

## DECLARATION

I, Heisanam Pushparani Devi, hereby declare that the thesis entitled “Antimutagenic and antigenotoxic activity of *Curcuma caesia* Roxb. against Cyclophosphamide” submitted to Assam University, Silchar for the degree of Doctor of Philosophy (Ph.D) has been done by me under the supervision of Professor Pranab Behari Mazumder, Department of Biotechnology, Assam University, Silchar and not submitted elsewhere for the award of any other degree.

Date:

Heisanam Pushparani Devi

Place:

## ACKNOWLEDGEMENT

First and foremost I bow my head as a mark of giving thanks to **Almighty God** who has been with me all along the way who gave everything to complete this work successfully.

I express my deep sense of gratitude to my respected supervisor Professor **Pranab Behari Mazumder** for guiding me insightfully. His inspiring and constructive criticism infused in me the spirit of hard work. I always feel proud and grateful to be associated with him.

I am fortunate enough to have had the seniors like Yumnam Romila Devi, Samim Ahmad Shah, Leimapokpam Shivadutta Singh, Laishram Priyadarshini Devi for their valuable guidance and suggestions when needed. Without them I could not have completed my thesis successfully.

Friends in need are friends indeed and I have no words to show my heartily gratitude towards my beloved friends Bisworjit N, Sarmistha Dey, Udaykumar Vandana, R.K. Suraj, Chandani, Anil Seram, Mohindro L, who all are my inspiration.

I extend my deep sense of gratitude to all the teaching, non teaching Staffs and all the research scholars of the Department of Biotechnology, Assam University, Silchar for their help and support during the study period.

It is my pleasure to express my sincere thanks to IMTECH, Chandigarh for providing me the specific strains of bacteria whenever I required.

I take this opportunity to thank Pasteur Institute, Shillong for providing me the animals whenever I required for my studies.

Above all, I owe the credit of this work to my family members: my Father: (Late) Heisanam Shyamsunder Singh, my Mother: Heisanam Pati Devi, my Sister: Heisanam Romibala Devi, my Brothers: Heisanam Sanjay Singh and Heisanam

Premananda Singh, Husband: Chingangbam Umashankar Singh, my Brother in law:  
Oinam Ajitkumar Singh, my Nephew: Oinam Royston Singh, my Uncle: Khundrakpam  
Debendro Singh and other relatives for their moral support, love, prayers, inspirations  
and constant encouragement throughout....

Heisanam Pushparani Devi

SL NO	CONTENTS	PAGE NO.
CHAPTER I	INTRODUCTION	1-7
CHAPTER II	REVIEW OF LITERATURE	9-33
2.1	Source of Oxidative stress and its implications	9-10
2.2	Lifestyle pattern as risk factors	11-12
2.3	Implications of oxidative stress	12
2.3.1	Oxidative DNA damage	12
2.3.2	Oxidative protein damage	12
2.3.3	Oxidative lipid damage	13
2.4	Mechanism of cancer Development and cancer progression	13
2.5	Chemoprevention by synthetic antioxidants and its side effects	13-14
2.6	Antimutagens	14
2.7	Preparation of plant samples for extraction	15
2.8	Extraction and choice of solvents	16
2.9	Antioxidant and Antimutagenic activity of plant extracts	19-20
2.10	In Vitro models for studying Antigenotoxicity activity	20-24
2.11	Plant secondary metabolites and its relationship to cytotoxic activities	24-28
2.12	Terpenes and antioxidant activity	28-32
2.13	Chemical constituents of <i>Curcuma caesia</i> Roxb.	32-33
2.14	Biological activities of <i>Curcuma caesia</i> Roxb.	33

CHAPTER III	MATERIALS AND METHODS	34-63
3.1	Materials	34
3.2	Chemicals and Reagents	34-37
3.3	Plant Material Collection and Extraction	37
3.3.1	Extraction	38
3.3.2	Percentage Yield	39
3.4	Preliminary phytochemical screening	39
3.4.1	Test for alkaloids	39
3.4.1.1	Dragendroff's test	39
3.4.1.2	Mayer's test	39
3.4.1.3	Hager's test	39
3.4.1.4	Wagner's test	40
3.4.2	Test for carbohydrates	40
3.4.3	Test for steroids	40
3.4.4	Test for Flavanoids	40
3.4.4.1	Aqueous NaOH test	40
3.4.4.2	Concentrated H <sub>2</sub> SO <sub>4</sub> test	41
3.4.4.3	Schinoda's test	41
3.4.5	Detection of Phenolic compounds	41
3.4.5.1	Ferric chloride test	41
3.4.5.2	Lead acetate test	41
3.4.6	Detection of Saponins	41
3.4.7	Test for Soluble Starch	41
3.4.8	Test for Reducing Sugars	41
3.4.8.1.	Fehling's Test for free reducing sugar	41
3.4.8.2	Fehlings test for combined reducing sugar	42
3.4.9	Test for Tannins	42
3.4.10	Test for terpenoids	42
3.5	Determination of total phenol contents in the	42

	plant extracts (Principle)	
3.5.1	Procedure	42-43
3.6	In Vitro Antioxidant Studies	43
3.6.1	DPPH radical scavenging activity (Principle)	43
3.6.1.1	Procedure	44
3.6.2	Reducing Power Assay (Principle)	44
3.6.2.1	Procedure	45
3.6.3	ABTS Radical Cation Decolourisation Assay (Principle)	45
3.6.3.1	Procedure	45-46
3.6.4	Superoxide anion radical scavenging assay (Principle)	46
3.6.4.1	Procedure	46-47
3.7	Antimutagenicity Assay (Principle)	47-48
3.7.1	Bacterial strains	48
3.7.2	S9 Preparation	49
3.7.3	Salmonella-microsome assay	49
3.7.4	Antimutagenicity testing	49-50
3.7.5	Statistical analysis	50
3.8	Acute Toxicity Study	50-51
3.9	Antigenotoxicity study	51
3.9.1	Experimental Design	51-52
3.9.2	Micronucleus assay (Principle)	52-53
3.9.2.1	Procedure	53
3.10	Biochemical Analysis	54
3.10.1	Serum Sample Collection	54
3.10.2	Determination of serum SGOT and SGPT (Principle)	54-55
3.10.2.1	Procedure	55

3.10.3	Quantitative assay for lipid peroxidation (Principle)	55-56
3.10.3.1	Procedure	56
3.10.4	Estimation of GSH level (Principle)	57
3.10.4.1	Procedure	57
3.10.5	Estimation of Glutathione Reductase (Principle)	57-58
3.10.5.1	Procedure	58
3.10.6	Estimation of protein concentration (Principle)	58-59
3.10.6.1	Procedure	59
3.11	Statistical analysis	59
3.12	Histopathological examination	59
3.13	Determination of cytotoxicity of the extracts	60
3.13.1	Cell lines and culture preparation	60
3.13.2	Procedure	61
3.14	GC-MS analysis (Principle)	62
3.14.1	Procedure	62
3.15	UV-Visible analysis	62-63
CHAPTER IV	RESULTS	64-119
4.1	Yield of <i>Curcuma caesia</i> Roxb. rhizome extracts	64
4.2	Preliminary Phytochemical Screening	64
4.3	Determination of total phenol contents in the plant extracts	66
4.4	Reducing power Assay	67
4.5	In Vitro Antioxidant Studies	68
4.5.1	DPPH Radical Scavenging Assay	68-69
4.5.2	ABTS <sup>+</sup> Radical Scavenging Activity	70
4.5.3	Superoxide Anion Radical Scavenging Assay	72-73
4.6	Antimutagenicity Assay	75-78

4.7	Acute toxicity studies	82
4.8	Antigenotoxicity Assay	83
4.9	Biochemical Analysis	87
4.9.1	SGOT and SGPT Analysis	87-88
4.9.2	Lipid Peroxidation and GSH Assay in the liver	89-90
4.9.3	GR and Protein content in the liver of mice	92
4.9.4	Lipid Peroxidation and GSH Assay in the Kidney of mice	94
4.9.5	GR and Protein Content in the kidney of mice	97
4.10	Histopathological Analysis	100
4.10.1	Histopathological Analysis of Liver	100
4.10.2	Histopathological analysis of kidney	104
4.11	Cytotoxicity Assay of the extracts against cancer cell lines	106
4.11.1	Cytotoxicity activity of the extracts against breast cancer cell lines MDAMB231	106-107
4.11.2	Cytotoxicity activity of the extracts against lung cancer cell lines Calu6	109
4.12	GC-MS analysis of the extracts	111
4.12.1	GC-MS analysis of EaECC	111
4.12.2	GC-MS Analysis of EECC	112
4.13	UV-Visible Spectrum of EaECC and EECC	117
4.13.1	UV-visible spectrum of EaECC of <i>Curcuma caesia</i> Roxb.	118
4.13.2	UV-Visible peak values of Ethanolic extract of <i>Curcuma caesia</i> Roxb.	119
CHAPTER V	DISCUSSION	122-136
5.1	In Vitro Antioxidant Studies	122
5.1.1	DPPH radical scavenging assay, Total phenol	122



	contents of the extracts	
5.1.2	Reducing power assay of the extracts	123
5.1.3	ABTS <sup>+</sup> radical scavenging activity	124
5.1.4	Superoxide Anion Radical Scavenging Assay	125
5.2	Antimutagenicity Assay	125-126
5.3	Acute toxicity studies	126-127
5.4	Antigenotoxicity Assay	128-129
5.5	Biochemical Analysis	131
5.5.1	SGOT and SGPT Analysis	131
5.5.2	Lipid Peroxidation Assay in both liver and kidney of mice	131-132
5.5.3	GSH, GR and Protein Assay in both liver and kidney of mice	132
5.6	Histopathological Analysis	132
5.6.1	Histopathological Analysis of liver	132
5.6.2	Histopathological Analysis of kidney	133
5.7	Cytotoxicity Assay of the extracts against cancer cell lines	134
5.8	GC-MS and UV-Visible Spectral Analysis of EaECC and EECC	135-136
CHAPTER VI	SUMMARY AND CONCLUSION	137-140
CHAPTER VII	REFERNCES	141-182

## LIST OF TABLES

Table No.	Description	Page No.
1	Solvents used in the extraction of active Compounds	17
2	Solvents used for active component extraction	18
3	Study of acute toxicity of rhizome extracts of <i>C. caesia</i> Roxb.	51
4	Yield of the extracts	64
5	Preliminary Phytochemical Screening	65-66
6	Absorbance of each extracts to find the total Phenol content	66
7	Total phenolics in the studied extracts	67
8	Reducing power Assay	68
9	DPPH radical scavenging activity	69
10	ABTS <sup>+</sup> radical scavenging activity of standard and EaECC extract of <i>Curcuma caesia</i> Roxb	71
11	ABTS <sup>+</sup> radical scavenging activity of EECC, MECC and AECC of <i>Curcuma caesia</i> Roxb.	71
12	Superoxide anion scavenging ability of Standard and Ethyl acetate extract of <i>Curcuma caesia</i> Roxb.	73
13	Superoxide anion scavenging ability of Standard and Ethanol, Methanol and Aqueous extract of <i>Curcuma caesia</i> Roxb.	74
14	Number of his <sup>+</sup> revertants in <i>Salmonella typhimurium</i> strains produced by <i>Curcuma caesia</i> Roxb. extracts against Cyclophosphamide	76-77
15	General behavior of animals	82
16	Mortality Observation (Acute Toxicity Study)	82
17	Body weight	83
18	The effect of treatment with EaECC on the micronuclei induced by CP in bone marrow cells of mice	84
19	The effect of treatment with EECC on the micronuclei induced by CP in bone marrow cells of mice	84
20	The effect of treatment with MECC on the micronuclei induced by CP in bone marrow cells of mice	85

21	SGOT and SGPT analysis	89
22	Lipid Peroxidation and GSH Assay in the liver	90
23	Level of GR and protein in the liver of CP treated and extract treated mice	93
24	Level of LPO and GSH in the kidney of CP treated and extract treated mice	95
25	Level of GR and protein in the kidney of CP and extracts treated mice	98
26	Cytotoxic activity of <i>C.caesia</i> extracts in MDAMB 231 cell lines	108
27	Cytotoxic activity of <i>C.ceasia</i> extracts in Calu 6 cell lines	110
28	GC-MS analysis of Ethyl acetate extract of <i>Curcuma caesia</i> Roxb.	113-114
29	GC-MS analysis of Ethanolic extract of <i>Curcuma caesia</i> Roxb.	115-117
30	UV Visible peak values of ethyl acetate extract of <i>Curcuma caesia</i> Roxb.	118
31	UV-Visible peak values of Ethanolic extract of <i>Curcuma caesia</i> Roxb.	119

## LIST OF FIGURES

Fig No.	Description	Page No.
1	Common source of ROS/RNS	11
2	Mechanisms of action of antimutagens	15
3	Extraction with Soxhlet apparatus	16
4	Schematic Representation of Successive extraction of the rhizomes of <i>Curcuma caesia</i> Roxb.	38
5	Mechanism of action of rhizome extracts in DPPH Assay	43
6	Mechanism of action of Polyphenols in reducing power assay	44
7	Scavenging of ABTS radical cation by the antioxidants	45
8	Schematic overview of Histidine biosynthesis in <i>Salmonella typhimurium</i>	48
9	Schematic representation of mice micronucleus assay and biochemical analysis	54
10	Reaction Catalyze by the SGPT	55
11	Reaction Catalyze by SGOT	55
12	Mechanism of peroxy/ alkoxyl radicals leading to the damage of lipids	56
13	Mechanism of Glutathione Reductase	58
14	Conversion of MTT into Formazan by mitochondrial enzyme	60
15	Cancer Cells growing in T-25 Cm2 Flask	61
16	Standard curve for Gallic acid to find out the total Phenol content	68
17	Reducing power assay of <i>C. Caesia</i> Roxb extracts	68
18	DPPH Assay of <i>Curcuma caesia</i> Roxb. rhizome extracts	70
19	ABTS <sup>+</sup> Assay for four rhizome extracts of <i>Curcuma caesia</i> Roxb.	72
20	Superoxide anion scavenging assay of the <i>C. caesia</i> rhizome extracts	75

21	Antimutagenic activity of all extracts in TA 98 in the absence of S9	78
22	Antimutagenic activity of all extracts in TA 100 in the absence of S9	79
23	Antimutagenic activity of all extracts in TA 98 in the presence of S9	79
24	Antimutagenic activity of all extracts in TA 100 in the presence of S9	80
25	Bacterial colonies in the petriplates	80-81
26	Micronuclei and Normal cells	86
27	Comparison of micronuclei formation in all extracts	87
28	Standard calibration curve for SGOT and SGPT	88
29	Lipid peroxidation in the liver of CP and extract treated mice	91
30	Level of GSH in the liver of CP treated and extract treated mice	91
31	Level of GR in liver	92
32	Level of protein in liver	94
33	Level of LPO in the kidney of CP treated and extract treated mice	96
34	Level of GSH in the kidney of CP treated and extract treated mice	96
35	Level of GR in the kidney of CP treated and extract treated mice	97
36	Level of protein in the kidney of CP treated and extract treated mice	99
37	Caliberation curve for standard BSA for the estimation of protein content in liver and kidney	99
38-40.	Histopathological analysis of liver	101-103
41-43.	Histopathological analysis of kidney	104-106
44.	Cytotoxic activity of <i>C.caesia</i> Roxb extracts against MDAMB 231 cell lines	109

45.	Cytotoxic activity of <i>C.caesia</i> Roxb extracts against Calu6 cell lines	111
46-47.	GC-MS chromatogram of EaECC and EECC	112
48-49.	UV-Visible spectrum of EaECC and EECC	118,120

## LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromole
nM	Nanomole
mM	Millimolar
mL	Mililitre
°C	Degree Celsius
hr	Hour
g	Gram
kg	Kilogram
b.wt	Body weight
M	Molar
rpm	Revolution per minute
ABTS	2, 2'-Azinobis (3-Ethylbenzthiazoline-6-Sulfonate)
AECC	Aqueous Extract of <i>Curcuma caesia</i> Roxb.
AUS	Assam University Silchar
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
<i>C.caesia</i> Roxb.	<i>Curcuma caesia</i> Roxb.
CFU	Colony Forming Unit
Conc.HCl	Concentrated Hydrochloric acid
Conc.H <sub>2</sub> SO <sub>4</sub>	Concentrated Sulphuric acid
CP	Cyclophosphamide
CuII Sulphate Pentahydrate	Copper(II) Sulphate Pentahydrate

Dil.HCl	Dilute Hydrochloric acid
DMSO	Dimethyl Sulphoxide
DPPH	(2, 2-Diphenyl-1-picryl hydrazyl)
DTNB	5, 5'-Dithiobis-(2-Nitrobenzoic Acid)
EaECC	Ethyl Acetate Extract of <i>Curcuma caesia</i> Roxb.
EDTA	Ethylene Diamine Tetra Acetic acid
EECC	Ethanolic Extract of <i>Curcuma caesia</i> Roxb
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FeCl <sub>3</sub>	Ferric Chloride
GAE	Gallic Acid Equivalent
GR	Glutathione Reductase
GSSG	Oxidized Glutathione
GSH	Reduced Glutathione
IARC	International Agency for Research Centre
IMTECH	Institute of Microbial Technology
IEC	International Ethics Committee
KCl	Potassium Chloride
KOH	Potassium Hydroxide
MgCl <sub>2</sub>	Magnesium Chloride
MDA	Malondialdehyde
MECC	Methanolic Extract of <i>Curcuma caesia</i> Roxb.
MTT bromide]	[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NADP	Nicotinamide Adenine Dinucleotide Phosphate



NaHCO <sub>3</sub>	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NaN <sub>3</sub>	Sodium Azide
NBT	Nitroblue Tetrazolium
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate Buffer Saline
PMS	Phenazine Methosulphate
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substance
TCA	Trichloroacetic Acid
US	United Staes
W HO	World Health Organisation