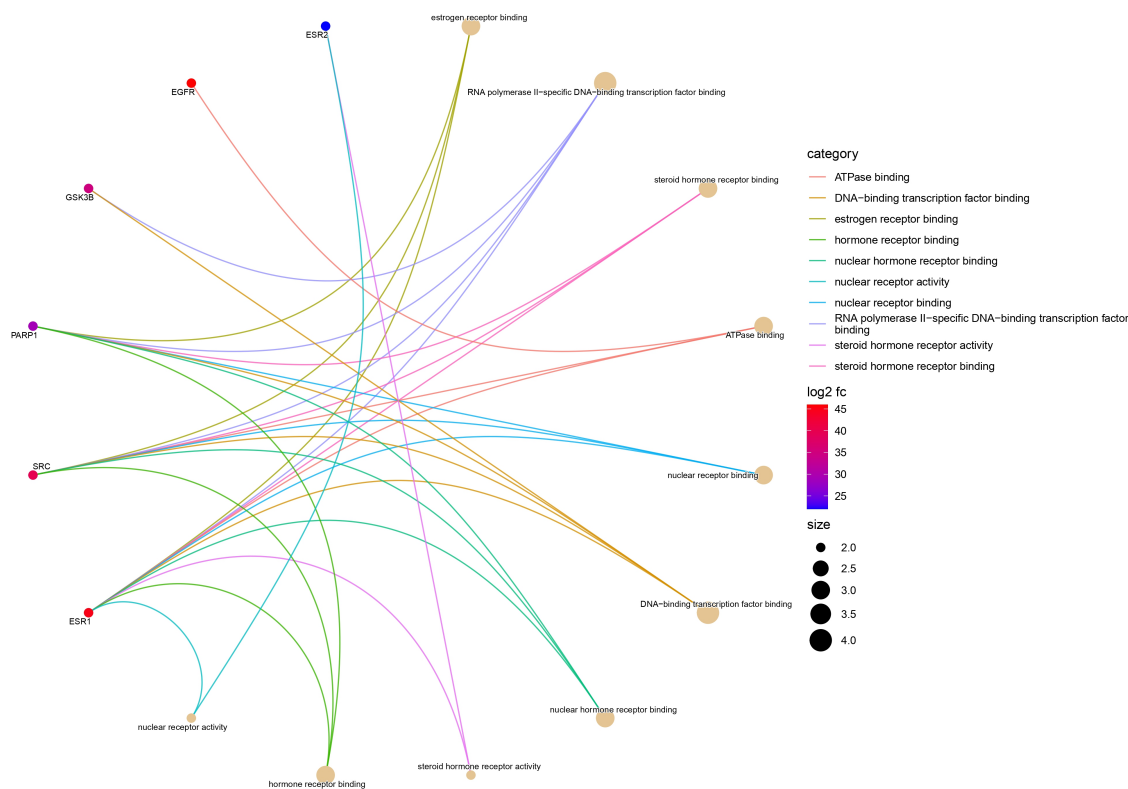


Figure 5.8. Net plot of Gene Ontology (Molecular Functions) analysis of core genes.

3.4. KEGG Pathway Enrichment Analysis

The KEGG pathway enrichment analysis further revealed that 6,2'-Dihydroxyflavone is primarily involved in the modulation of 150 distinct signalling pathways ($p < 0.01$). This extensive pathway involvement emphasizes the compound's potential multi-targeted mechanism of action within cellular systems. Notably, the analysis indicated that the core molecular targets of 6,2'-Dihydroxyflavone are significantly enriched in several key signalling pathways known to play crucial roles in the initiation, progression, and metastasis of colorectal cancer. These include, but are not limited to, the ErbB signalling pathway, Estrogen signalling pathway, the Prolactin signalling pathway, and the Relaxin signalling pathway (Figure 5.9). Each of these pathways is associated with critical biological processes such as cell proliferation, apoptosis, angiogenesis, and alteration of the tumour microenvironment. The significant enrichment of these pathways suggests that 6,2'-Dihydroxyflavone may exert its therapeutic effects by targeting multiple molecular mechanisms simultaneously, thereby contributing to the suppression of tumour growth and progression in colorectal cancer. A further comprehensive analysis of the signalling pathways involved suggests that 6,2'-Dihydroxyflavone may exert its anticancer effects in colorectal carcinoma predominantly through the modulation and inhibition of key oncogenic pathways, specifically the EGFR/RAS/ERK and EGFR/PI3K signalling cascades. These pathways are critically implicated in the regulation of cellular proliferation, survival, and metastasis in colorectal cancer. By targeting these signalling axes, particularly the EGFR protein, 6,2'-Dihydroxyflavone may effectively suppress tumour growth, inhibit metastatic potential, and overcome resistance mechanisms associated with colorectal carcinoma progression (Figure 5.10).

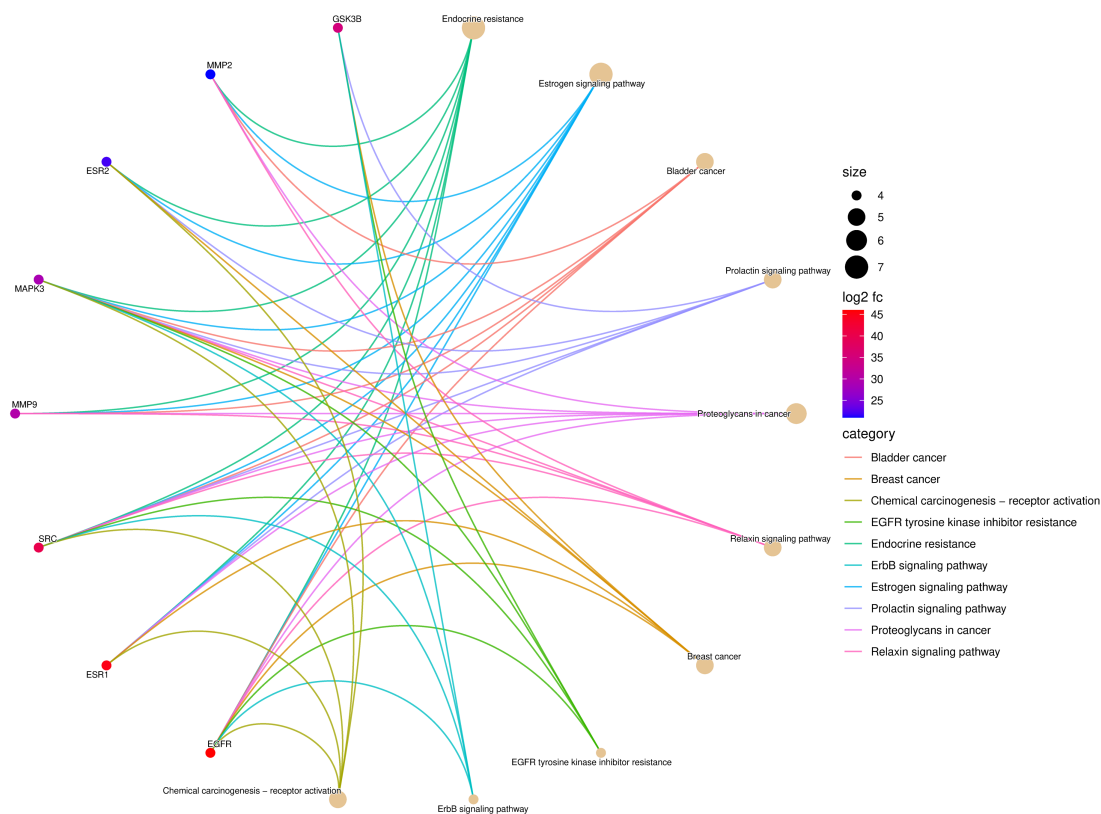


Figure 5.9. KEGG Pathway enrichment analysis of core genes

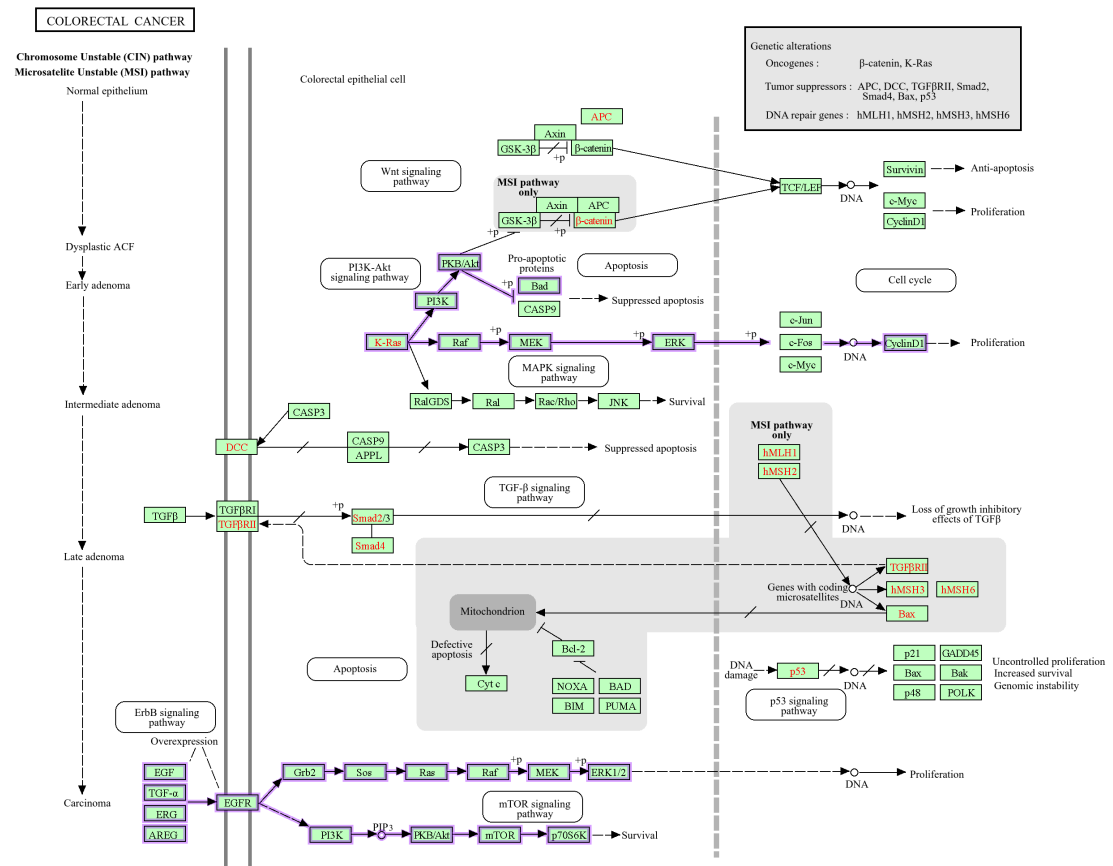


Figure 5.10. 6,2'-Dihydroxyflavone may exert its anticancer effect via inhibition of the EGFR/RAS/ERK and the EGFR/PI3K/mTOR pathway in colorectal carcinoma.

3.5. Molecular docking

Based on the core target screening and KEGG pathway enrichment analysis, the Epidermal Growth Factor Receptor (EGFR) was identified as a potential key molecular target for 6,2'-Dihydroxyflavone. Given EGFR's critical role in promoting tumour growth and survival, it was hypothesized that 6,2'-Dihydroxyflavone may exert its anticancer effects through direct interaction and inhibition of this target. To evaluate the binding affinity and interaction profile between 6,2'-Dihydroxyflavone and EGFR, molecular docking studies were performed. The binding results were subsequently compared with Afatinib, a known EGFR inhibitor, which served as the positive control to assess the relative binding efficiency and potential inhibitory activity of 6,2'-Dihydroxyflavone. The docking study revealed that the control ligand Afatinib exhibited a marginal superior binding affinity compared to 6,2'-Dihydroxyflavone with EGFR. Afatinib demonstrated a binding energy of -10.86 kcal/mol and an inhibition constant of 10.98 nM (Figure 5.12), whereas 6,2'-Dihydroxyflavone presented a binding energy of -9.21 kcal/mol and an inhibition constant of 177.7 nM (Figure 5.11). The interactions for 6,2'-Dihydroxyflavone included hydrogen bonds with THR(A:766) and MET(A:769), along with hydrophobic interactions involving residues such as LEU(A:764) and MET(A:742). Conversely, Afatinib exhibited hydrogen bonding with ASP(A:831) and THR(A:766), among other significant interactions.

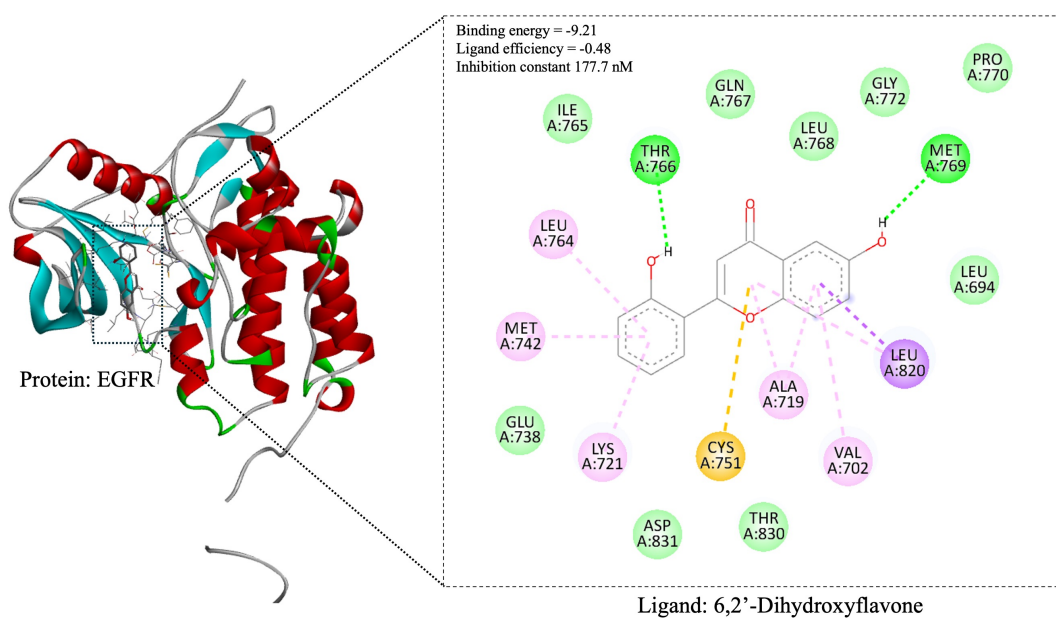


Figure 5.11. Docking interaction of 6,2'-Dihydroxyflavone with EGFR.

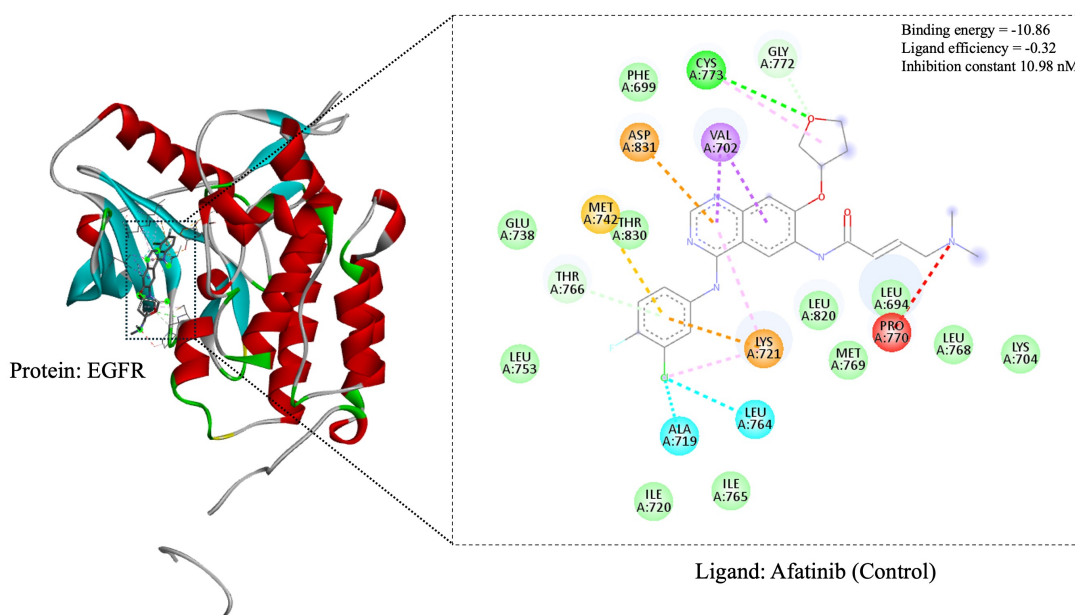


Figure 5.12. Docking interaction of Afatinib (control) with EGFR.

3.6. Molecular Dynamic Simulation

3.6.1. Root Mean Square Deviation (RMSD)

The RMSD analysis was performed to assess the stability and structural integrity of the Afatinib-bound complex (control) and the ligand, 6,2'-Dihydroxyflavone-bound complexes during the molecular dynamic simulation (Figure 5.13). The Afatinib control demonstrated a stable RMSD profile, consistently maintaining values between 1–2 Å during the simulation, signifying robust conformational stability. The 6,2'-Dihydroxyflavone complex also exhibited a similar RMSD pattern, stabilizing within an analogous range following an initial phase of volatility. This pattern indicates that ligand 6,2'-Dihydroxyflavone successfully preserved its binding state, akin to the recognized stability of Afatinib. The negligible RMSD fluctuations noted in both complexes validate the system equilibration and structural integrity.

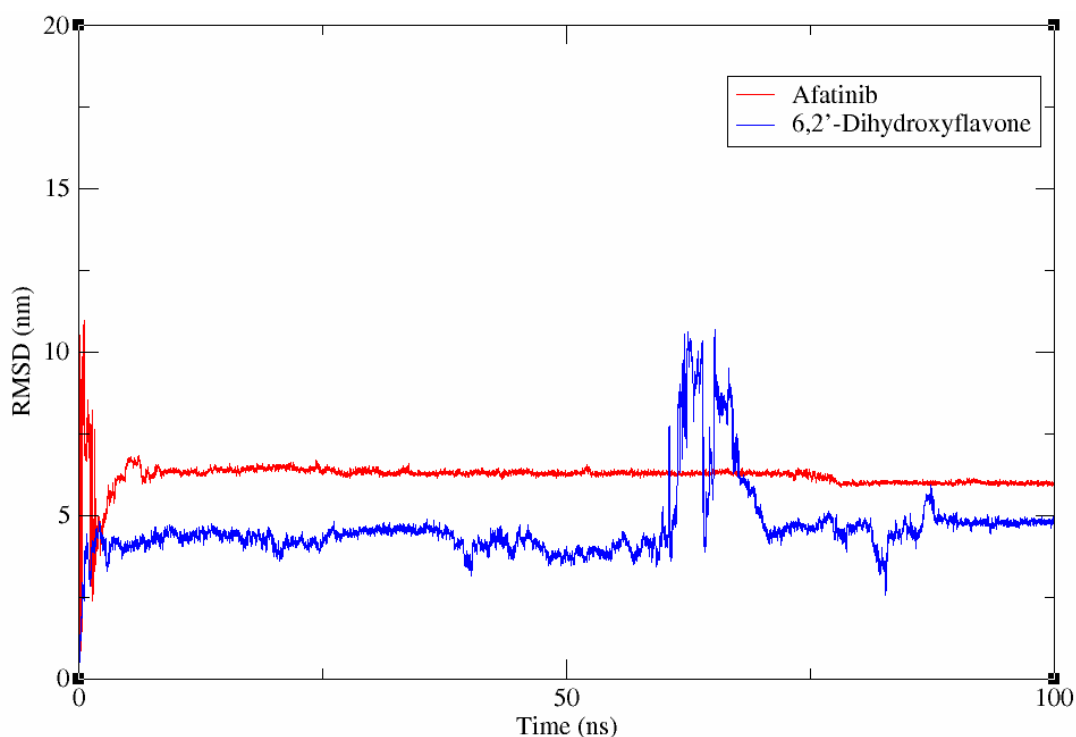


Figure 5.13. RMSD profile of EGFR-6,2'-Dihydroxyflavone complex compared to control EGFR-Afatinib complex for 100 ns of MD simulation.

3.6.2. Root Mean Square Fluctuation (RMSF)

The RMSF analysis was conducted to evaluate the flexibility of specific residues in the Afatinib-bound complex (control) and the ligand 6,2'-Dihydroxyflavone bound complex throughout the molecular dynamic simulation. The plot demonstrates that the Afatinib complex displayed consistently reduced RMSF values for most residues, signifying enhanced structural stiffness and stable interactions. Conversely, the ligand 6,2'-Dihydroxyflavone -bound complex exhibited somewhat greater variations in specific areas, notably in the C-terminal region, indicating enhanced flexibility in these parts (Figure 5.14). The Afatinib combination exhibited RMSF values below 0.4 nm for most residues, but the ligand 6,2'-Dihydroxyflavone complex demonstrated fluctuations beyond 1.5 nm in the terminal region. Such fluctuations may indicate areas with diminished structural limitations or adaptable loop sections. The RMSF profile indicates that Afatinib establishes a stiffer and more stable complex, whereas ligand 6,2'-Dihydroxyflavone exhibits adequate stability with localized flexibility, potentially affecting binding interactions and dynamic activity at the target site.

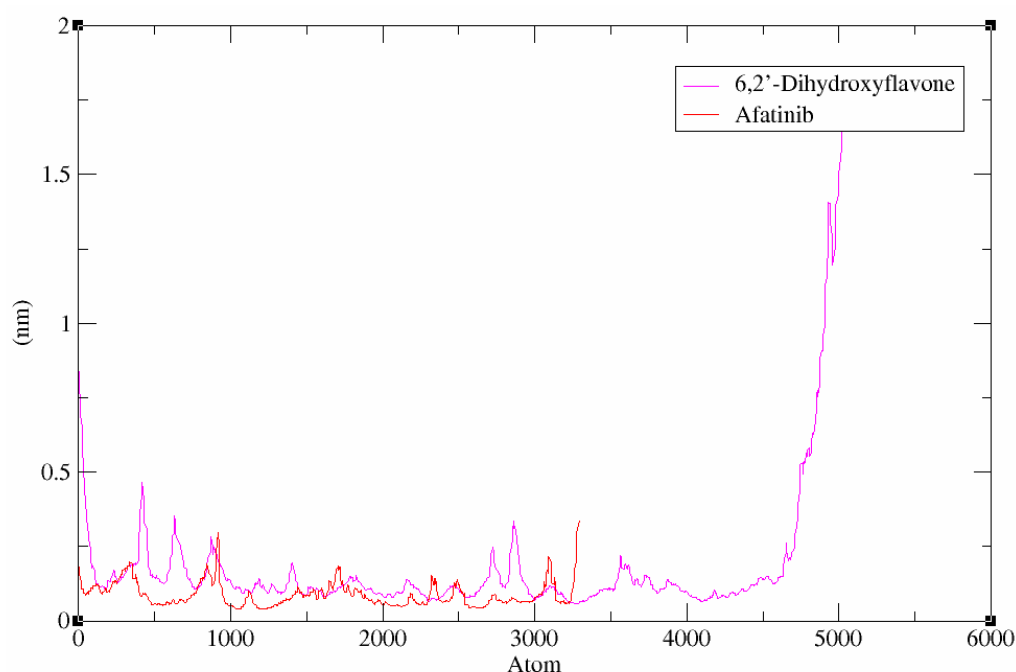


Figure 5.14. RMSF profile of EGFR-6,2'-Dihydroxyflavone complex compared to control EGFR-Afatinib complex for 100 ns of MD simulation.

3.6.3. Hydrogen Bond (H-Bond)

The study of hydrogen bonds was performed to assess the stability and interaction strength of the Afatinib-bound (control) and ligand 6,2'-Dihydroxyflavone-bound complexes during the molecular dynamic simulation. The plot illustrates that both systems sustained stable hydrogen bond formation during the 100 ns simulation (Figure 5.15). The Afatinib complex exhibited robust hydrogen bonding patterns, primarily sustaining 1-2 hydrogen bonds continuously throughout the simulation duration. In contrast, the ligand 6,2'-Dihydroxyflavone complex demonstrated more dynamic behaviour, with variable hydrogen bond interactions that occasionally escalated to 3-4 bonds during specific intervals. The Afatinib complex exhibited a more consistent and enduring hydrogen bond profile, hence confirming its stable binding mode. Ligand 6,2'-Dihydroxyflavone's capacity to generate several hydrogen bonds at different sites indicates flexible interactions that could improve target engagement. The variable hydrogen bond arrangement in ligand 6,2'-Dihydroxyflavone may suggest flexibility within the binding site, thereby affecting its overall stability and efficacy. These data indicate that whereas Afatinib facilitates consistent connections, ligand 6,2'-Dihydroxyflavone may utilize adaptable hydrogen bonding to preserve substantial binding affinity.

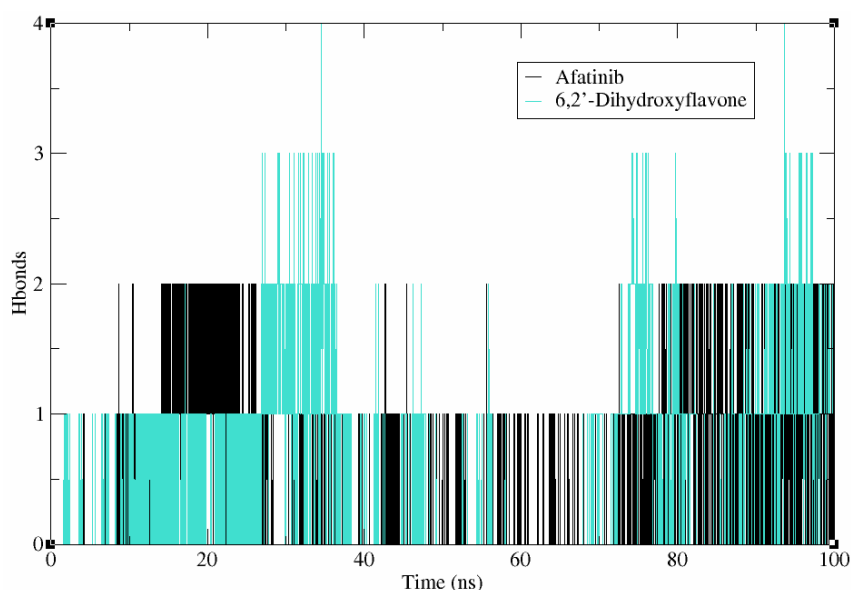


Figure 5.15. Hydrogen bond formation of EGFR-6,2'-Dihydroxyflavone complex compared to control EGFR-Afatinib complex for 100 ns of MD simulation.

3.6.4. Solvent Accessible Surface Analysis (SASA)

The SASA study was conducted to assess the extent of exposure of the protein-ligand complexes to the solvent environment throughout the 100 ns molecular dynamics simulation. The SASA profile for the Afatinib-bound (control) and ligand 6,2'-Dihydroxyflavone-bound systems demonstrates notable disparities in solvent exposure (Figure 5.16). The Afatinib complex exhibited a consistently decreased and stable SASA value of approximately 110-120 nm², signifying a more compact structure with diminished solvent accessibility. This indicates a deeply embedded and stable binding pocket with limited exposure to the surrounding solvent. Conversely, the ligand 6,2'-Dihydroxyflavone complex exhibited consistently elevated SASA values ranging from 175 to 185 nm², indicating increased solvent exposure. The elevated SASA implies that ligand 6,2'-Dihydroxyflavone adopts a binding conformation that facilitates more surface area interaction with the solvent, potentially signifying a more flexible or dynamic binding mode in contrast to Afatinib. The consistent SASA profiles for both systems demonstrate structural integrity during the simulation. The comparatively lower SASA in the Afatinib complex corresponds with its steady hydrogen bonding pattern and less flexibility, reinforcing its recognized function as a regulator. Conversely, the elevated SASA for the ligand 6,2'-Dihydroxyflavone may indicate flexible interactions, possibly affecting its bioavailability or binding efficacy.

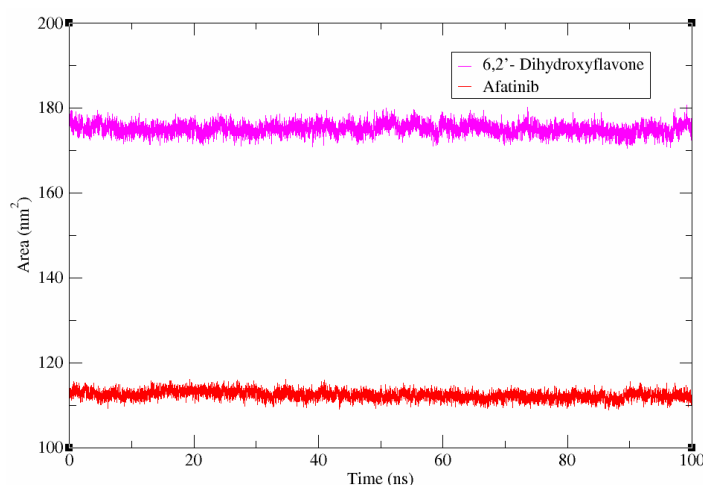


Figure 5.16. Solvent Accessible Surface Area of EGFR-6,2'-Dihydroxyflavone complex compared to control EGFR-Afatinib complex for 100 ns of MD simulation.

3.7. gmx_MMPBSA

The gmx_MMPBSA binding free energy of the EGFR-6,2'-Dihydroxyflavone complex utilizing gmx_MMPBSA elucidates the stability and contact affinity of the ligand with the protein during the MD simulation (Figure 5.17). The gas-phase energy (GGAS) is significantly advantageous, exhibiting substantial electrostatic and van der Waals contributions to binding. Nonetheless, the solvation energy (GSOLV) is predominantly adverse, indicating the desolvation cost during complex formation. The total binding free energy (ΔG_{bind}) oscillates around 0 to -20 kcal/mol, signifying a persistent and energetically advantageous association between the ligand and EGFR. The results indicate that 6,2'-Dihydroxyflavone demonstrates a robust binding affinity, positioning it as a potential candidate for colon cancer therapy aimed at targeting EGFR.

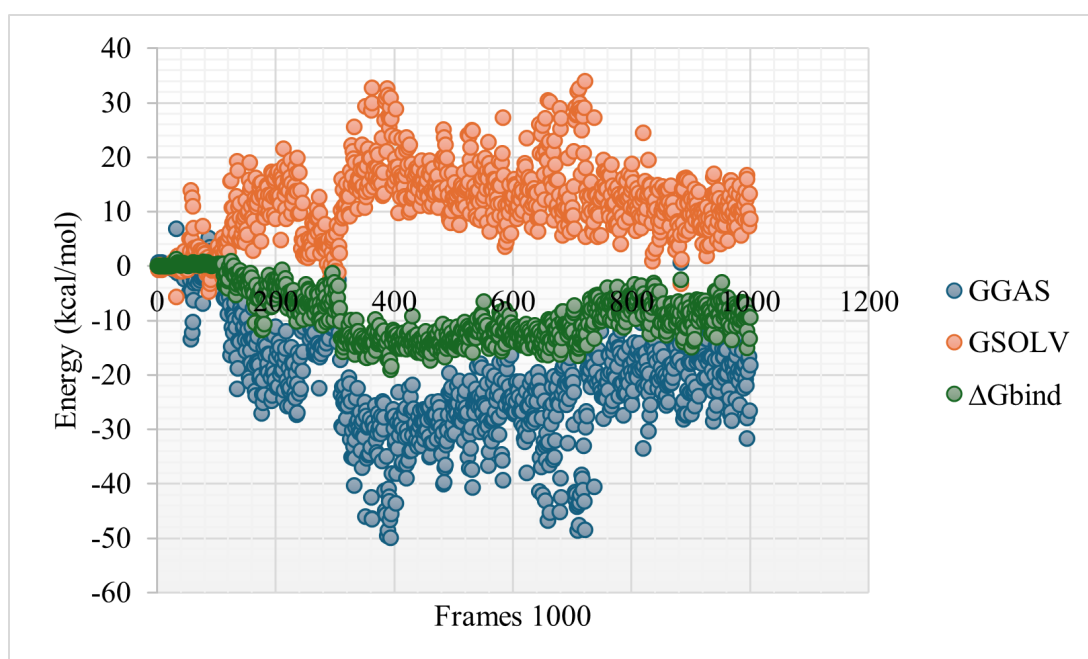


Figure 5.17. gmx_MMPBSA binding free energy of the EGFR-6,2' Dihydroxyflavone complex

4. DISCUSSION

The growing intersection of computational science and biology has garnered increasing attention with the advancement of modern bioinformatics. Data mining techniques play a critical role in extracting meaningful information from large and complex datasets, thereby facilitating more targeted and impactful research efforts (Gauthier *et al.*, 2019). Network pharmacology, with its capacity to address complex biological systems and multi-target, multi-mechanism interactions, offers a valuable approach to investigating the potential mechanisms underlying clinical treatments and disease prevention. Leveraging extensive experimental data and clinical trial outcomes, network pharmacology provides a systematic framework for elucidating therapeutic pathways and optimizing intervention strategies (Zhang *et al.*, 2019). Therefore, network pharmacology has been widely employed for the identification of bioactive compounds, the construction and analysis of interaction networks connecting pharmacological components with disease-associated genes, and the prediction of potential drug target proteins.

In this present study, the molecular mechanism underlying the anticancer and pro-apoptotic potential of 6,2'-Dihydroxyflavone isolated from *E. caudata* against colorectal carcinoma was systematically investigated by employing network pharmacology. A total of 98 key proteins were identified in colorectal carcinoma that could be the possible targets of 6,2'-Dihydroxyflavone. Most of these proteins belong to enzymes, kinases, receptors, etc., and therefore a protein-protein interaction (PPI) network was constructed to analyse their interconnections. Protein-protein interaction complexes refer to the assemblies of genes and proteins that interact with one another to facilitate various biological processes, molecular functions, and cellular components (Wong *et al.*, 2023). It is essential for understanding various physiological and biochemical processes, elucidating disease mechanisms, and discovering prospective therapeutic targets, and an abnormal PPI is the basis of many pathological conditions (Wong *et al.*, 2009).

Genes and proteins were represented as nodes, while edges illustrated the interactions and signalling events connecting them within the protein-protein interaction (PPI) network. These connections reflect the activation or repression of signal transduction pathways from one gene or protein to another (Belenahalli *et al.*,

2019). The 98 identified genes were ranked according to their degree and the number of interconnecting edges within the PPI network using Cytoscape software. Among these, EGFR, ESR1, PTGS2, SRC, GSK3B, MMP9, PARP1, MAPK3, ESR2, and MMP2 emerged as the core target genes of 6,2'-Dihydroxyflavone, with EGFR exhibiting the highest degree value, indicating its central role as the primary molecular target.

The core target genes were subsequently subjected to Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses to construct a pathway-target interaction network. These analyses revealed that the core targets are significantly enriched in multiple cellular signalling pathways implicated in colorectal cancer pathogenesis, including the ErbB signalling pathway, Estrogen signalling pathway, Prolactin signalling pathway, and Relaxin signalling pathway, among others.

Notably, the upregulation of prolactin receptors (PRLR) in colorectal carcinoma has been strongly correlated with the activation of the JAK2-STAT and JAK2-ERK1/2 signalling cascades, which collectively contribute to enhanced tumorigenesis, metastasis, and the development of chemoresistance (Neradugomma *et al.*, 2014). Similarly, the Relaxin signalling pathway exerts a pro-tumorigenic influence in colorectal cancer by promoting cellular invasion, metastasis, angiogenesis, and remodelling of the tumour microenvironment. This effect is mediated through the activation of Relaxin Family Peptide Receptor 1 (RXFP1), which subsequently triggers downstream signalling pathways such as cAMP/PKA, PI3K/AKT, ERK1/2, and nitric oxide (NO) production (Kotwal *et al.*, 2024).

The ErbB signalling pathway is a primary mediator of colorectal cancer progression via its engagement with many downstream proteins and signalling cascades such as RAS/RAF/MEK/ERK (MAPK) Pathway (enhancing chemoresistance), PI3K/AKT/mTOR Pathway (evasion of apoptosis), JAK/STAT Pathway (promoting tumour progression and metastasis), PLC γ /PKC Pathway (promoting cell motility, invasion, and angiogenesis), and Src Family Kinases (cell adhesion, motility, and proliferation; promotes metastasis) (Fang and Richardson, 2005; Hynes and MacDonald, 2009; Koveitypour *et al.*, 2019; Wan *et al.*, 2020; Wang *et al.*, 2022).

Comprehensive understanding of the molecular dynamics associated with these pathways offer critical insights for the development of targeted therapies and the mitigation of resistance mechanisms in colorectal carcinoma. Collectively, these findings suggest that 6,2'-Dihydroxyflavone may exert its anticancer effects by modulating these carcinogenic pathways, potentially through the inhibition of key protein-protein interaction (PPI) networks involved in colorectal cancer progression.

Further analysis suggests that 6,2'- Dihydroxyflavone may exert its anticancer effects in colorectal carcinoma by targeting the EGFR/RAS/ERK and EGFR/PI3K signalling cascades. By regulating these pathways, especially through EGFR inhibition, the compound may suppress tumour growth, reduce metastasis, and help overcome therapeutic resistance (Figure 5.10). Furthermore, EGFR has been reported to be overexpressed in over 80% of colorectal cancer, and is linked with poor prognosis (Pabla *et al.*, 2015). Therefore, molecular docking was performed to analyse the inhibitory potential of 6,2'- Dihydroxyflavone with EGFR. The docking analysis revealed that while Afatinib exhibited a stronger binding affinity and a lower inhibition constant, 6,2'-Dihydroxyflavone also demonstrated significant interactions with critical residues within the EGFR active site. Notably, the formation of hydrogen bonds and hydrophobic interactions with residues such as THR(A:766), MET(A:769), and LEU(A:764) supports its potential as an EGFR inhibitor. Although Afatinib displayed superior binding parameters, 6,2'-Dihydroxyflavone achieved a binding energy of -9.21 kcal/mol, which is well below the general threshold of -5.5 kcal/mol commonly associated with effective inhibitors such as Osimertinib and Dacomitinib (Abd El-Lateef *et al.*, 2024). These findings suggest that 6,2'-Dihydroxyflavone possesses promising inhibitory activity against EGFR and warrants further investigation as a potential therapeutic candidate for colorectal cancer treatment.

The molecular dynamics simulation outcomes offer significant insights into the structural stability, flexibility, and binding characteristics of the control drug Afatinib and the test ligand 6,2'-Dihydroxyflavone in association with the target protein. The RMSD readings for both complexes indicated stable trajectories throughout the 100 ns simulation. Afatinib exhibited a consistently reduced RMSD value, signifying robust structural stability and negligible departure from its original conformation. Conversely, the ligand 6,2'-Dihydroxyflavone complex demonstrated

marginally increased RMSD values, indicating substantial structural variations. This disparity may suggest a less stable binding conformation for ligand 6,2'-Dihydroxyflavone in comparison to Afatinib. The RMSF measurements further validated the RMSD observations. Afatinib exhibited little variations throughout the protein's residue index, indicating a tight and stable binding environment. Conversely, the ligand 6,2'-Dihydroxyflavone exhibited greater variations, especially in the loop areas, signifying enhanced flexibility. This implies that 6,2'-Dihydroxyflavone's binding method may entail more dynamic interactions, possibly resulting in a less stable complex in comparison to Afatinib.

An examination of hydrogen bonding underscored a crucial element of ligand stability. Afatinib frequently preserved one to two hydrogen bonds during the simulation, enhancing its stable binding profile. 6,2'-Dihydroxyflavone exhibited a variable hydrogen bond profile, with intermittent maxima surpassing three bonds. The elevated quantity of hydrogen bonds for ligand 6,2'-Dihydroxyflavone may imply stronger transient contacts. This inconsistency indicates diminished stability and a less resilient binding profile. The SASA analysis revealed a considerable differentiation between the two complexes. Afatinib exhibited a diminished SASA value ($\sim 110\text{-}120\text{ nm}^2$), signifying a compact conformation with decreased solvent accessibility. Ligand 6,2'-Dihydroxyflavone had an elevated SASA value ($\sim 175\text{-}185\text{ nm}^2$), indicating a more solvent-exposed and potentially variable binding conformation. The reduced SASA in the Afatinib complex corresponds with its stable hydrogen bond characteristics and low RMSF values, hence affirming its stable binding conformation.

This study provides a comprehensive investigation into the potential anticancer mechanisms of 6,2'-Dihydroxyflavone against colorectal carcinoma using a network pharmacology approach integrated with molecular docking and molecular dynamics simulations. The compound demonstrated interactions with several key genes implicated in multiple cancer-related signalling pathways, with EGFR identified as a central target. 6,2'-Dihydroxyflavone exhibited strong inhibitory potential against EGFR, thereby disrupting downstream signalling cascades and promoting apoptotic pathways in colorectal cancer cells. Therefore, 6,2'-Dihydroxyflavone isolated from *E. caudata* may serve as a promising candidate for future drug development as a novel EGFR inhibitor for the management of colorectal carcinoma. However, further

validation of these *in silico* findings through in-depth *in vitro*, *in vivo*, and clinical studies remains essential to confirm its therapeutic potential.

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ABBREVIATIONS

5FU	5-Fluorouracil
ABTS	2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
ANOVA	Analysis of Variance
AO	Acridine Orange
ASA	Ascorbic Acid
BP	Biological Processes
BSA	Bovine Serum Albumin
CAD	Caspase Activated DNase
CASP3	Caspase 3
CC	Cellular Component
CPCSEA	Control and Supervision of Experiments on Animals
CRC	Colorectal Carcinoma
CTP	Compound Target Pathway
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EC-AE	<i>Elaeagnus caudata</i> Aqueous Extract
EC-ME	<i>Elaeagnus caudata</i> Methanolic Extract
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
g	gram
GAE	Gallic Acid Equivalent
Gmx_MMPBSA	Gromacs-Molecular Mechanics Poisson-Boltzmann Surface Area

GO	Gene Ontology
GRB2	Growth Factor Receptor-bound protein 2
GROMACS	GROningen MOlecular Simulation package
GSH	Glutathione
GSK3 β	Glycogen synthase kinase 3 β
GST	Glutathione S-Transferase
h	Hour
HAMP	Horticulture And Aromatic Plants
hERG	Human Ether-a-go-go-related gene
HSP90	Heat Shock Protein 90
IC ₅₀	Inhibitory Concentration
ICAD	Inhibitor of Caspase Activated DNase
JAK2	Janus Kinase 2
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-HRMS	Liquid Chromatography-High Resolution Mass Spectrometry
LD ₅₀	Lethal Dose
LMPA	Low Melting Point Agarose
LPO	Lipid Peroxidation
MAPK1	Mitogen-Activated Protein Kinase 1
MAPK3	Mitogen-Activated Protein Kinase 3
MDA	Malondialdehyde
MDM2	Murine Double Minute 2
MEM	Minimum Essential Medium
MF	Molecular Functions
mg	milligram
min	Minute
ml	Millilitre
MMP2	Matrix Metalloproteinase 2
MMP9	Matrix Metalloproteinase 9
MTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH	Nicotinamide Adenine Dinucleotide (NAD ⁺) Hydrogen
NBT	Nitroblue tetrazolium
NCBI	National Center for Biotechnology Information
nm	Nanometre
NMPA	Normal Melting Point Agarose
NSCLC	Non-Small Cell Lung Carcinoma
OCT2	Organic Cation Uptake Transporter 2
PARP1	Poly (ADP-ribose) Polymerase 1
PBS	Phosphate Buffered Saline
PDA	Photo Diode Array
PDB	Protein Data Bank
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit
PMS	Phenazine Methosulfate
pNA	Para-nitroaniline
PTGS2	Prostaglandin-Endoperoxide Synthase 2
QE	Quercetin Equivalent
RBC	Red Blood Cell
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SASA	Solvent Accessible Surface Area
sec	Second
SEM	Standard Error of the Mean
SMILES	Simplified Molecular Input Line Entry System
SOD	Superoxide Dismutase
SPSS	Statistical Package for the Social Sciences
STRING	Search Tool for Retrieval of Interacting Genes/Proteins
TCA	Trichloroacetic Acid
TLC	Thin Layer Chromatography
UALCAN	University of Alabama Cancer Database

UCSF	University of California, San Francisco
WHO	World Health Organisation
μg	Microgram
μl	Microlitre
μmol	Micromolar

BIO-DATA OF THE CANDIDATE

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Educational Qualification:

Exam Passed	Year	Board/ University	Subject	Division	Percentage/ CGPA
HSLC	2009	MBSE	General	Distinction	84.4 %
HSSLC	2013	MBSE	General	Second	55 %
B. Sc	2017	MZU	Zoology	Distinction	89.08%
M. Sc	2019	MZU	Zoology	First	7.898/10
NET	2019	CSIR	Life Science	NA	AIR 101
GATE	2022	IIT	Life Science	NA	89.12 percentile
GATE	2022	IIT	Biotechnology	NA	64.2 percentile

**MBSE – Mizoram Board of School Education; MZU – Mizoram University;
 CSIR – Council of Scientific and Industrial Research; IIT – Indian Institute of Technology*

AWARDS & DISTINCTIONS

1. Awarded ***College Proficiency Award*** by Lunglei Government College for securing 3rd University rank in the Bachelor of Science (Zoology) examination conducted by Mizoram University in the 2017.
2. Awarded ***University Gold Medalist*** for securing First Position in the Master of Science (Zoology) examination conducted by Mizoram University in the year 2019.
3. Awarded the ***Innovation in Science Pursuit for Inspired Research (INSPIRE)*** Fellowship by the Department of Science and Technology, Government of India (Junior Research Fellowship, JRF) in the year 2020 and upgraded to Senior Research Fellow (SRF) in the year 2022.

CONFERENCES, SEMINARS AND SYMPOSIUMS ATTENDED

International

1. International Conference on ‘Bioeconomy from Bioresources: Promoting Traditional Resources of NER for Viksit Bharat’ and 11th Convention of Society for Ethnopharmacology, India, Gangtok, Sikkim, 15th – 16th November 2024.
2. Asia-Pacific Organisation for Cancer Prevention (APOCP) International Conference on Cancer Prevention and 11th General Assembly of APOCP, Kolkata, India, 8th to 10th December, 2022.
3. International Conference on Biotechnology for Environment and Health (ICBEH) and 15th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) and 25th to 27th November 2021.
4. International Seminar on Recent Advances in Science and Technology (ISRAST) and 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST), 16th -18th November 2020.
5. International Conferences of Recent Advances in Animal Sciences (ICRAAS) organized by the Department of Zoology, Pachhunga University College, November 6-8, 2019.

National

1. National symposium on “Avian Biology & Comparative Physiology” organized by the Department of Zoology, Mizoram University on 22nd – 24th October 2018.

State Level

1. State-level seminar on ‘*Climate change adaptation and Disaster risk reduction*’ organized by Mizo Academy of Sciences (MAS) in collaboration with the Department of Environment, Forest & Climate change, Climate change cell of MISTIC and the Department of Disaster management and rehabilitation, Gov’t of Mizoram on 3rd November, 2017

WORKSHOPS AND TRAINING ATTENDED

1. DBT-Sponsored workshop on ***“Biostatistics in Biomedical Research”***, organized by DBT Advanced State Biotech Hub, Mizoram University from 17th to 18th December 2024.
2. DBT-Sponsored workshop on ***“Ensuring safety in drug development: A workshop on Drug discovery and pharmacovigilance”***, organized by DBT Advanced State Biotech Hub, Mizoram University from 9th to 10th December 2024.
3. DBT-Sponsored training on ***“Hands-On workshop on Real-Time PCR and Droplet Digital PCR”***, organized by DBT Advanced State Biotech Hub, Mizoram University and BioRad Laboratories from 24th to 25th September 2024.
4. SERB – Karyashala sponsored High-end hands-on workshop on ***“Diagnostic and Translational Research using miRNA (Non-coding RNA) in Human Induced Pluripotent Stem Cells (HiPSCs) for Personalized Medicine”***, Department of Zoology, Central University of Punjab, Bathinda from 11th to 20th March, 2024.
5. State Level Two Day Training on ***‘Impact of Forest Fire on Flora & Fauna’*** organised by Zoological Survey of India, on 29th – 30th April, 2019 at Aizawl.
6. Training on ***‘Animal Handling, Maintenance & Care’*** organized by Department of Zoology, Mizoram University on 26th March, 2018

PAPERS PRESENTED IN CONFERENCES, SEMINARS, AND SYMPOSIUMS

1. **F. Nghakliana**, Zothansiam (2024) “*Identification of novel HSP90 inhibitors from Elaeagnus caudata for targeting colorectal carcinoma and non-small cell lung adenocarcinoma using network pharmacology and molecular dynamic simulation*” International Conference on ‘Bioeconomy from Bioresources: Promoting Traditional Resources of NER for Viksit Bharat’ and 11th Convention of Society for Ethnopharmacology, India, Gangtok, Sikkim, 15th – 16th November 2024.
2. **F. Nghakliana**, C. Lalmuansangi, Mary Zosangzuali, Marina Lalremruati, Zothansiam (2022) “*Methanolic extract of Elaeagnus caudata (Sarzukpui) induces caspase-mediated apoptosis in human adenocarcinomic basal epithelial cells, A549*” Asia-Pacific Organisation for Cancer Prevention (APOCP), Kolkata, India, 8th to 10th December 2022.
3. **F. Nghakliana**, C. Lalmuansangi, Mary Zosangzuali, Marina Lalremruati, Zothansiam (2021) “*In vitro antioxidative and anticancer activity of Silverberry, Elaeagnus caudata Schltl. against Type II Human Lung Adenocarcinoma cells, A549 via alteration of redox homeostasis*” 15th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) and International Conference on Biotechnology for Environment and Health (ICBEH), 25th to 27th November 2021
4. **F. Nghakliana**, Zothansiam (2020) “*Evaluation of the in-vitro free radical scavenging activities and ex-vivo antioxidative potential of Silverberry, Elaeagnus caudata Schltl.*” 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) and International Seminar on Recent Advances in Science and Technology (ISRAST), 16th -18th November 2020.

5. **F. Nghakliana**, Jacqueline Lalbiakhlui Fanai, Lucy Lalmuanpuii, Zothansiam (2019) “*Phytochemical composition, anti-oxidative potential and anti-cancer activity of Callicarpa arborea Roxb. Against Human Lung Adenocarcinoma (A549)*” International Conference on Recent Advances in Animal Sciences (ICRAAS-2019), November 6-8, 2019, Seminar Hall, Pachhunga University College, Aizawl, Mizoram, India.

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1. **F. Nghakliana**, L. Tochhawng, H. Sailo, L. Ralte, F. Lalsangpuii, M. Iyer, V. Palanivel, B. Vellingiri, K.R. Kharat, Zothansiam (2025). *In Vitro* Evaluation of Anticancer and Apoptotic Activity of *Elaeagnus caudata* Schltdl. with computational identification of a novel HSP90 inhibitor. *Chemistry & Biodiversity*, e00679
2. C. Lalmuansangi, Lalfakawmi, **F. Nghakliana**, Hmingremhlua Sailo, Lalchhandami Tochhawng, Amit Kumar Trivedi, Kiran R. Kharat, Balachandar Vellingiri, Nachimuthu Senthil Kumar, Zothansiam (2025), Anticancer activity of *Stemona tuberosa* (wild asparagus) against type-II human lung adenocarcinoma (A549) cells and identification of SRC inhibitor using integrated network pharmacology and molecular dynamic simulation. *Discover Oncology*. 16, 429.
3. F. Lalsangpuii, Samuel Lalthazuala Rokhum, **F. Nghakliana**, VL Ruatpuia, Lalchhandami Tochhawng, Amit Kumar Trivedi, R. Lalfakzuala, Zothansiam (2024). *Mikania micrantha* silver nanoparticles exhibit anticancer activities against human lung adenocarcinoma via caspase-mediated apoptotic cell death. *Artificial Cells, Nanomedicine, and Biotechnology*. 52(1):186-200.
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2. Mary Zosangzuali, Marina Lalremruati, C. Lalmuansangi, **F. Nghakliana** and Zothansiamia (2023). *Pharmacological importance and chemical composition of Mallotus roxburghianus Muell.Arg.* Pullaiah, T (Eds). Phytochemistry and Pharmacology of Medicinal Plants Volume 2, Pg. 413-421, Apple Academic Press.
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5. **F. Nghakliana** and Zothansiamia (2020). Qualitative Disorders. J. Vonk, T. K. Shackelford (eds.), *Encyclopedia of Animal Cognition and Behavior*. Springer Nature Switzerland AG.

PARTICULARS OF THE CANDIDATE

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ABSTRACT

BIOASSAY-GUIDED ISOLATION OF THE ANTI-NEOPLASTIC COMPOUND FROM *ELAEAGNUS* *CAUDATA* SCHLTDL.

**AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

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**DEPARTMENT OF ZOOLOGY
SCHOOL OF LIFE SCIENCES**

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ABSTRACT

BIOASSAY-GUIDED ISOLATION OF THE ANTI-NEOPLASTIC
COMPOUND FROM *ELAEAGNUS CAUDATA* SCHLTDL.

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In partial fulfillment of the requirements of the degree of Doctor of
Philosophy in Zoology of Mizoram University, Aizawl

CONSOLIDATED ABSTRACT

Despite continual progress in theranostic, cancer persists as a significant health issue, with 19.9 million cases and 9.7 million fatalities globally in 2022. The burden is further intensified by the anticipated 47% increase in new cancer cases by 2040. Despite recent advancements in cancer prognosis attributable to radical therapies such as organ transplantation, chemotherapy, radiation, immunotherapy, and surgical resection, cancer treatment continues to be impeded by the non-specificity and harmful side effects of these treatments. Moreover, cancer treatments have persistently encountered significant challenges due to the emergence of resistance and recurrence in many patients. Consequently, despite all these significant progresses in cancer therapy, the limitations of current conventional treatments persistently drive the quest for more targeted and less hazardous therapeutic agents and methodologies. Among the numerous compounds assessed for anticancer properties, natural compounds from medicinal plants appear most promising due to their safety, efficacy, and reduced side effects relative to synthetic medications. Historically, medicinal plants have been primary sources for anticancer drug discovery, with plant-derived compounds such as vinblastine, vincristine, etoposide, paclitaxel, docetaxel, topotecan, and irinotecan being among the most effective chemotherapeutics available for cancer treatment in modern medicine. Therefore, this study was oriented towards the identification and isolation of a natural compound with promising prospects for future anticancer therapy from the unexplored medicinal plant *Elaeagnus caudata*. **Chapter 1** describes the preparation and extraction of *E. caudata* using various solvents using Soxhlet apparatus followed by the estimation of their phytochemical composition and their antioxidative potential. In **Chapter 2**, we performed the preliminary investigation of the *in vitro* anticancer and pro-apoptotic activity of *E. caudata* extracts against human non-small cell lung carcinoma cell line A549. **Chapter 3** emphasizes the fractionation of the methanolic extract of *E. caudata* and the comprehensive investigation of the *in vitro* anticancer and pro-apoptotic potential of the most potent fraction against A549 and human colorectal carcinoma cell line HCT116 followed by *in silico* analyses of molecular targets of the selected fraction. **Chapter 4** then focuses on the isolation and identification of a lead compound from the selected fraction using a bioassay-guided

approach followed by the in-depth *in vitro* study of the anticancer and apoptotic potential of the isolated compound against the HCT116 cell. Finally, **Chapter 5** employs various *in silico* tools to identify the molecular targets and signalling pathways involved in the anticancer and pro-apoptotic activity of the isolated compound.

Chapter 1: Preparation of *Elaeagnus caudata* extracts, estimation of their phytochemical content, and free radical scavenging activity.

Elaeagnus caudata, a medicinal plant traditionally used in Mizo folk medicine, was investigated for its phytochemical composition and antioxidative properties using various *in vitro* and *ex vivo* assays. Methanolic and aqueous extracts were prepared via Soxhlet extraction, and their phytochemical content were analysed using standard protocols. The methanolic extract exhibited a higher extraction yield (22.24%) than the aqueous extract (18.35%). The quantitative phytochemical analysis revealed significantly higher total phenolic content in the aqueous extract (259.32 ± 5.18 mg GAE/g) than in the methanolic extract (157.92 ± 4.1 mg GAE/g). Likewise, the flavonoid content was also higher in the aqueous extract (1869.25 ± 25.9 mg QE/g) than in the methanolic extract (1000 ± 12.93 mg QE/g). The antioxidative potential of *E. caudata* was also assessed using various assays. The aqueous extract possessed higher scavenging activities than the methanolic extract for DPPH, superoxide anions and ABTS^{•+} radicals with an IC₅₀ of 61.48 ± 1.06 µg/ml, 335.7 ± 0.61 µg/ml, and 372.2 ± 16.17 µg/ml, respectively. Consistently, the aqueous extract was also found to exhibit higher ferric-reducing power than the methanolic extract and the standard ascorbic acid. *Ex vivo* assays further validated the antioxidative effects. Anti-haemolytic activity showed the aqueous extract inhibiting 80.77% of haemolysis, significantly higher than the methanolic extract (43.48%). Lipid peroxidation inhibition in liver homogenates demonstrated an inhibition rate of 81.58% for the aqueous extract and 61.63% for the methanolic extract. These findings indicate that *E. caudata* possesses potent antioxidant properties, likely attributed to its rich phenolic and flavonoid content. The study highlights its potential as a source of natural antioxidants for pharmaceutical and nutraceutical applications. Therefore, further research is warranted to explore its bioactive compounds and other pharmacological significance of *E. caudata*.

Chapter 2: Preliminary screening of the anticancer activity of *Elaeagnus caudata*.

Elaeagnus caudata has been extensively used to treat various ailments including cancer in the traditional medicine of North-East India. Additionally, several bioactive compounds with potent anticancer properties have been isolated from other species of the *Elaeagnus* genus, suggesting that *E. caudata* may also contain similar or novel anticancer compounds. Therefore, a preliminary study was conducted to determine the anticancer activity of *E. caudata* using various *in vitro* assays. MTT assay revealed a dose and time-dependent cytotoxic effect of *E. caudata* extract with the methanolic extract (EC-ME) possessing stronger cytotoxicity as compared to the aqueous extract. Fluorescent AO/EtBr staining confirmed apoptotic induction, with EC-ME-treated cells exhibiting a significantly higher apoptotic index (86.7%) compared to the untreated control (1.2%). Antioxidant assays demonstrated a significant increase in the level of glutathione (GSH) and activities of glutathione S-transferase (GST), and superoxide dismutase (SOD) levels, while lipid peroxidation, measured via malondialdehyde (MDA) level, was significantly reduced. The genotoxic potential of EC-ME was also assessed using the Comet assay, which showed increased DNA damage, indicated by enhanced comet tail length and olive tail moment. Further, caspase 6 activity was significantly elevated (2.3 fold increase) in EC-ME-treated cells, confirming apoptotic cell death. As a preliminary study, these findings suggest that *E. caudata* exhibits promising anticancer properties through induction of apoptosis, modulation of antioxidant levels, and induction of DNA damage in A549 cells. However, further in-depth studies, including compound isolation are necessary to fully elucidate its therapeutic potential and mechanisms of action.

Chapter 3: Bioassay-guided fractionation of crude extract of *Elaeagnus caudata* and anticancer activity of the active fraction.

Despite its extensive traditional use in treating various illnesses, including cancer, *E. caudata* remains neglected and relatively unexplored in terms of its pharmacological potential. The methanolic extract of *E. caudata* leaves was fractionated using column chromatography, yielding 17 fractions. The B2 fraction was identified as the most cytotoxic, with the lowest IC₅₀ against A549 and HCT116 cells. A higher apoptotic index further supported the B2 fraction activities, increased caspase 3/6 activity, DNA damage, and elevated lipid peroxidation with decreasing antioxidant levels. LC-HRMS analysis revealed 12 major compounds in the B2 fraction, with Amorphigenin emerging as a promising drug candidate based on the SwissADME and pkCSM analysis. Network pharmacology identified HSP90 as the primary target of Amorphigenin, with a binding affinity of -11.11 kcal/mol, which is stronger than the control Geldanamycin. Molecular dynamic simulation reveals an average RMSD of 1.0 nm and lower RMSF value with the generation of a significant amount of hydrogen bonds indicating a more stable binding conformation of the Amorphigenin-HSP90 complex than with the control. The Amorphigenin complex also exhibited elevated SASA values than the control oscillating between 110 and 115 nm². The gmx_MMPBSA calculation reveals the binding energy of the complex varies between -35 and -17 kcal/mol, with an average of about -25 kcal/mol. Thus, our findings suggest that *E. caudata* and its bioactive compounds, particularly Amorphigenin, hold potential for the development of novel targeted therapies for non-small cell lung carcinoma and colorectal cancer.

Chapter 4: Isolation, identification, and anticancer activity of the lead compound of *Elaeagnus caudata*

Natural products have long been integral to the discovery of novel therapeutic agents, particularly in oncology, where plant-derived natural compounds contribute significantly to chemotherapeutic development. This study focuses on the isolation of a novel compound from *Elaeagnus caudata* which may hold promise for a new anticancer chemotherapy. A flavonoid, 6,2'-Dihydroxyflavone (MW 254.24 g/mol) was isolated from the methanolic extract of *E. caudata* guided by bioassay and confirmed using FTIR, CHNS(O) analysis, LC-HRMS, and ^1H and ^{13}C NMR. MTT assay revealed that 6,2'-Dihydroxyflavone inhibited HCT116 cell proliferation in a dose- and time-dependent manner, significantly superior to 5-fluorouracil (5-FU) in efficacy at higher concentrations. The mode of cellular death via apoptosis was observed via increased apoptotic index as evident from the AO/EtBr dual staining, increased DNA damage and confirmed by the increased Caspase 3 and 6 activities, upregulation of pro-apoptotic genes and subsequent downregulation of anti-apoptotic genes. Oxidative stress markers were also evaluated, demonstrating a decrease in glutathione (GSH) levels and antioxidant enzyme activities, including glutathione S-transferase (GST) and superoxide dismutase (SOD). Conversely, lipid peroxidation (MDA levels) increased significantly, suggesting a redox imbalance contributing to apoptosis. These findings suggest that 6,2'-Dihydroxyflavone from *E. caudata* exhibits significant cytotoxic and pro-apoptotic properties against HCT116 cells via oxidative stress-mediated apoptosis and modulation of apoptotic gene expression. The compound's efficacy in comparison to 5-FU highlights its potential as a novel anticancer agent. Further studies, including *in vivo* evaluations and insight into its mechanism, are warranted to explore its therapeutic applications in cancer treatment.

Chapter 5: Identification of molecular targets of a lead compound in colorectal carcinoma using integrated network pharmacology and Molecular dynamic simulation

Colorectal carcinoma remains a leading cause of cancer-related mortality worldwide, often compounded by chemoresistance and adverse effects from conventional therapies. This chapter employed an integrated *in silico* approach combining network pharmacology, molecular docking, and molecular dynamics (MD) simulations to investigate the anticancer potential of 6,2'-Dihydroxyflavone, a flavonoid isolated from *E. caudata*, against colorectal carcinoma. Target prediction and gene-disease association studies identified 98 common targets between 6,2'-Dihydroxyflavone and colorectal carcinoma. Protein-protein interaction (PPI) network analysis revealed critical hub genes, including EGFR, ESR1, PTGS2, and SRC, with EGFR emerging as the central target. Gene Ontology and KEGG pathway enrichment analyses indicated that 6,2'-Dihydroxyflavone potentially modulates several cancer-related pathways, particularly the ErbB, Estrogen, Prolactin, and Relaxin signalling pathways, which play vital roles in tumour progression, metastasis, and therapeutic resistance. Molecular docking analysis confirmed a strong interaction between 6,2'-Dihydroxyflavone and EGFR, with a binding energy of -9.21 kcal/mol, supporting its potential as an EGFR inhibitor. Comparative docking with Afatinib, a known EGFR inhibitor, revealed a slightly higher binding affinity for Afatinib. Subsequent 100-nanosecond MD simulations validated the stability of the 6,2'-Dihydroxyflavone-EGFR complex, with root mean square deviation (RMSD) and hydrogen bonding analysis confirming sustained interactions. Although 6,2'-Dihydroxyflavone exhibited greater flexibility and solvent exposure than Afatinib, binding free energy calculations (GMX_MMPBSA) demonstrated favourable energetics, supporting stable complex formation. Overall, this study suggests that 6, 2'-Dihydroxyflavone exerts anticancer effects by targeting EGFR and disrupting critical oncogenic signalling pathways in colorectal carcinoma. These findings highlight the therapeutic potential of 6,2'-Dihydroxyflavone as a promising candidate for colorectal cancer treatment targeting the EGFR pathway, meriting further experimental validation through *in vitro*, *in vivo*, and clinical studies.