DECLARATION

I, **Laishram Priyadarshini Devi**, hereby declare that the thesis entitled **"Phytochemical screening and molecular characterization of anti-hyperglycemic plant *Cassia alata* Linn."** submitted to Assam University, Silchar for the degree of Doctor of Philosophy (Ph.D) is the findings of my own research work carried out under the joint supervision of Dr. P.B. Mazumder and Dr. M.D. Choudhury. The results presented here have not been previously submitted to my University or Institution for any degree, deploma, and associate fellowship whatsoever.

Date: Laishram Priyadarshini Devi

Place: Assam University, Silchar

ACKNOWLEDGEMENT

This thesis represents a milestone in my academic life, which has been seen through completion with the tremendous support and encouragement from my well wishers. The thesis sounds incomplete without a word of thanks, so here is a pleasant task to express my thanks to those who contributed in many ways to the success of this study.

Special appreciation goes to my supervisor **Dr. P. B. Mazumder** for his sincere contribution towards shaping this thesis. His guidance, advice, supports and encouragement, as a mentor, during my doctoral research endeavour was beyond price and without which the work would not have been possible. I consider myself fortunate enough for trusting on me.

Similar profound gratitude goes to my co-supervisor **Dr. M. D. Choudhury**. His valuable observations and comments is the reason behind the establishment of better thesis. I specially thank him for encouraging me. His encouragements have further motivated my academic life.

I gratefully acknowledge (UGC), government of India, New Delhi, for the financial assistance in the form of **Rajeev Gandhi National Fellowship (RGNF)** for SC/ST scheme which strengthened me to perform my work at ease.

I owe a great deal of gratitude to **Dr. P. Kumar** (Department of Life Sciences, Manipur University) for the recommendation of the plant sample and for providing the long-established information related to the plant. I further extend my sincere gratitude to **Dr. Rajmahon** (Professor, Department of Chemistry, Manipur University), for lending his helping hand and allowing me to work in his laboratory for some part of my work.

I acknowledge **Pasteur Institute**, Shillong for providing me the required experimental mice from time to time. I would like to thank **Botanical Survey of India** (BSI), Shillong for the plant identification. I am thankful to **Skanda Life Sciences** Pvt Ltd and **Chromous Biotech** Pvt Ltd, Bangalore for their technical help.

It's my moment to specially acknowledge **Dr. Bonani Mazumder** Ma'am for her generosity, support and encouragement. Words fail me to express my gratitude for the homely feeling that she gave me at Silchar.

I extend my gratefulness to all the faculty members, technical staffs and research scholars of the Department of Biotechnology, Assam University, Silchar, for their kind support and co-operation.

Words are short to express my deep sense of gratitude to my lab-mates and friends who have shared together the sweet and bitter taste of PhD journey. I owe a great respect to Shamim Ahamad Shah, L. Shivadutta and R.K. Suraj for their moral support and encouragement. I sincerely thank my friends and labmates Komuha, Romila, Bijaya, Chandani, Pushparani, Biswajit, Anil, Mohindro, Ranjana, Jaya, Uday, Sharmista, Priyadarshani and Piyali who were always beside me during my hard/happy moments to motivate me.

With heart and soul, I acknowledge my Grandfather Late Laishram Yaimatombi for his heavenly blessings that showered upon me, my Grandmother Laishram Damayenti who have always blessed me and never forget to pray for me. With heart and soul, I further acknowledge my grandparents Late Moirangthem Achouba and Late Moirangthem Shakhi for their boundless love, care and heavenly blessings.

The present thesis is also the blessings showered upon me by my relatives. Special regards goes to my Uncles L. Kora, L.Ebohal, M. Ratan, L. Pishak, L. Gouramani, M. Sharat, S. Gyanil, M. Jiten, M. Gogen, M. Muhin, M, Shinghajit and M. Nanao. The same regards goes to my Aunties L. Leima, L. shakhi, M. Loidang, L. Memma, M. Khambi, M. Memcha, M. Bino, N. Memma, L. Jamuna, S. Amubi, M. Naobi and M. Appe. I further acknowledge the love and care from my cousin sisters K. Thaba, M. Sharda, M. Chaoba, M. Jini, L. Geetabali, L. Sarjubala, L. Geetarani, L. Madhubala, L. Priya and L. Nanao. I thank my brothers L. Pritamjit and L. Priyobarta for their love and help. I sincerely thank my cousin brothers L. Thabal, L. Meimu, K. Ebungomacha, M. Robit, M. Rajiv and L. Premchand.

Last but not least, with great love, I acknowledge my parents **Laishram Birbasu Singh** and **Laishram Memma Devi**. They are the root cause of my success. They strengthen me with prayers, bless me with love and encourage me with hope. Words fail me to express my love for them, so here I dedicate by thesis to my parents.

Laishram Priyadarshini Devi

CONTENTS

Sl. No.		Description	Page No.
1		**Introduction**	1-19
1.1.		Diabetes	1
1.2.		History of diabetes: discovery, description and treatment	1
	1.2.1.	Ancient records of clinical features of diabetes	1
	1.2.2.	Diagnostic period	2
	1.2.3.	Experimental road to discovery of insulin.	3
	1.2.4.	Chemical and structural identification of insulin	5
	1.2.5.	The birth of Human Insulin	6
	1.2.6.	Stem cell research: future insulin production	7
1.3.		Glucose homeostasis: Glucose metabolism and regulation	7
1.4.		Classification of diabetes	8
	1.4.1.	Type 1 Diabetes Mellitus	8
	1.4.2.	Type 2 Diabetes Mellitus	8
	1.4.3.	Other specific types of diabetes mellitus	9
	1.4.4.	Gestational diabetes mellitus	9
1.5.		Etiology and pathogenesis of Diabetes Mellitus	9
	1.5.1.	Etiology and pathogenesis of Type 1 Diabetes Mellitus	10
	1.5.2.	Etiology and pathogenesis of Type 2 Diabetes Mellitus	11
1.6.		Molecular genetics of Diabetes Mellitus	11
1.7.		Complications of diabetes	13
	1.7.1.	The microvascular complications	14
	1.7.2.	Macrovascular complications	15
1.8.		Prevalence of diabetes	15
1.9.		Current treatment approaches to diabetes mellitus: success and shortcomings	16

1.10.			Antihyperglycemic plants: Source for better antidiabetic drugs of tomorrow	18
1.11.			*Cassia alata* Linn.: An ethnomedicinal antihyperglycemic plant	18
	1.11.1.		Taxonomic hierarchy of Cassia alata Linn.	19
1.12.			Objectives of the work	19
2			Review of literature	20-56
2.1.			Significance and scope of the research area	20
2.3.			Theory and practical base literature reviews relevant to the research	20
	2.3.1.		Techniques of plant analysis	20
		2.3.1.1.	Botanical identification of the plant	21
		2.3.1.2.	Plant extracts preparation techniques	21
		2.3.1.3.	Choice of suitable extraction solvent and procedure	21
		2.3.1.4.	Methods of separation of phytochemicals	23
	2.3.2.		Antioxidant activity of an antidiabetic plant	25
	2.3.3.		In vitro models for assessing antidiabetic activity of a plant	30
	2.3.4.		Animal models in diabetes research	38
		2.3.4.1.	The ethics of animal research	38
		2.3.4.2.	OECD guidelines for toxicity test	38
		2.3.4.3.	Strain, age and sex of mice	38
		2.3.4.4.	Factors to consider during metabolic test	39
	2.3.5.		Induction of diabetes	40
	2.3.6.		In vivo metabolic test	42
	2.3.7.		Molecular characterization of medicinal plants	52
3			**Material and methods**	57-79
3.1.			Plant material	57
3.2.			Chemicals and reagents	57
3.3.			Plant sample/extract preparation	60
	3.3.1.		Successive solvent extraction of the plant leaves	61

3.4.			Preliminary phytochemical screening	62
3.5.			In vitro antioxidant activity assessment	64
	3.5.1.		DPPH free radical scavenging assay	64
	4.5.2.		Reducing power assay (RPA)	64
	3.5.3.		Ferric reducing/ antioxidant power (FRAP) assay	65
	3.5.4.		Nitric oxide radical scavenging assay (NRSA)	65
3.6.			Quantification of Total Phenolic Content (TPC)	66
3.7.			In vitro Antidiabetic activity assessment	66
	3.7.1.		α-Amylase inhibitory test:	66
	3.7.2.		α-glucosidase inhibitory test:	66
	3.7.3.		Insulin secretion test using MIN6 pancreatic β-cell line	67
3.8.			Experimental animals	67
3.9.			Toxicity test	68
3.10.			Induction of diabetes in mice	69
3.11.			Oral glucose tolerance test (OGTT)	69
3.12.			In-vivo antidiabetic activity assessment.	70
	3.12.1.		Study Design for in vivo antidiabetic activity assessment	70
	3.12.2.		Measurement of biochemical parameters	71
		3.12.2.1.	Estimation of serum triglyceride (TG)	71
		3.12.2.2.	Estimation of serum total cholesterol (TC)	72
		3.12.2.3.	Estimation of serum HDL-cholesterol	72
		3.12.2.4.	Serum total protein was estimated by Biuret method	73
		3.12.2.5.	Estimation of Serum Glutamic Oxaloacetic Transaminase (SGOT)	73
		3.12.2.6.	Estimation of Serum Glutamic Pyruvic Transaminase (SGPT)	73
		3.12.2.7.	Estimation of liver glycogen content	74
	3.12.3.		Histological studies	74

3.13.		GC-MS analysis of the extract	74
3.14.		Molecular characterization of the plant	75
	3.14.1.	Genomic DNA isolation	75
	3.14.2.	RAPD fingerprinting	76
3.15.		Statistical Analysis	77
3.16.		Graphical presentation of the methodology	78
4		**Results**	80-115
4.1.		Successive solvent extraction of the plant leaves	80
4.2.		Preliminary phytochemical screening	80
4.3.		Quantification of total polyphenols.	82
4.4.		Antioxidant activity assessment	83
	4.4.1.	DPPH free radical scavenging assay	83
	4.4.2.	Reducing power assay (RPA)	83
	4.4.3.	Ferric Reducing Antioxidant Power (FRAP) assay	85
	4.4.4.	Nitric oxide radical scavenging assay (NRSA)	86
	4.4.5.	Correlation between the DPPH activity, FRAP value, Reducing power activity, and NO radical scavenging activity with Total Phenolic Content of CAEE, CAME and CAAE.	88
4.5.		In vitro antidiabetic activity assessment	89
	4.5.1.	Effect of CAEE, CAME and CAAE on α-amylase activity	89
	4.5.2.	Effect of CAEE, CAME and CAAE on α-glucosidase activity	91
4.6.		Acute oral toxicity test of the extracts	93
4.7.		Oral Glucose Tolerance Test (OGTT)	93
	4.7.1.	Effect of CAEE, CAME and CAAE on OGTT in normal mice	93
	4.7.2.	Effect of CAEE, CAME and CAAE on OGTT in STZ induced diabetic mice	94
4.8.		In-vivo antidiabetic activity assessment of the	96

		extracts	
	4.8.1.	In vivo antidiabetic effect of extracts on fasting BGL in STZ induced diabetic mice	96
	4.8.2.	Lipid profile and total protein changes in serum showing the effect of extracts	98
	4.8.3.	Changes in SGOT and SGPT on serum and Hepatic glycogen in the liver tissue homogenate of normal and diabetic mice after 28 days of treatment with CAME, CAEE and CAAE	99
	4.8.4.	Histological studies	101
4.9.		Effects of CAME on secretory function of pancreatic MIN6 β-cells	103
4.10.		GC-MS profile study of CAME	104
4.11.		Molecular characterization of the plant	110
5		**Discussion**	115
6		**Summary and Conclusion**	130
6		**Reference**	136

LIST OF TABLES

Table No.	Description	Page No.
2.1	Polarity and chemical profiles of most of the common extraction solvents	22
2.1	Summary of rodent models of type 1 diabetes	41
4.1	Percentage yield of extracts	80
4.2	Result of preliminary phytochemical screening of CAEE, CAME and CAAE	81
4.3	Total Phenolic Content (TPC) of CAEE, CAME and CAAE.	82
4.4	Influence of different concentrations of CAEE, CAME, CAAE and Acarbose on α-amylase activity expressed in terms of %inhibition and IC_{50}	89
4.5	Influence of different concentrations of CAEE, CAME, CAAE and Acarbose on α-glucosidase activity expressed in terms of %inhibition and $IC_{50.}$	91
4.6	Effect of extracts on oral glucose tolerance test in normal group mice.	94
4.7	Effect of extracts on oral glucose tolerance test in STZ induced diabetic mice group.	95
4.8	*In vivo* antidiabetic effect of extracts on fasting BGL in STZ induced diabetic mice after 28 days of treatment.	96
4.9	Lipid profile and total protein changes in serum showing the effect of extracts.	98
4.10	Changes in SGOT and SGPT on serum and Hepatic glycogen in the liver tissue homogenate of normal and diabetic mice after 28 days of treatment with CAEE and CAAE	100
4.11	Phytochemicals identified in CAME by GC-MS.	106

LIST OF FIGURES

Figure No.	Description	Page No.
3.1	General scheme of extract preparation	60
3.2	Successive solvent extraction of the plant leaves using solvents of ascending polarity	61
3.3	Graphical presentation of the methodology (phase 1)	77
3.4	Graphical presentation of the methodology (phase 2)	78
3.5	Graphical presentation of the methodology (phase 3)	78
3.6	Graphical presentation of the methodology (phase 4)	79
3.7	Graphical presentation of the methodology (phase 5)	79
4.1	Graph showing yield of the extracts	80
4.2	Gallic acid standard curve	82
4.3	Graph showing DPPH scavenging activity of the extracts; Graph showing IC_{50} for DPPH scavenging activity of the extracts; Percent inhibition of extracts versus log (extract concentration) curve.	84
4.4	Reducing Power Assay (RPA)	85
4.5	Calibration curve of $FeSO_4.7h_2O$ for determination of FRAP value	86
4.6	FRAP value of CAEE, CAME and CAAE	86
4.7	Graph showing NRSA activity of the extracts; Graph showing IC_{50} for NRSA activity of the extracts; Percent inhibition of extracts versus log (extract concentration) curve.	87
4.6	Graphs demonstrating correlation between the Total Phenolic Content (TPC) and DPPH activity, FRAP value, RPA and NRSA	88
4.9	Linear regression graph for α-amylase IC_{50} calculation a) CAEE, b) CAME, c) CAAE and d) A.	90
4.10	Linear regression graph for α-glucosidase IC_{50} calculation	92
4.11	Graph of Inhibitory effect of extracts (IC_{50}) on α-amylase and α-glicosidase activity.	92
4.12	Effect of acute oral toxicity test of extracts on body weight of mice	93
4.13	Effect of extracts on OGTT in normal group mice.	94
4.14	Effect of extracts on OGTT in diabetic group mice	95
4.15	Percentage (%) change in BGL in NC, DC, EETDM, METDM, AqETDM and GTDM.	97
4.16	Micrograph of mice pancreatic tissue	101

4.17	Micrograph of liver sections	102
4.18	Micrograph of kidney sections	102
4.19	Effects of CAME on secretory function of pancreatic MIN6 β-cells	103
4.20	The Ca^{2+} dependency of the insulinotropic of CAME and gliclazide	104
4.21	Chromatogram of CAME by GC-MS	105
4.22	The compounds identified by matching the spectra with those found in the NIST 05 library	110
4.23	Electrophoresis of total DNA extracted from the three samples	110
4.24	RAPD profile of the three samples	111
4.25	The dendrogram derived from RAPD profiles	111
4.26	RAPD patterns of F1 (A), F2 (B) and F3 (C) using primer 1	112
4.27	RAPD patterns of F1 (A), F2 (B) and F3 (C) using primer 2	113
4.28	RAPD patterns of F1 (A), F2 (B) and F3 (C) using primer 3	114

LIST OF PLATES

Plate No.	Description	Page No.
1	World prevalence of diabetes	16
2	*Cassia alata* Linn.	19
3	Herbarium of *Cassia alata* Linn	57
4	Swiss albino mice	67
5	Intraperitoneal injection of STZ	69

ABBREVIATION

%	Percentage
µ	Micro
ADA	American Diabetes Association
AGE	Advanced Glycosylated End products
AMP	Adenosine Monophosphate
AMPK	AMP-dependent Kinase
ATP	Adenosine Tri-phosphate
BCE	Before Common Era
BGL	Blood Glucose Level
BSA	Bovine Serum Albumin
CA	*Cassia alata*
CAAE	CA aqueous extract
CAEE	CA ethanol extract
CAME	CA methanol extract
CD	Cluster of Differentiation
CTLA	Cytotoxic T-lymphocyte Associated-protein
CVD	Cardiovascular Disease
DAN	Diabetic Autonomic Neuropathy
dL	Decilitre
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
DNA	Deoxyribonuclic Acid
DNS	Dinitrosalicylic acid
DNTP	Deoxynucleotide
DPPH	2,2- Diphenyl-1-Picrylhydrazyl
DR	Diabetic Retinopathy
DSP	Diabetic Sensorimotor Polyneuropathy

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
ERK	Extracellular signal Regulated Kinases
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry
GDM	Gestational Diabetes Mellitus
GLUT	Glucose Transporter
GSIS	Glucose Stimulated Insulin Secretion
HDL	High Density Lipoprotein
HLA	Human Leukocyte Antigen
HMC	Major Histocompatibility Complex
IDDM	Insulin Dependent Diabetes Mellitus
IDF	International Diabetes Federation
IL	Interleukin
INSR	Insulin Receptor Genes
IP	Intra-Peritoneal
IRS	Insulin Receptor Substrate
KRB	Krebs-Ringer Bicarbonate
LDL	Low Density Lipoprotein
ml	Millilitre
MODY	Maturity-onset Diabetes of the Youth
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NDDG	National Diabetes Data Group
NIDDM	Non Insulin Dependent Diabetes Mellitus
nm	Nano-Meter
PKC	Protein Kinase-C
PNPG	Para-Nitrophenyl-α-D-Glucopyranoside
PPAR	Peroxisome Proliferator-activated Receptor

RAPD	Random Amplified Polymorphic DNA
rDNA	Recombinant Deoxyribonuclic Acid
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SNP	Single Nucleotide Polymorphism
STZ	Streptozotocin
T1D	Type 1 Diabetes
T2DM	Type 2 Diabetes Mellitus
TBE	
TCA	Trichloroacetic acid
TE	
TNF	Tumor Necrosis Factor
TPTZ	2,4,6-Tripyridyl-s-Triazine
UPGMA	Unweighted Pair Group Method with Arithmatic mean
VLDL	Very Low Density Lipoprotein
VNTR	Variable Number Tandem Repeat
w/v	Weight per Volume
WHO	World Health Organization