Antidiabetic Evaluation of Medicinal Plant of Cucurbitaceae Family

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Statement of Originality

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Preface

The present thesis entitled "***Antidiabetic Evaluation of Medicinal Plant of Cucurbitaceae Family***" comprises the work done by the author in Division of Pharmacology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata for the degree of Doctor of Philosophy in Pharmacy.

Over the past two decades, there has been an enormous growth in the use of medicinal plants in the therapeutic management of various diseases. Drug corporations and research institutions are focusing their efforts on the untapped potential of the plant kingdom in their search for novel medications and lead chemicals. The traditional healthcare system which is mostly based on botanical sources is still used by about 80% of the worldwide population.

Plant extracts have been recommended as therapies in the Indian traditional healthcare system from ancient times. All significant medical traditions including Ayurveda, Unani and homoeopathy depend primarily on medications of plant origin. The basis of the Indian traditional system is made up of low toxicity, simple availability of herbal products and frequent use of practitioners an ingrained faith particularly in rural regions and a strong public desire to employ medicinal plants for therapeutic purposes. Some medicinal food plants were selected from the Cucurbitaceae family which depending on the potency of herbal research and are traditionally of use in the North-East region as a treatment for a wide range of diseases and problems.

In the present study, some medicinal food plant fruits were selected for isolation, *in-vitro*, *in-vivo*, and molecular mechanisms through modulation of the oxidative stress and glycosylated haemoglobin (HbA1c) of antidiabetic evaluation with various animal models.

Before the pharmacological evaluation, LD_{50} of extracts has been determined, to fix the dose levels for pharmacological activities. The fruit fractions of the Cucurbitaceae family plants were also evaluated for *in-vitro* anti-diabetic activity and the efficacious fraction was used in an experimental *in-vivo* anti-diabetic animal model produced by high-fat diet and low dose of streptozotocin. Thus the thesis covered the above mentioned studies in a logical sequence with related references annexed to each chapter. In conclusion the detailed study has been linked up in a manner to justify the relation of the work to establish the antidiabetic potency of Cucurbitaceae family plants especially to explore the molecular mechanism through modulation of the oxidative stress and glycosylated haemoglobin (HbA1c).

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List of Abbreviation

$^1O_2$	-	Singlet oxygen
ACE	-	Angiotensin I-converting enzyme
ACN	-	Acetonitrile
AESH	-	Aqueous Ethanol Extract *Solena heterophylla*
AGI	-	Alpha Glucosidase Inhibitors
ALP	-	Alkaline Phosphatase
ALT	-	Alanine Transaminase
ANOVA	-	Analysis of Variance
AST	-	Aspartate Transaminase
ATP	-	Adenosine triphosphate
BHA	-	Butylated hydroxyanisole
BHA	-	Butylated Hydroxy Anisole
BHT	-	Butylated hydroxytoluene
CAM	-	Complementary and alternative medicine
CAT	-	Catalase
CFCP	-	Chloroform Fraction of *Cyclanthera pedata*
CFSH	-	Chloroform Fraction *Solena heterophylla*
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experiments on Animals
CuB	-	Cucurbitacin B
DNA	-	Deoxyribonucleic acid
DPP4	-	Dipeptidyl peptidase 4
DPPH	-	1,1-diphenyl-1-picrylhydrazyl
DTNB	-	5, 5′-dithio bis-2-nitro benzoic acid
EACP	-	Ethyl acetate Fraction of *Cyclanthera pedata*
EASH	-	Ethyl Acetate Fraction *Solena heterophylla*
EASH	-	Ethyl acetate of *Solena heterophylla*
EDTA	-	Ethylene diamine tetra acetic acid
EFCP	-	Ethanolic fraction of *Cyclanthera pedata*
EFSH	-	Ethanol Fraction *Solena heterophylla*
EFSH	-	Ethanolic fraction of *Solena heterophylla*
FBG	-	Fasting Blood Glucose

GAE	-	Gallic acid equivalent
GC-MS	-	Gas chromatography–mass spectrometry
GH	-	Glycoside hydrolase
GLP-1	-	Glucagon-like peptide
GLUT2	-	Glucose transporter 2
GPX	-	Glutathione peroxidase
GSH	-	Glutathione
H_2O_2	-	Hydrogen peroxide
HbA1C	-	Glycosylated hemoglobin
HFCP	-	Hexane Fraction of *Cyclanthera pedata*
HFD	-	High Fat Diet
HFSH	-	Hexane Fraction *Solena heterophylla*
HO	-	Hydroxyl
HOCl	-	Hypochlorous acid
HPLC	-	High-performance liquid chromatography
HPTLC	-	High-performance thin layer chromatography
IDDM	-	Insulin Dependent Diabetes Mellitus
IDF	-	International Diabetes Federation
LC-MS	-	Liquid chromatography-mass spectrometry
LC-MS/MS	-	Liquid chromatography-mass spectrometry-mass spectrometry
LC-QTOF-MS	-	Liquid Chromatography-Quadrupole Time-of-Flight Tandem Mass Spectrometry
MDA	-	Malondialdehyde
MGAM	-	Maltase-Glucoamylase
NADH	-	Reduced nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dinucleotide
NaOH	-	Sodium Hydroxide
NBT	-	Nitrobluetetrazolium chloride
NIDDM	-	Non-Insulin Dependent Diabetes Mellitus
NIDDM	-	Non-insulin-dependent diabetes mellitus
NMR	-	Nuclear Magnetic Resonance
NO	-	Nitric Oxide
O^2	-	Superoxide
OECD	-	Organisation for Economic Co-operation and Development

OGTT	-	Oral Glucose Tolerance Test
PKC	-	Protein Kinase C
PMS	-	Phenazinemethosulphate
RNS	-	Reactive Nitrogen Species
RO	-	Alkoxyl
ROO	-	Peroxyl
ROS	-	Reactive Oxygen Species
SALP	-	Serum Alkaline Phosphatase
SEM	-	Standard Error of Mean
SGLT1	-	Sodium/glucose transporter
SGOT	-	Serum glutamic oxaloacetic transaminase
SGPT	-	Serum glutamic pyruvic transaminase
SHF	-	*Solena heterophylla* Fraction
SI	-	Sucrase-isomaltase
SOD	-	Super oxide Dismutase
STZ	-	Streptozotocin
T1DM	-	Type 1 Diabetes Mellitus
T2DM	-	Type 2 Diabetes Mellitus
TBA	-	Thiobarbituric acid
TBARS	-	Thiobarbituric Acid Reactive Substances Assay
TCA	-	Trichloroacetic Acid
UV	-	Ultraviolet
WHO	-	World Health Organization

Dedicated
to
My Family
&
My Guide

Chapter 1

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1. Introduction

1.1. Diabetes overview

An international public health issue is diabetes mellitus, a chronic metabolic condition. According to data provided by the World Health Organisation (WHO), as of the year 2000, there were roughly 171 million people living with diabetes globally. This number is predicted to rise gradually to 366 million by 2030 (Nair et al, 2013; Bachhawat et al, 2011).

Type-2 diabetes mellitus, the most prevalent type of this disease, affects more than 95% of the diabetic population worldwide.

The salient features of diabetes are as follows:

- A condition that is diverse and has an ever-declining insulin response because to insulin resistance (Lebovitz et al, 1997). The condition known as insulin resistance occurs when the pancreatic beta-cells' insulin is unable to initiate the absorption of glucose by the muscle and adipose tissues. The insulin receptors in those cells have been gradually downregulated, which is the cause of this pathological disease. There may or may not be an insulin shortage in this situation. (Ahamad et al, 2011).

- β-cells have to work harder in order to make up for the Insulin resistance, thus giving rise to free radicals and oxidative stress (Zhou et al, 2012). Free radicals, which are molecules with an unpaired electron, and reactive oxygen species, or ROS, which are molecules with an unpaired oxygen atom, eventually kill beta cells because the body's natural antioxidant defences are insufficient. This can happen as a result of either an excess of ROS generation or the inactivation of antioxidants (AO), which will tip the ROS/AO balance in favour of stress. (Tripathi et al, 2013).

- The pancreatic -cells eventually degrade because of increased oxidative stress brought on by the blood ongoing production of free radicals. (Zhou et al, 2012).

One of the main issues with type-2 Diabetes Mellitus is postprandial hyperglycemia. With the use of pharmaceuticals and herbal therapies that may interfere with our gut's machinery for metabolising carbohydrates, novel therapeutic options are being developed to control this condition. These methods have been shown to be effective in both the prevention and treatment of chronic vascular problems. (Sim et al, 2010). Doctors suggest food control and sufficient exercise for managing diabetes before turning to pharmacological therapy. Exercise

provides the unique benefit of conserving insulin during glucose entry into muscle cells. So, in addition to pharmacological therapy, this is a crucial step in controlling hyperglycemia. (Zhou et al, 2012). There is no particular diet for regulating blood sugar levels in a diabetic patient, according to the American Diabetes Association, a professional organisation of diabetologists. The diet needs to be adjusted based on each person's needs and objectives. Diabetes dietary management requires a combination of:

- Blood urea and serum creatinine were routinely evaluated together with other metabolic indicators in long-term patients, such as blood pressure, lipids, and glycated haemoglobin.
- Retaining a healthy body weight.
- Lifestyle management (Tripathi, 2013).

Current strategy for management of Type- 2 Diabetes Mellitus targets two chief enzymes present in human intestine, which are responsible for breaking down large polysaccharides and simplifying them to glucose. They are as follows:

- Pancreatic Alpha-amylase: This enzyme belongs to the GH 13 superfamily of carbohydrate metabolizing enzymes. Their role is to break down the complex polysaccharides in to smaller oligosaccharides, known as the “Limit Dextrin’s” (Annison & Topping, 1994; Kearsley & Sicard, 1989).
- Intestinal brush border Alpha-Glucosidases: These enzymes belong to the GH 31 superfamily of carbohydrate metabolizing enzymes. They metabolize the “Limit Dextrin’s” in to glucose molecules. These liberated Glucose molecules gradually get absorbed into the blood circulation through the Intestinal epithelium (Van Beers et al., 1995).

The α-Glucosidase and α-Amylase inhibitors have been proved to delay the glucose absorption through the intestinal epithelia. As a result, the undigested glucose gets transported to the distal jejunum, ileum and Colon region (Chahal et al, 2017). The result is decrease in the post prandial hyperglycaemia.

AGIs may be used as first-line, second-line, or third-line therapy alternatives, according to the International Diabetes Federation (IDF) guidelines. AGIs may be used in conjunction with Metformin, Sulfonylurea, DPP4 inhibitors, Thiazolidinediones and Insulin treatment in

clinical settings. The American Association of Clinical Endocrinology advises using AGIs (Alpha Glucosidase Inhibitors) in all phases of diabetes regardless of the initial HbA1c level (Flint et al., 2008). Although the relative safety of this class of compounds has been established, they are less effective than the conventional medicinal medicines. The National Guidelines from China, Japan, Taiwan, Korea, and Singapore, which are similar to the recommendations from the Asian-Pacific Panel, all advocate the usage of AGIs (Henrissat, 1991). Alpha Glucosidase Inhibitors are often prescribed in China and Japan, two of the most severely diabetic nations. They are regarded as untapped diamond agents. AGIs are not among the first-line or second-line medications, despite the advantages of newer therapies. The correct explanation has to be determined. (Kalra et al, 2014). Marketed α-glucosidase inhibitors like Acarbose and Miglitol are said to cause loose stools, bloating, and diarrhoea, which are all ascribed to the colon's undigested glucose. These unfavourable side effects were shown to be the primary reasons for the early cessation of this class of medications in randomised control studies. Determining new α -glucosidase inhibitors from plant sources has so been the focus of study Natural, plant-based medications have been shown to be less likely to cause adverse effects and to be more effective and safe than synthetic medications. Once again, because plant-derived medicines are high in flavonoids and other phenolic, they are effective antioxidants. Because of this, they also function in vivo as agents that scavenge free radicals. As a consequence, they are able to fight against various Diabetes Mellitus problems (Ibrahim et al, 2014). The fundamental shortcoming of current therapies for diabetes mellitus has been the inability to control the progression of the pathological alterations. Due to their abundance of Flavonoids and phenolic, which are significant α-glucosidase inhibitors, naturally derived plant extracts and fractions serve as significant and efficient diabetes treatment agents (Zhou et al, 2012).

It has been demonstrated that alpha glucosidase inhibitors decrease both postprandial hyperglycemia and the discrepancy between the maximum and minimum postprandial glucose levels. This decrease in postprandial hyperglycaemia lowers the HbA1c level, which is able to assist individuals with NIDDM over the long run. In NIDDM patients, this management leads to improved glucose regulation. (Cai et al, 2017).

1. It has been demonstrated that alpha glucosidase inhibitors decrease both postprandial hyperglycemia and the discrepancy between the maximum and minimum postprandial glucose levels. This decrease in postprandial hyperglycemia lowers the HbA1c level, which is able to assist individuals with NIDDM over the long run. In NIDDM

patients, this management leads to improved glucose regulation. The recommended dosage for acarbose is 100 mg taken three times per day, for a total of 300 mg per day.

2. Acarbose can cause a postprandial drop in plasma glucose levels of around 50%.
3. 3. Researchers have discovered that the mean HbA1c decline ranges from 0.6% to 1.4% on average.
4. It was shown that the mean drop in fasting plasma glucose was 21.8 mg/dL.

In terms of its ability to reduce blood sugar, acarbose monotherapy has been contrasted with other oral anti-diabetic medications (Bischoff, 1994).

In comparison to single medication treatment regimens, the addition of alpha-glucosidase inhibitors to other oral hypoglycemic medicines results in enhanced glycaemic control**. Table 1.1** shows the clinical study reports supporting the above statement. Patients with NIDDM who are using sulfonylurea, metformin, or insulin have better glycaemic control when they also take acarbose or miglitol. They lower HbA1c by around 1.1% when added to sulfonylureas, 0.8% when added to metformin, and 0.5% when added to insulin. **Table 1.1** compares the efficacy of Acarbose with marketed anti-diabetic drugs working through other mechanisms (Lebovitz et al, 1997):

Table 1.1 Clinical study data for Alpha-Glucosidase inhibitors in combination with other anti-diabetic agents

Duration (Weeks)	Antidiabetic drug	Dose (mg/day)	Number of patients	Change in blood glucose		Change in HbA1c	Reference
24	Acarbose	300	32	- 29	- 60	-1.3	Hoffman and Spengler, 1994
	Metformin	1700	32	-25	-34	-1.1	Hoffman and Spengler, 1994
24	Acarbose	32	67	- 22	-49	-0.76	Coniff et al, 1995
	Tolbutamide	750-3000	66	-36	-59	-1.27	Coniff et al, 1995
24	Acarbose	300	28	-25	-40	-0.1.1	Hoffman and Spengler, 1994
	Glibenclamide	3.5-10.5	27	-29	-34	-0.9	Hoffman and Spengler, 1994

Regarding a pharmaceutical agent anti-diabetic effect, there are two more variables. They are GLP-1 (glucagon-like peptide) secretion and post-prandial GIP secretion; both measured 30-

240 minutes after eating. While increasing GLP-1 secretion, alpha-glucosidase inhibitors decrease GIP secretion. The presence of carbohydrates in the ileum after therapy with an alpha-glucosidase inhibitor may be the cause of this effect (Bayraktar et al, 1996). The post-prandial studies detailed GLP-1 level is varied in kind. By causing the pancreatic β-cells to produce more insulin, it is a crucial trigger in lowering blood glucose levels. Additionally, according to Braun et al, 1996; alpha-glucosidase inhibitors can lower blood triglyceride levels. However, alpha-glucosidase inhibitors are unable to affect the main metabolism of triglycerides or cholesterol. They have a minimal impact on body weight. Clinical investigations have demonstrated that alpha-glucosidase inhibitors can have some extra metabolic benefits (Ceriello et al, 1996). It is possible for postprandial hyperglycemia to initiate coagulation. This happens as a result of an increase in the plasma concentrations of prothrombin fragments 1 and 2, then the fibrinogen δ-diamer. By reducing post-prandial hyperglycemia, prevention of Alpha-Glucosidase-mediated carbohydrate metabolism can stop blood coagulation. As a result, they can stop thromboembolic problems from occurring (Lebovitz, 1997; Chiasson et al, 1994; Chiasson et al, 1996; Coniff et al, 1995).

1.2. Detailed physiology of Carbohydrate metabolizing enzymes

In contemporary culture, plant starches including wheat, rice, flour, cereals, tubers, fruits, and vegetables serve as the main source of glucose, the primary metabolite required for energy synthesis, in the human diet (Marks & Flatt, 1989). 500–600 glucose residues are organised in a long chain and connected by α -1, 4 linked glucose units to generate amylose.

Amylopectin is a highly branching and substantial molecule. A number of α-1, 4 linked glucose chains made up of 12-70 glucose residues are present in it. About 5% of the links are likewise of the α-1, 6 sorts.

These things have been schematically represented in **Figure 1.1**. The complicated, massive di- and oligo- saccharides must first be transformed into simple glucose molecules since they cannot be carried over the intestinal epithelia. The intestinal enzymatic activity is essential for this goal. There are two categories of carbs: digestible and non-digestible carbohydrates (MacGregor et al., 2001). Simple mono- and di-saccharide sugars, such as glucose and sucrose, are among the available carbohydrates (Annison & Topping, 1994; Sajilata et al., 2006). According to Brayer et al., 2000, the disaccharides and bigger molecules are reduced into monosaccharide components and absorbed from the upper digestive tract. Fibrous, polyol and inulin carbohydrates are not readily accessible. Endogenous gut enzymes cannot

breakdown these fibres and bacterial enzymes in the colon ferment those (Brayer et al., 2000). The significance of the various carbohydrate kinds will be covered in the section after, with an emphasis on readily accessible carbs (Ao et al., 2007). It has been discovered that two alpha-amylases and four alpha-glucosidases work together to completely convert starch into glucose in mammals. The small intestine contains many starch-digesting enzymes, including the sucrase-isomaltase (SI system) and maltase-glucoamylase (MGAM systems), which work together to extract glucose from complex polysaccharides. Following that, it is absorbed by the intestinal epithelium (Englyst & Englyst, 2005). The final catalysts needed to liberate glucose from these polysaccharides are these enzymes (Messer & Kerry, 1967). The human small intestine's two carbohydrate-metabolizing systems, MGAM and SI, are both intricate protein complexes made up of N- and C-terminal domains. These domains complement the structures of the substrates, which are oligosaccharides in composition, according to their structural elucidation (Naumoff, 2007). The use of alpha-glucosidase inhibitors in the treatment and prevention of Type-2 diabetes through regulation of postprandial blood glucose levels has been demonstrated to be extremely beneficial (Kearsley & Sicard, 1989).

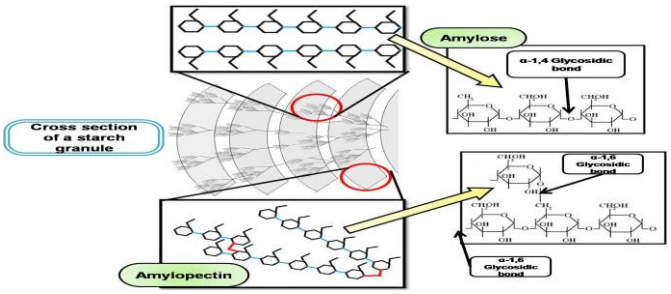

Figure 1.1. Structure of a starch granule

Salivary and pancreatic alpha-Amylases (EC 3.2.1.1), which are endohydrolases in nature, break the internal α-1, 4 bonds of the Amylose and Amylopectin structures in the starch molecule. They are divided into Limit Dextrins, which are shorter linear and branching dextrin chains (Semenza, 1986). Through hydrolytic cleavage, the resulting combination of dextrins is further transformed into glucose **Figure 1.2** through the exohydrolytic cleavage activities of MGAM (Maltase-Glucoamylase; EC 3.2.1.20 and 3.2.1.3) and Sucrase-Isomaltase (SI; EC 3.2.148 and EC 3.2.10), before they are absorbed in to the bloodstream (Nichols et al., 2003). The sodium/glucose transporter SGLT1 and the fructose transporter (GLUT 5) are the two primary transporters that carry sugar through the intestinal epithelia (Sim et al, 2010).

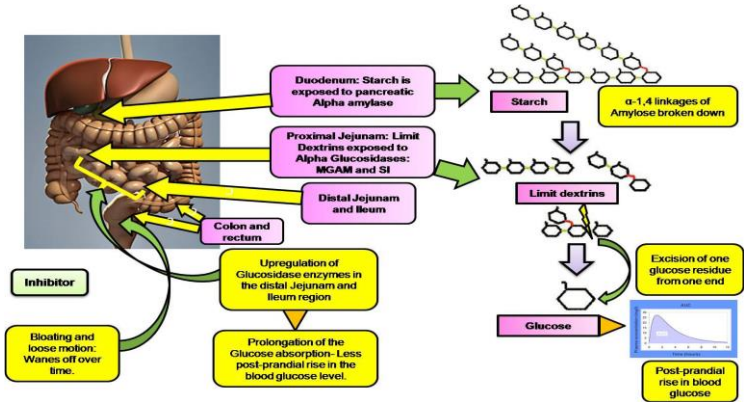

Figure 1.2. Schematic representation of the carbohydrate digestion site and process.

Resistant Starches and fibres can avoid being processed in the small intestine. They are subsequently sent to the colon, where bacteria ferment them (Naim et al., 1988). The human large intestine contains hundreds of distinct bacterial species, which together form a sophisticated microflora. Although gramme positive (anaerobic rods and cocci) and gramme negative (Bacteroides) bacteria can both be found in the colon, the Gramme positive species predominates. Despite the lack of clarity on the structure/function links between the various microbiota components, it has been proven that this system is crucial for preserving the homoeostasis of the organism. The synthesis and metabolism of short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate, are carried out by the microbiota. Although the bulk of the chemoheterotrophic species in the colon obtain their energy through anaerobic respiration, most of the organisms do so via fermentation. They do this by phosphorylating substrates at the cellular level. The fermentation process produces metabolic by products from the starting substrate that serve as the electron acceptors (Macfarlane et al, 1999). Numerous fermenting enzymes are linked to the intestinal bacteria and used by them to metabolise the undigested carbohydrates. One of these is *Bacteroides thetaiotaomicron,* which possesses a cluster of genes for starch utilisation and 236 glycoside hydrolases. The following provides illustrations of the several types of starch-digesting enzymes:

1.2.1. Glycoside hydrolases (GH)

All starch-digesting enzymes are classified as Glycoside Hydrolases (GH), including α-amylase, SI, and MGAM. One of the strongest bindings in natural polymer chemistry, the glycosidic link between two glucose residues in a carbohydrate molecule, may be broken by these enzymes (Gunther et al., 1996). Additionally, the cleavage occurs 1017 times more quickly than in the case of an uncatalyzed cleavage process. Thus, GHs are regarded as one

of the most effective enzymes. There are 115 distinct GH families in all, according to the CAZY (Carbohydrate Active Enzyme) database. According on the amino acid sequence similarity profiles of the enzymes, this categorization was done. This classification's main goal is to show how similarly each family members fold and serve. Substrates are frequently shared between two families of enzymes, and the substrate specificities within a family of enzymes are frequently highly diverse. Pancreatic and salivary α-amylases are members of the GH13 family, whereas SI and MGAM are members of the GH31 family.

1.2.2. Alpha amylases

The most significant enzymes in the GH13 family are α-glucosidase, α-amylase, pullulanase, and cyclomaltodextinase, each of which performs a variety of tasks. The GH13 family of enzymes has been the subject of several structural and kinetic researches, particularly on the α-amylase. According to research by starch hydrolysis via the retention mechanism is catalysed by GH13 pancreatic and salivary α-amylase enzymes (Gunther et al., 1996; Heymann & Gunther, 1994; Noren et al., 1986). The α-amylase system of enzymes cleaves all of the α-1, 4 links of amylose and amylopectin, but spares the α-1, 6 linkages of amylopectin. The sequence similarity between the pancreatic and salivary amylases is substantial (97%). The pancreatic amylase performs its job in the stomach since the salivary amylase cannot operate in the presence of the stomach acidic environment (Heymann & Gunther, 1994). The catalytic domain of α-amylase has a $(\beta/\alpha)_8$ structure. It comprises of a substrate binding site that resembles a trench. There are multiple sub sites in this substrate binding site. One glucose residue from the substrate interacts with each of these sub sites. The substrate is cleaved between sub sites -1 and +1, one of the pancreatic α-amylase five binding sub sites. The ideal substrate binding and cleavage can occur when a Maltose residue serves as the leaving group at sites +1 and +2, in addition to three sugars at the -1, -2, and -3 sites (Brayer et al., 2000). Even a thorough digestion of the eaten starch is unable to generate a sizable amount of glucose. Therefore, the cleavage of the Dextrin residues and release of the Glucose depend on the intestinal brush-border α-glucosidases.

1.2.3. Alpha Glucosidases

Following the breakdown of the starch, the released digestive products are further broken down into glucose by the enzymes sucrase-isomaltase (SI) and maltase-glucoamylase (MGAM) (Rossi et al., 2006). The alpha-glucosidases act as exohydrolases, i.e., break the limit-dextrins from one end, freeing the Glucose residues one at a time from the substrate,

whereas the alpha-amylases can cleave the α-1, 4 bonds of the substrate, i.e., the complex polysaccharides. Maltose, maltotriose, and other short, branching substrates make up Limit Dextrins, commonly known as the α-Limit Dextrins (Rossi, 2004). Because the small intestine alpha-amylase starch digestion is not compartmentalised, the brush border glucosidases (SI and MGAM) are likely to be exposed to bigger intermediates than α-limit dextrins (Frandsen & Svensson, 1998). Researchers have really noted that the substrate composition of the SI and MGAM differs over the small intestine whole length. The cause is that as starch travels through the small intestine, it gradually breaks down into less Dextrins (Ernst et al., 2006). As a result, the concentration of the accessible substrate steadily rises via the intestine. It has been demonstrated that MGAM has significantly improved hydrolytic activities with starch that has been pre-treated with -amylase but exhibits very little activity for starch by itself (Davies & Henrissat, 1995). Given that the MGAM and SI are members of the same superfamily of genes, they have several characteristics in common. The GH 31 super family of proteins, which includes the n- and c-terminal domains, includes the proteins MGAM and SI (Quaroni & Semenza, 1976). They are stalked intrinsic membrane proteins that live in the human small intestine mucosa's borderland. While the extracellular intestine luminal side of the protein serves as the catalytic domain, its N-terminal portion serves as an anchor (Okuyama et al., 2001). In the past, the terms "sucrase" (-1, 2), "isomaltase" (-1, 6), "maltase" (-1, 4) and "glucoamylase" (-1, 4) were each connected to a specific catalytic domain of the GH31 protein (Lovering et al., 2005). Given that the Maltase activities are present in all alpha-glucosidase domains, the name "Maltase" appears to be a little unclear. Despite the fact that SI exhibits isomaltase activity, isolmaltose is not a byproduct of the starch digestion by Amylase. Therefore, the alpha-glucosidases are unlikely to use isomaltase as a substrate (Messer & Kerry, 1967). To prevent misunderstanding over the related domain activities, we shall refer to the individual MGAM and SI domains here as either N-terminal (ntSI and ntMGAM) for the domain closest to the membrane anchor or C-terminal (ctSI and ctMGAM) for the luminal domain **(Figure 1.3.)**.

1.2.3.1. Sucrase-isomaltase

Nearly 10% of all the proteins in the intestinal membrane are made up of SI, sucrose-isomaltase. The intermediate ileum is where the majority of the enzymatic activity takes place, whereas the distal ileum exhibits 20–30% less activity (Quaroni & Semenza, 1976). Though the small intestine hosts the majority of the SIs, some of them can be detected in the colon during the early stages of development (Okuyama et al., 2001The SI gene is located on

chromosome 3, and the 210 kDa protein it codes for is synthesised as a single polypeptide with a size range of 24-260 kDa (Lovering et al., 2005). With N- and O-linked carbohydrates, it is glycosylated.

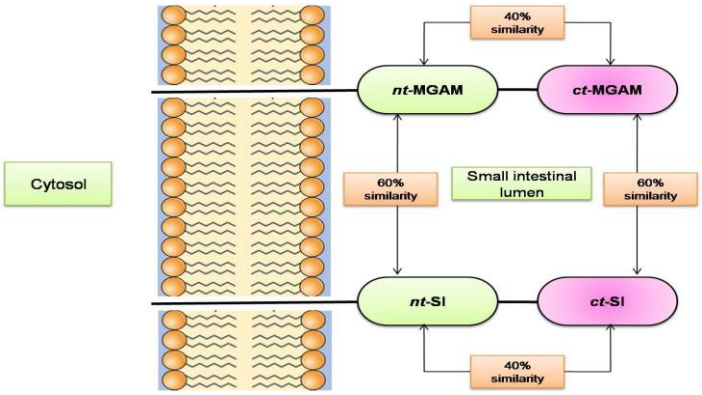

Figure 1.3. Schematic representations of the Sucrase-Isomaltase (SI) and Maltase-Glucoamylase (MGAM) enzyme systems

The enzyme is transported and inserted into the intestinal enterocytes' apical membrane after being glycosylated. Extracellular pancreatic proteases then break down the enzyme to produce free ctSI and membrane-bound ntSI subunits (Saphiro et al., 1991). Although they are first cut apart by proteolytic cleavages, the N- and C-terminal subunits are still connected by non-covalent connections (Ernst et al., 2006). The initial kinetic evaluation of SI was completed using crude enzymatic preparations from the intestinal scrapings of the pig, rat, and human. It was shown that SI has relatively little action towards bigger oligosaccharides, but accounts for 80% of the total intestinal maltase activity, practically all of the isomaltase activity, and all of the sucrase activity (Semenza, 1987). Even while the SI's N- and C-terminal domains both exhibit Maltase activity, several subtle differences are extremely noticeable (Ernst et al., 2006).The ctSI exhibits Sucrase activity, whereas the ntSi is responsible for isomaltase activity. Through the isolation and purification of the individual ntSI, ctSI, and intact Sis, first efforts have been made to differentiate the distinct activities in rat SI (Holman et al., 1999). Additionally, their actions with limit dextrins were contrasted. The following details can be used to describe the N- and C-terminal carbohydrate-digesting mechanisms:

- ntSI has α-1,6 debranching activity
- ctSI has linear α-1,4 activity (Krentz & Bailey, 2005).

Another mixed substrate incubation investigation showed that the ctSI component hydrolyzes sucrose, maltose, and isomaltose while the ntSI hydrolyzes isomaltose. These investigations subsite affinity estimations further imply that the N- and C-terminal subunits have distinct binding patterns for maltooligosaccharides. While ctSI only has two subsites, ntSI has four (Mooradian & Thurman, 1999).There is one point of contention regarding the ntSI α-1, 4 hydrolytic functions. The isolation of the ntSI domain using recombinant DNA technology made it feasible to characterise the α-1, 4 and α-1, 6 kinetic characteristics of the domain (Krentz & Bailey, 2005; Asano, 2003).

1.2.3.2. Maltase-glucoamylase

MGAM has a lower population density than the Si. Up to 2% of all membrane proteins are represented by it. Although it is present throughout the whole small intestine, the distal ileum is where it is most active (Chiasson et al., 2002). In contrast to the SI, MGAM has a varied tissue distribution since it may be found in the colon, kidneys, and small intestines as well. A 210 kDa protein is produced by the MGAM, which is encoded on chromosome 7 (Chehade & Mooradian, 2000). After being synthesised, MGAM goes through a maturation phase in which it is glycosylated to create a mature protein with a molecular weight of 335 kDa. Contrary to SI, no reports of proteolytic processing have been made for MGAM, and its N- and C-terminal subunits are still intact. It's conceivable that the MGAM protein's high glycosylation shields it from proteolytic cleavage (Bischoff, 1994).

MGAM has less accessible kinetic studies than SI does. The MGAM is responsible for 20% of the intestinal maltase activity, very little isomaltase activity, and 100% glucoamylase activity (Chehade & Mooradian, 2000). MGAM is made up of a single chain as opposed to SI's cleaved structure, which is connected via a non-covalent contact (Rabasa-Lhoret & Chiasson, 1998). The SI subunits may be divided up and separately studied, it should be emphasised. It was not possible to evaluate the functions of the intestinal scrapings since the Maltase-Glucoamylase protein was previously examined using an intact protein that was immunopurified from them (Stuttz, 1999). The cativity of the N-terminal has also been elucidated by heat denaturing the C-terminal, a more thermolabile one in comparison to the N-terminal (Berland et al., 1995; Withers et al., 1999). It has been proposed that the activities of N-terminal and C-terminal domains are similar. According to research on the fully intact human enzyme, malto-oligosaccharides are degraded by a series of subsites, each of which has a particular affinity for a glucose moiety from the substrate (Li et al., 2005). These

experiments showed that the substrate-binding cleft of MGAM can accept four glucose residues and that maltotriose and maltotetraose, which are present in larger concentrations, impede substrate activity, although maltose and other malto-oligosaccharides do not (Breitmeier et al., 1997). Since the SI subunits display different kinetic and substrate characteristics, and MGAM and SI have shown to be closely related, further molecular characterization was merited to test whether MGAM subunits actually do exhibit identical activities. Recent work described the recombinant expression of ntMGAM from human, which represent the first attempt to characterize individual MGAM domain without harsh effects of heat inactivation.

The anomeric centres of the substrate are either retained or not by the GH catalytic processes. GH31 family members work by using the maintaining catalytic mechanism. Through the traditional Koshland double-displacement method, which involves the creation and subsequent hydrolysis of a glycosyl-enzyme intermediate, glycosidic bond breakdown happens in this manner.

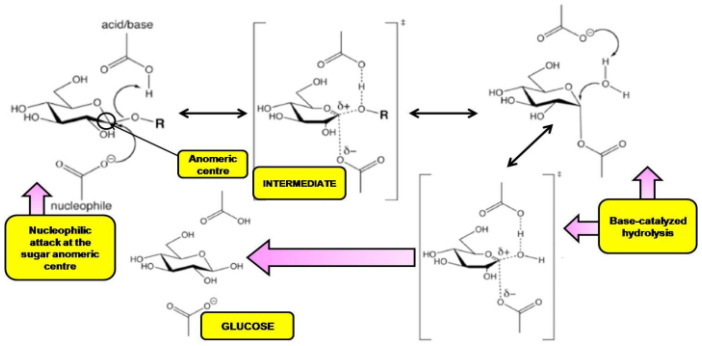

Figure 1.4. Catalytic mechanisms of Glycoside hydrolases

An active site carboxylic residue attacks the sugar anomeric centre nucleophilically to form this intermediate. Water then attacks the anomeric centre of this intermediate, causing a general base-catalyzed hydrolysis that releases the product anomeric structure while netly maintaining it (Sim et al, 2010). The catalytic mechanism of GHs has been represented in **Figure 1.4.**

1.3. Quality evaluation of medicinal plants

To prevent, diagnose, or treat ailments as well as to maintain good health, medicinal plants are employed in a variety of forms (leaves, flowers, fruits, woods, barks, roots, gums, resins, essential oils, etc.) and formulations (extracts, tinctures, etc.). Over 80% of people worldwide

have used herbal medicines, and these products play a crucial role in health-care systems, according to the WHO (Rivera-Mondragón et al., 2017). Accurate chemical information is crucial for the quality, safety, and effectiveness of the finished herbal products since the phytochemical contents and quality of such compounds influence the therapeutic potential of medicinal plants. Various sources, plant species or sections used, extraction techniques, formulation, and chemical components are only a few of the variables that might change the chemical homogeneity in medicinal plants (Agatonovic- Kustrin & Morton, 2017). Despite being extensively used and generally regarded as safe; herbal medications have been associated with a variety of unfavourable side effects. In reality, the quality, effectiveness, and safety of the product can be affected by variations in the bioactive content, the presence of toxic pollutants, pesticides, microbiological contamination, and adulteration with other plant species or synthetic drugs (Rivera-Mondragón et al., 2017). Plants have been shown to have a wide range of elements, and it is sometimes difficult to identify the precise component responsible for the therapeutic effect (Mukherjee et al., 2013). To assess the potency of therapeutic plants, phytomarkers are crucial. Chemical makers have also been employed in other sectors of research, including the validation of original species, the search for innovative goods and materials or substitutes, the improvement of extraction and purification processes, structural elucidation, and quality evaluation. Scientifically employing chemical markers can aid in the identification and development of new drugs (Li et al., 2008). The analytical chemistry of medicinal plants has developed during the past few decades. Marker profiling of medicinal herbs, often known as "fingerprinting," has become a practical substitute for conventional analytical techniques (Kharyuk et al., 2018). Today, a variety of marker profiling approaches are used to guarantee the efficacy of herbal medicines. A single medicinal plant has a large number of chemical markers, according to marker profiling. In fact, it is preferable for chemical producers to be unique components that support the pharmacological effects of herbal medicines. A chemical marker's concentration in a herbal medication is used to assess its quality (Li et al., 2008). Marker substances are primarily divided into two categories: molecular markers and phytochemical markers. Analytical markers and active markers are two categories that the phytochemical markers often fall under. The biomarkers, also known as active markers, are in charge of having pharmacological effect (Desai & Tatke, 2019; Mukherjee et al., 2011). Different technologies have been developed. Authorised by numerous organisations including the WHO and USFDA to detect and characterise the marker component in herbs and also herbal medications (Agatonovic-Kustrin & Morton, 2017). Either qualitative or quantitative

methods must be used for herbal product quality control. Numerous techniques, including UV and IR, have been widely employed to detect qualitative traits. While LC-MS/MS, HPLC, GC-MS, and HPTLC are used to quantitatively determine medicinal plants for quality control analysis (Balekundri & Munnur, 2020). Therefore, chemo profiling might be used for product standardisation and validation to confirm plant material, find the amounts of active components, and relate chemical attributes to bioactivity.

1.3.1. Chromatographic techniques for validation of medicinal plants

Alkaloids, saponins, phenolic acids, flavonoids, terpenoids, and other phytoconstituents with substantial therapeutic potential and clinical benefits were among the numerous phytoconstituents detected in the natural products. Therefore, for natural product-based medication screening processes, phytoconstituent screening and assessment are crucial. Due to the frequently complicated structures of these compounds, it might be difficult to identify their chemical structures because of their low sensitivity (Zhang et al., 2017). The interpretation of the molecular variability of the natural products has been done using metabolic fingerprinting. This information may be utilised to assess medicinal effectiveness and link it with other taxonomically similar plants that have been studied (Mendez-Lopez et al., 2020). The field of metabolomics, which has grown over the past two decades, is defined in terms of all tiny molecules (1.5 kDa) in natural products (Roca et al., 2021). Metabolite profiling not only detects metabolites in relation to their distribution of phytomolecules but also evaluates the nature of chemicals (Mukherjee et al., 2016). Metabolomics is now used to check the quality of food supplements, nutrition, and botanicals (Li et al., 2019). The fundamental purpose of metabolomics is to link changes in the chemical profile to a shift in macroscopic phenotype caused by a perturbation (Kellogg and Kang, 2020). The different analytical methods have been developed for metabolomic profiling such as mass spectrometry (MS), NMR spectroscopy and infrared and Raman spectroscopy (Scalbert et al., 2014). In addition to having orders of magnitude more sensitivity than NMR spectroscopy, MS-based metabolomics methods also have the ability to link to liquid chromatography (LC) or gas chromatography (GC) directly (Fujimura et al., 2011). The most popular technique for analysing chemical variation in natural goods is metabolomics, which uses liquid chromatography-mass spectrometry (LC-MS) (Mukherjee et al., 2016; Méndez-López et al., 2020). Untargeted and targeted metabolomics are the two main alternatives used to assess LC-MS-based metabolomics investigations. High-resolution mass spectrometers with

quadrupole time of flight (Q-ToF) have tested the untargeted approach (Roca et al., 2021). The LC–ToF–MS technology provides excellent resolution and enhanced sensitivity for the analysis of targeted metabolites. In the study of natural products and natural therapies, TLC and planar chromatography are widely used as analytical tools (Agatonovic-Kustrin & Morton, 2017). HPTLC (High Performance Thin Layer Chromatography) is a type of planar chromatography which is the highly sophisticated type of analytical TLC and extensively used as a low-cost tool for analyzing sample mixes quickly (Mukherjee, 2019). HPTLC plates offer faster and sharper sample separation compared to conventional TLC plates (Agatonovic-Kustrin & Morton, 2017). Both normal phase and reversed-phase silica based materials are the most usually applied as sorbents in HPTLC plate which allows the study of natural products with a wide range of polarity (Galal et al., 2015). It has a number of benefits, including the ability to test many samples concurrently on the same chromatographic plate and under the same experimental conditions. Additionally, it is the only chromatographic method that enables results to be shown as a vibrant picture (Agatonovic-Kustrin & Morton, 2017). HPTLC is frequently used in the analysis of natural products and their formulation. HPTLC system can be integrated with bio-autography/bioassays for identifying certain bioactivities of natural products; and HPTLC combined with mass spectrometry (MS)/NMR for dereplication of natural products (Galal et al., 2015). In the phytochemical analysis of crude plant products, quick and efficient identification of bioactive phytoconstituents is important. Plant extracts are efficiently screened by several chromatographic approaches such as HPLC (High-Performance Liquid Chromatography) in accompanied by a variety of testing systems (Kumar, 2017). HPLC is one of the most widely used, complexes, effective, and varied chromatographic separation techniques for separating the bioactive components in natural products and their production. Additionally, it is used to discover and measure potentially harmful compounds like adulterants (Mukherjee, 2019). In practice, RP-HPLC (Reversed-phase High Performance Liquid Chromatography) is the most extensively utilized HPLC for separating analytes depending on their hydrophobicity. In RP-HPLC system, the mobile phase is used polar and stationary phase is non-polar which just opposite to normal phase chromatography (Mukherjee, 2019). Research has been done extensively on the mechanism of RP retention (adsorption and partition model), which is mostly based on hydrophobic interactions between eluting molecules and the stationary phase. The most often employed stationary phases are either octadecyl (C18) or octyl (C8) phases that are silica- or polymer- bonded. RP C18 columns, however, are the most often used for a range of tasks, including the study of bioactive components within complicated chemical classes (Cavaliere

et al., 2018). In RP-HPLC system the polar compounds travel through the column more quickly and efficiently. The identification of the bioactive chemicals found in natural goods and products generated from them is frequently done using the RP-HPLC technology (Mukherjee, 2019; Cavaliere et al., 2018).

1.4. Medicinal plants in the management of diabetes

Hyperglycemia and hyperinsulinemia are two aberrant physiological characteristics that characterise the metabolic disorder diabetes mellitus (DM) (Xu et al., 2018). One of the most common non-communicable illnesses worldwide, it is. Around 46.3 billion individuals are affected by it globally as of 2019, and by 2045, that figure is projected to increase to about 70 billion (Papoutsis et al., 2021). The occurrence among adult adults is 8.5 percent worldwide, with a greater rate of increase in low- and middle-income nations (Xu et al., 2018). DM has been categorized into three types, namely type I diabetes mellitus, type II diabets mellitus and gestational diabetes melliutus (Ahmed et al., 2020). Children are more prone to developing type I diabetes mellitus, which is caused by the systemic autoimmune destruction of the insulin-secreting beta cells in the pancreas (Teng and Chen, 2017). Because of improper insulin utilisation by the body, type II diabetes results in hyperglycemia (Ahmed et al., 2020). It is thought that 90–95 percent of all cases of diabetes is caused by insulin resistance and is characterised by high and variable blood glucose levels (Trinh et al., 2016). In Gestational diabetes the different degrees of hyperglycemia persist during the pregnancy period (Ahmed et al., 2020). A healthy diet, regular exercise, the right medicines, and frequent monitoring of the condition can help control and lessen the consequences of diabetes mellitus (Alam et al., 2020). Lowering blood glucose levels and reducing insulin resistance are the main targets of diabetes mellitus therapy. This restores metabolic balance and guards against subsequent diabetic problems (Tundis et al., 2010). Oral anti-diabetic medications have been associated with a number of undesirable side effects, such as hypoglycemia, peripheral edoema, osteoporosis, and heart attacks, which has restricted their therapeutic use. New antidiabetic options with fewer side effects must be created to treat diabetes mellitus (Xu et al., 2018). Thus, research into noble antidiabetic medicines derived from medicinal plants is becoming more important (Tundis et al., 2010).

1.4.1. Diabetes and its management

The largest source of carbohydrates for both humans and other living things is starch, which is digested by the enzymes α-amylase and α-glucosidase (Yang et al. 2019). Following

carbohydrate consumption, they are initially broken down into oligosaccharides or disaccharides in saliva or the pancreas by -amylase (EC 3.2.1.1) (Teng and Chen, 2017). The α-amylases broke the polysaccharides α-(1-4) link, resulting in minute oligosaccharides or disaccharides. In the small intestine, the enzyme α-glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) breaks down the hydrolytic glycoside link of polysaccharides to monosaccharides and creates glucose molecules like glucose and fructose **Figure 1.5** (Papoutsis et al., 2021). Blood glucose levels can rise in some cases, such as when these enzymes are overactive, when insulin is deficient, or when insulin resistance is present, resulting in hyperglycemia (Yang et al. 2019). The inhibition of α-amylase and α-glucosidase can greatly minimize the postprandial glucose level and hence can be a useful approach for control of blood glucose levels in type 2 diabetes patients (Chen and Kang, 2013). As a result, drugs that aim to lower postprandial hyperglycemia are advised for managing diabetes and avoiding problems associated with it (Chai et al., 2015).

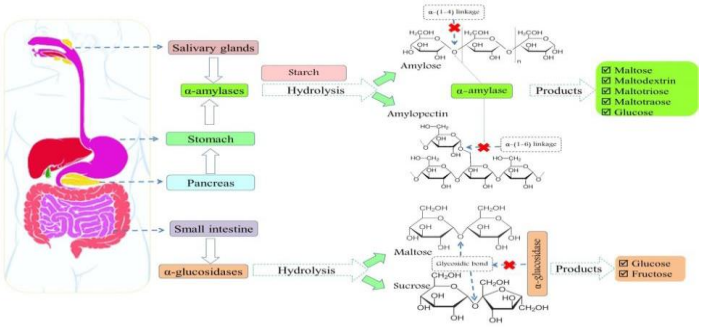

Figure 1.5. Role and site of carbohydrate metabolizing enzymes (α-amylase and α-glucosidase)

The therapies for diabetes mellitus management are aimed at controlling blood glucose levels in order to delay or avoid diabetic consequence (Somtimuang et al., 2018). The different drugs have been authorized and are routinely used in the management of diabetes (Alam et al., 2021; Tripathy et al., 2021). The most often used pharmaceuticals are biguanides, thiazolidinediones, α-glucosidase inhibitors, and glucagon-like peptide-1 agonists. However, a number of side effects (such as nausea, bloating, gas, diarrhoea, and flatulence) and the expensive cost of many of these anti-diabetic medications have restricted their usefulness (Somtimuang et al., 2018). The medical management of diabetes mellitus involves the use of nine different types of medications **Table 1.2**. Depending on their pathogenetic differences, diabetics are prescribed these drugs (Alam et al., 2021; Tripathy et al., 2021).

Table 1.2. List of the current therapeutics used to treat diabetes mellitus

Sl No.	Therapeutic Class	Generic name	Mechanism of action	Side effects
1	Amylin analogues	Pramlintide	↓ glucagon release	Hypoglycemia, nausea
2	Biguanide	Metformin	↑ the effects of insulin	Weight loss, lactic acidocis, diarrhea, abdominal cramps
3	Gliptins	Sitagliptin Vildagliptin Linagliptin Alogliptin	↑ incretin levels, which inhibit glucagon release	Upper respiratory tract infection, headache, dizziness
4	Glucagon-like peptide-1 agonists	Albiglutide, Dulaglutide, Semaglutide, Lixisenatide	↑ glucagon like peptide-1 receptor	Bloating, headache, nausea, indigestion
5	Meglitinides	Nateglinide, Repaglinide	Increase insulin secretion from pancreatic β-cells	Weight gain, risk of hypoglycemia
6	SGLT-2 inhibitors	Canagliflozin, Dapagliflozin, Empagliflozin	Increased glucosoria byinhibiting SGLT-2 in the kidney	Urinary tract infection, polyuria, dehydration,
7	Sulfonylureas	Glyburide, Glimperide	Enhances insulin secretion from pancreatic β-cells	Hemolysis, weight gain
8	Glitazones	Alogliptin/Piogli tazone, Rosiglitazone	Activates peroxisome proliferator-activated receptors (PPARs)	Weight gain, heart failure, macular edema
9	α-glucosidae inhibitors	Acarbose, Miglitol, Voglibose	Reduced intestinal glucose absorption	Flatulence, diarrhea

1.4.2. Herbals for the management of diabetes

Although pharmaceutical agents have been developed for the treatment of diabetes, natural treatments including herbal preparations and botanicals have been the mainstay of diabetes treatment for ages under different ideologies and practises (Xu et al., 2018). Additionally, herbal remedies are a common component of complementary and alternative medicine (CAM), which is used by many diabetics (Seetaloo et al., 2018). The plant species include a number of active compounds that have anti-diabetic benefits and have very few, if any, detrimental side effects that have been observed. Numerous herbal extracts and phytoconstituents have demonstrated potent hypoglycemic properties (Shori, 2015). Tradition has passed down the knowledge through the years, and it is now fueling an increase in study into phytochemicals with therapeutic potential (Franco et al., 2018). The WHO has supported use of such herbs for treating diabetes since of their therapeutic potential (Somtimuang et al., 2018). According to ethnopharmacological research, approximately 800 herbs and their bioactive compounds may have anti-diabetic properties (Arulselvan et al., 2014). For instance, bitter gourd (Momordica charantia L) fruit and leaf juices are traditionally used to lower blood sugar levels in a variety of nations, including the Indian subcontinent and many parts of Africa. Because they have minimal undesirable side effects, herbs and food plants can thus be used instead of or in addition to conventional treatments (Seetaloo et al., 2018). Over the last few decades, professionals working in the field of diabetes research have investigated the potential therapeutic benefits of several natural products and phytochemical substances (Arulselvan et al., 2014). Two prominent examples of anti-diabetic medications are galegina and pycnogenol, which are extracted from Pinus pinaster Aiton and Galega officinalis L., respectively. Since it has been established that certain bioactive compounds, such as phenolic acids, polyphenols, terpenoids, alkaloids, saponins, quinones, etc., are the least cytotoxic and cause the fewest adverse reactions, researchers have increasingly focused on elucidating the molecular pathways of these molecules on different targets (Xu et al., 2018).

It has been demonstrated that bioactive compounds found in medicinal plants, such as terpenes and polyphenols (flavonoids and phenolic acids), have the ability to inhibit digestive enzymes. Additionally, due to their possible radical scavenging properties, flavonoids and phenolic acids can potentially aid in reducing non-enzymatic glycosylation (Franco et al., 2018).Some of the most well-known herbs that have been researched for diabetes mellitus are included in **Table 1.3**.

Table1.3. List of antidiabetic medicinal plants

Botanical name	Family	Extract/Plant parts used	Phytoconstituents	Reference
Abelmoschus moschatus	Malvaceae	Leaf, seed, root	Myricetin, Farnesol	Liu et al.,2010
Abroma augusta	Malvaceae	Leaf, bark, root	Friedelin, Choline, Betaine	Mir et al., 2013
Acacia catechu	Mimosaceae	Leaf, stem, bark	α, β, γ catechin, Quercetin, Kaempferol	Ikarashi et al., 2011
Acacia nilotica	Mimosaceae	Leaf, stem, seed, gum, root	Tannins, Betulin, Gallic acid, αamyrin, β-sitosterol	Ali et al., 2012
Acacia polyacatha	Mimosaceae	Seed	Tryptamines, βcarbolins	Okpanachi et al., 2010
Achyranthes aspera	Amaranthaceae	Leaf, stem, root, seed	Flavonoids	Chhetri et al., 2005
Aegle marmelos	Rutaceae	Leaf, fruit, seed, root	Ageline, Lupeo, Eugenol,	Maity et al., 2009
Agrimonia eupatoria	Rosaceae	Whole plant	Luteolin, Quercetin, Apegenin, Kaempferol	Al-snafi, 2015
Albizia odoratissima	Mimosaceae	Leaf, bark, seed	Flavonoids, Tannins, Fixed oils	Rajan et al., 2011a
Allium cepa	Amaryllidaceae	Bulb, seed	Allicin, Arginine, Choline, Pectin, Rutin	Kelkar et al., 2001
Allium sativum	Amaryllidaceae	Bulb	Allicin, Terpenoids, Phenols	Sharma et al., 2013
Alpinia	Zingiberaaceae	Rhizome	Galagin, Alpinin,	Shetty &

galangal			Quercetin, Kaempferol	Monisha, 2015
Anacardium occidentale	Anacardiaceae	Leaf, Bark	Kaemferol, Oxalate, Saponin	Obaineh & Shadrach, 2013
Andrographis paniculata	Acanthaceae	Root	β-Sitosterol	β-Sitosterol
Annona muricata	Annonaceae	Leaf	Corossolin, Muricine, Emodin, Chlorogenic acid	Florence et al. 2014, CoriaTéllez et al., 2018
Arctium lappa	Asteraceae	Leaf	Liognans, sesquiterpene lactones, phytosterols, flavonoids	Bahmani et al., 2014; Alsnafi, 2014
Areca catechu	Arecaceae	Leaf, fruit	Quercetin, βSitosterol, Tannins, Alkaloids	Mondal et al., 2012
Botanical name	Family	Extract/Plant parts used	Phytoconstituents	Reference
Bacopa monnieri	Plantaginaceae	Aerial part	Bacosides, Betulinic acid, β-Sitosterol, Herpestine	Al-snafi, 2013
Bauhinia forficata	Fabaceae	Leaf	Kaempferol, Quercetin-O-glycoside, Flavonoids	Bolson et al., 2015; Da Cunha, et al., 2010
Benicasa hispida	Cucurbitaceae	Fruit	Uronic acid, βsitosterin, Carotenes	Mahatma et al., 2014
Berberis aristata	Berberidaceae	Stem, root	Saponins, Glycosides	Upwar et al., 2011

Bombax ceiba	Malvaceae	Leaf, bark, seed, root	Lupeol, Stigmasterol, Amyrin, Tannins	Antil et al., 2013
Brassica juncea	Brassicaceae	Leaf, flower, stem, seed	Brassicasterol, βSitosterol, Isothiacynates, Camfesterol	Thirumalai et al., 2011
Calamus rotang	Arecaceae	Leaf, fruit, root	Flovens, Phenylpropanoids, Steroids, Ligans	Ripa et al., 2015
Camellia sinensis	Theaceae	Leaf	Theanine, Myricetin, Quercetin, Kaempferol	Tang et al., 2012, Passos et al., 2018
Cannabis sativa	Cannabaceae	Leaf, root	Cannabinoids, Caryophyllene, Myrcene, Linaloo	Levendal & Frost, 2006
Carica papaya	Caricaceae	Leaf, fruit	Papain, βcaeroene, Carposide, Carpinine, Kaempferol	Maniyar & Bhixavatimath, 2012
Cassia fistula	Caesalpiniaceae	Flower, Bark	Fistulic acid, Fistuladin, Sennosides A, B, Emodin, Lupel	Jarald et al., 2013
Chamomilla recutita	Asteraceae	Flower	Camazulene, eucalyptol, Coumarin, Camphor	Semenya et al., 2012; Ghizlane & Aziz, 2016
Citrullus colocynthis	Cucurbitaceae	Leaf, fruit, root	Flavonoids, Glycosides	Hussain et al., 2014
Curcuma longa	Zingiberaceae	Rhizome	Curcumin, Tumerone, Sesquiterpenoids	Nishiyama et al., 2005

Dioscorea alata	Dioscoreaceae	Tuber	γ-tocopherol, Ip[omeanol	Maithili et al., 2011
Echinodorus grandiflorus	Alismataceae	Leaf	Chicoric acid, Fertaric acid, Steroids, Cembrane	Marques et al., 2017; Garcia et al., 2016
Eugenia jambolana	Myrtaceae	Stem, seed	Eugenin, Myricetin, Betulinic acid, Ellagic acid	Ayyanar and Subash-babu, 2012
Ficus	Moraceae	Bark	Meso-inositol, β-amyrin, Lupeol	Gayathri and Kannabiran, 2008
Ficus racemosa	Moraceae	Bark, fruit, root	α-amyrin acetate, Lupenol, Stigmasterol	Gul-E-Rana et al., 2013
Girardinia diversifolia	Urtcaceae	Root	Flavonoids, Tannins, Glycosides	Baig et al., 2014
Hibiscus rosasinensis	Malvaceae	Leaf, flower	Cyanidin, Thiamine, Niacin, Stigmasterol	Venkatesh et al., 2008
Hydnocarpus laurifolia	Flacourtiaceae	Seed	Sterol, Flavonolignans, Flavonoids	Rao et al., 2014
Ipomoea batatas	Conovolvulaceae	Leaf, root	Caffeoylquinic acid, Anthocyanins, Flavonoids	Panda & Sonkamble, 2012
Lagenaria siceraria	Cucurbitaceae	Aerial part, fruit, seed	Triterpenes, Saponins, Flavonoids	Prajapati et al., 2010

Luffa acutangula	Cucurbitaceae	Fruit	Triterpenes, Anthroquinone glycoside	Pimple et al., 2011
Mangifera indica	Anacardiaceae	Leaf, peel	Anthroquinone, cardiac glycosides, flavonoids, steroids	Nwankwo & OsaroMathew, 2014
Mentha piperita	Lamiaceae	Leaf	Phenolic acids, Saponins, Tannins	Jose et al., 2013
Momordica charantia	Cucurbitaceae	Fruit	Resins, Phenolic compounds, Glycosides, Saponins	Joseph & Jini, 2013
Momordica charantia	Cucurbitaceae	Leaf	Charantin, vicine, Ploypepetide	Fuangchan et al., 2011, Basch et al., 2003
Murraya koenigii	Rutaceae	Leaf	Elemol, Myrcene, Linalool, αTerpinene	Elemol, Myrcene, Linalool, αTerpinene
Nelumbo nucifera	Nelumbonaceae	Rhizome	Myricetin-3-Oglucopyranoside, Quercetin-3-Oglucopyranoside, Kaempferol-3-Oglucopyranoside	Mukherjee et al., 1997
Ocimum sanctum	Lamiaceae	Aerial part	Ursolic acid, Apigenin, Isovitexin, Luteolin, Circineol	Patil et al., 2011
Panax pseudoginseng	Araliaceae	Rhizome, root	Ginsenosides, Triterpene saponins	Jia & Zhao, 2009

Phyllanthus emblica	Phyllanthaceae	Leaf, fruit	Coumarins, Steroids, Flavanoid	Nain et al., 2012
Plumbago indica	Plumbaginaceae	Leaf	Anthraquinones, Coumarins, Triterpenoids, Naphthoquinones	Sunil et al., 2012
Rosmarinus officinalis	Lamiaceae	Leaf	α-pinene, 1,8-Cineole, Camphor, Camphene	Bakirel et al., 2008; Kadri et al., 2011
Rotula aquatica	Boraginaceae	Root, flower	Tannins, Steroids, Flavonoids	Tullibali et al., 2013
Solanum melongena	Solanaceae	Fruit	Anthracenocides, Coumarin, Hydroxycinnamic acid	Nwanna et al., 2013
Swertia chirayita	Gentianaceae	Whole plant	Xanthone, Serol, Glycosides, Triterpenoids	Roy et al., 2015
Syzygium cumini	Myrtaceae	Leaf	Anthocyanins, Glucoside, Kaempferol, Ellagic acid	TrojanRodrigues et al., 2012; Ayyanar et al., 2012
Terminalia arjuna	Combretaceae	Bark	Tannins, Terpenoids, Steroids, Alkaloids	Ragavan et al., 2006
Tinospora cordifolia	Menispermaceae	Stem, root	Diterpenoid lactones, Alkaloids, Steroids, Glycosides	Nadig et al., 2012

Withania somnifera	Solanaceae	Leaf, root	Triterpenoids, Steroidal lactones, Tannins	Udayakumar et al., 2009
Zingiber officinale	Zingiberaceae	Rhizome	Gingerols, Paradols, Shogaols	Daily et al., 2015; Mao et al., 2019
Ziziphus nummularia	Rhamnaceae	Leaf	Flavonoids, Alkaloids, Tannins, Saponins	Rajasekaran et al., 2012

1.4.2.1. Herbals as carbohydrate enzyme inhibitors

It has long been believed that the digestive enzymes α-amylase and α-glycosidase play a significant role in regulating gluconeogenesis into the circulation. It has long been believed that the digestive enzymes α-amylase and α-glycosidase play a significant role in regulating gluconeogenesis into the circulation (Franco et al., 2018). While α-glucosidase hydrolyzes starch and disaccharides into glucose and fructose, α- amylase converts the polysaccharide into maltose, maltriose, and limit dextrins (Ahmed et al., 2020). As a result, by reducing the intestinal absorption of glucose molecules following digestion, these enzymes can help avoid both postprandial hyperglycemia and hyperlipidemia (Franco et al., 2018). The carbohydrate enzyme inhibitors such as acarbose, miglitol and voglibose are used clinically for the treatment of type II diabetes (Ahmed et al., 2020). However, these drugs cause unpleasant side effects such bloating, abdominal pain, diarrhoea, and flatulence (Alqahtani et al., 2019; Ahmed et al., 2020). The bulk of these inhibitors also contain sugar molecules, making their manufacturing time-consuming and multistep-intensive. Therefore, less harmful non-sugar inhibitors are required (Ahmed et al., 2020). Currently, multiple investigations on α-amylase and α-glucosidase inhibitors from medicinal plants have already been conducted (Trinh et al., 2016). According to several studies, eating fruits and vegetables high in polyphenol compounds can help prevent the activity of enzymes that break down carbohydrates, such as -amylase and α-glucosidase (Mihaylova et al., 2021).

1.4.2.1.1. Role of α-amylase inhibition

The majority of hydrolyzing enzymes, including α-amylases (1, 4-D-glucan-glucanohydrolase, EC 3.2.1.1), are present in both living and non-living species, including

fungus. However, polysaccharides like starch and glycogen which catalyses the hydrolysis of 1, 4-glucan bonds to yield glucose and maltose (Mukherjee, 2019; Tundis et al., 2010). As shown in **Figure 1.5**, the pancreas secretes pancreatic α-amylases into the small intestine, while the salivary glands release salivary amylases (also known as salivary amylase or ptyalin) into the mouth (Papoutsis et al., 2021; Kim et al., 2014; Robyt, 2008, Van Beers et al., 1995). The most common storage carbohydrate in plants is starch which consist of two types of polysaccharides such as amylose [a linear α- (1 → 4) linked glucan] and amylopectin [α- (1 → 6) branched linkages]. Typically, α-amylases break down α-(1-4) -glycosidic bonds in starch to produce glucose, maltose, maltotriose, maltotetraose and maltodextrins (Robyt, 2008). Therefore, blocking α-amylase may be an effective strategy for restricting carbohydrate breakdown and delaying the time needed to digest all carbohydrates, which will significantly reduce the amount of glucose absorbed and avoid postprandial blood glucose levels (Bhutkar and Bhise, 2012; Mukherjee,2019).

1.4.2.1.2. Herbals as α-amylase Inhibitors

It is well known that plant metabolites generated from medicinal plants have α-amylase inhibiting effects. Ethnomedicine has provided descriptions of many herbal medications that are used to manage diabetes and is recognised as a reputable source of anti-diabetic chemicals. Diabetes is treated with -amylase inhibitors because they stop the metabolism of starch, which lowers postprandial hyperglycemia (Mukherjee et al., 2020). The α-amylase inhibitory qualities of the conventionally employed herbs and herbal lead compounds, respectively, are highlighted in **Tables 1.4 and 1.5.**

Table1.4. Medicinal plants having α-amylase inhibitory property

Plant name	Family	Parts used	IC50 value	Reference
Abelmoschus esculentus	Malvaceae	Peel	132.63 µg/ml	Sabitha et al., 2012
Amaranthus cruentus	Amaranthaceae	Leaf	230 µg/ml	Oboh et al., 2013
Amaranthus spinosus	Amaranthaceae	Leaf	46.02 µg/ml	Rahmatullah et al., 2009
Andrographis lineata	Acantthaceae	Leaf	50.9 ± 0.17 mg/ml	Ayyanar et al., 2008

Artocarpus altilis	Compositae	Fruits	118.88 ± 11.14 µg/ml	Mootoosamy & Mahomoodally, 2014
Artocarpus altilis	Moraceae	Fruit	118.88 µg/ml	Nair et al., 2013
Artocarpus heterophyllus	Moraceae	Leaf	70.58 µg/ml	Nair et al., 2013
Asystasia gangetica	Acanthaceae	Leaf	3.75 µg/ml	Suvarchala et al., 2012
Calendula officinalis	Asteraceae	Leaf	72.60 ± 2.61 µg/ml	Kumar et al., 2012
Camellia sinesis	Theaceae	Leaf	20.7 ± 0.54 µg/ml	Nickavar and Yousefian, 2011
Cinnamomum verum	Lauraceae	Leaf	40 µg/ml	Ponnusamy et al., 2011
Cistus salviifolius	Cistaceae	Aerial part	217.10 ± 0.15 µg/ml	Sayah et al., 2017
Crataeva magna	Capparaceae	Bark	143.8 µg/ml	Loganayaki and Manian, 2012
Curcuma longa	Zingiberaceae	Rhizome	7.4 µg/ml	Abo et al., 2008
Cynarascolymus	Asteraceae	Leaf	95.78 µg/ml	TrojanRodrigues et al., 2012
Eleusine coracana	Poaceae	Seed	35.5 µg/ml	Sobana et al., 2009
Ficus benghalensis	Moraceae	Bud	4.4 µg/ml	Ponnusamy et al., 2011
Ficus racemosa	Moraceae	Fruits	46.7 ± 23.6 µg/ml	Trinh et al., 2016
Fucus vesiculosus	Fucaceae	Whole plant	59.1 µg/ml	Loradn et al., 2012
Luffa acutangula	Cucurbitaceae	Fruit	135 µg/ml	Pimple et al., 2011

Mallotus japonicus	Euphorbeaceae	Leaf	8.4 µg/ml	Indrianingsih et al., 2015
Momordica charantia	Cucurbitaceae	Leaf	66.64 ± 2.94% (at 1.25 mg/ml concentration)	Nag et al., 2015
Momordica charantia	Cucurbitaceae	Fruit	72.12 ± 6.97 µg/ml	Shaheen et al., 2017
Myrcia guianensis	Myrtaceae	Leaves	7.80 ± 0.92 µg/ml	De Cássia Lemos Lima et al., 2018
Myrcia rubella	Myrtaceae	Leaves	4.36 ± 0.08 µg/ml	De Cássia Lemos Lima et al., 2018
Myrcia torta	Myrtaceae	Leaves	5.31 ± 0.28 µg/ml	De Cássia Lemos Lima et al., 2018
Myrcia variabilis	Myrtaceae	Leaves	3.17 ± 0.25 µg/ml	De Cássia Lemos Lima et al., 2018
Myrcia vestita	Myrtaceae	Leaves	13.60 ± 0.96 µg/ml	De Cássia Lemos Lima et al., 2018
Myrcia virgate	Myrtaceae	Leaves	6.29 ± 0.65 µg/ml	De Cássia Lemos Lima et al., 2018
Myristica fragans	Myrtaceae	Fruit	0.85 mg/ml	Patil et al., 2011
Nerium indicum	Apocynaceae	Leaves	> 200 µg/ml	Barkaoui et al., 2017
Passiflora subpeltata	Passifloraceae	Fruit pulp	32.63 µg/ml	Shanmugam et al., 2018
Phaleria macrocarpa	Thymelaceae	Fruit	2.40 ± 0.23 µg/ml	Winarno, 2010

Phaseolus vulgaris	Fabeaceae	Beans	64.12 ± 2.12 µg/ml	Tan et al., 2017
Phyllanthus nirruri	Phyllanthaceae	Aerial parts	3.7 ± 1.1 µg/ml	Beidokhti et al., 2017
Piper betel	Piperaceae	Bark, fruit	96.56 µg/ml	Nair et al., 2013
Plectranthus esculenta	Lamiaceae	Tubers	128.89 ± 4.85 µg/ml	Eleazu et al., 2016
Plumbago zeylanica	Plumbaginaceae	Root	3.46 ± 0.53 µg/ml	Bachawat et al., 2011
Podocarpus macrophyllus	Podocarpaceae	Leaf	45.2 µg/ml	Indrianingsih et al., 2015
Punica grantum	Punicaceae	Leaf	94.25 µg/ml	Wu et al., 2009
Quercus dentate	Fabeaceae	Leaf	42.2 µg/ml	Indrianingsih et al., 2015
Quercus gilva	Fagaceae	Leaf	110.0 µg/ml	Indrianingsih et al., 2015
Quercus glauca	Fagaceae	Leaf	44.7 µg/ml	Indrianingsih et al., 2015
Quercus phillyraeoides	Fagaceae	Leaf	9.8 µg/ml	Indrianingsih et al., 2015
Raphia farinifera	Palmaceae	Leaves	99.96 ± 4.31 µg/ml	Dada et al., 2017
Rubia cordifolia	Rubeaceae	Roots	253.42 ± 4.88 µg/ml	Bachawat et al., 2011
Santalum spicatum	Santalaceae	Bark	0.9 µg/ml	Gulati et al., 2012
Saussurea lappa	Asteraceae	Rhizome	401.76 ± 5.17 µg/ml	Bachawat et al., 2011
Scabiosa arenaria	Dipsacaceae	Flowers and fruits	0.11-0.22 mg/ml	Hlila et al., 2015
Sechium edule	Cucurbitaceae	Leafy shoots	51.49 ± 2.13%	Nag et al., 2015
Solanum surrattense	Solanaceae	Leaf	43.66 µg/ml	Manjusha et al., 2013

Swertia chirata	Gentianaceae	Leafy shoots	4.72 ± 0.02 µg/ml	Nag et al., 2015
Symplocos racemosa	Symplocaceae	Bark	8.16 ± 0.28 µg/m	Bachawat et al., 2011
Syzygium aromaticum	Myrtaceae	Flower	0.8 ± 0.4 µg/ml	Ali-Shtayeh et al., 2012
Terminalia arjuna	Combretaceae	Bark	0.69 ± 0.08 µg/ml	Bachawat et al., 2011
Trichosanthes cucumerina	Cucurbitaceae	Leafy shoots	61.91 ± 0.96%	Nag et al., 2015
Trigonella foenumgraecum	Liguminosaeae	Seed	14,4 ± 1.6 µg/ml	Salehi et al., 2013
Xanthium strumarium	Compositae	Fruits	15.25 µg/m	Ingwale et al., 2018
Xylosoma congestum	Salicaceae	Leaf	182.3 µg/ml	Indrianingsih et al., 2015
Zea mays	Poaceae	Flower	0.93 mg/ml	Ezuruike and Prieto, 2014

Table 1.5. List of α-amylase inhibitors obtained from medicinal plants

Compound name	**IC50 value**	**Reference**
Betulin	125.89 ± 1.59 µg/ml	Somtimuang et al., 2018
Betulinic acid	56.23 ± 1.54 µg/ml	Somtimuang et al., 2018
Gnemonoside C	840 µg/ml	Kato et al., 2009
Gnemonoside D	277 µg/ml	Kato et al., 2009
Gnetin C	203 µg/ml	Kato et al., 2009
Isorhamnetin-3-O-glucoside	0.619 mM	Tundis et al., 2007
Isorhamnetin-3-Orutinoside	0.129 mM	Tundis et al., 2007
Linoleic acid	22.8 µg/ml	Teng and Chen, 2016

Lupeol	19.95 ± 1.06 µg/ml	Teng and Chen, 2016
Oleic acid	97.3 µg/ml	Somtimuang et al., 2018
Palmitic acid	38.7 µg/ml	Teng and Chen, 2016
Rosmarinic acid	1.4 mM	Eddouks et al., 2002
Scopoletin	15.86 ± 2.53 µg/ml	Somtimuang et al., 2018
α-amyrin	13.80 ± 1.56 µg/ml	Somtimuang et al., 2018
β-amyrin	28.18 ± 1.82 µg/ml	Somtimuang et al., 2018

1.4.2.1.3. Role of α-glucosidase inhibition

Glucosidases (EC 3.2.1.20) are carbohydrate hydrolytic enzymes that may be found in the tissues of microbial, plant, animal, and human beings. As seen in **Figure 1.5**, the small intestine secretes α- glucosidase from the luminal surface of enterocytes (Papoutsis et al., 2021; Wang et al., 2013). The main enzyme α-glucosidase is responsible for hydrolytically cleaving the disaccharides maltose and sucrose into monosaccharides glucose and fructose, which are then absorbed by specialised transporters like GLUT2 and SGLT1 transporters and released into the bloodstream (Kim et al., 2014; Kumar et al., 2011, Bischoff, 1994; Scheen, 2003). Inhibiting α-glucosidase activity might therefore prevent blood sugar from rising too quickly and reduce postprandial hyperglycemia (Papoutsis et al., 2021; Kim et al., 2014; Kumar et al., 2011), which may increase insulin sensitivity and reduce stress on β-cells (Mukherjee, 2019; Bischoff, 1994; Scheen, 2003).

1.4.2.1.4. Herbs as α-glucosidase Inhibitors

The α-glucosidase inhibitors compete with one another for glycosidase activity, halting the breakdown of sugar and so regulating blood sugar levels. Many of the many α-glucosidase inhibitors that have been isolated from plants are therapeutically important. Surprisingly, inhibiting α-glucosidases has become a common treatment for diabetes (Teng and Chen, 2017; Seetaloo et al., 2018; Mukherjee et al., 2020). **Table 1.6 and Table 1.7** highlight α-glucosidase inhibitory properties of the traditionally used herbs and herbal lead compounds respectively.

Table 1.6. Medicinal plants having α-glucosidae inhibitory property

Plant name	Family	Parts used	IC50 value	Reference
Abelmoschus esculentus	Malvaceae	Peel	142.69 µg/ml	Sabitha et al., 2012
Aloe vera	Asphodelaceae	Leaf	> 200 µg/ml	Shah et al., 2013
Amaranthus cruentus	Amaranthaceae	Leaf	190 µg/ml	Oboh et al., 2013
Artocarpus altilis	Moraceae	Bark and fruit	129.85 µg/ml	Nair et al., 2013
Artocarpus heterophyllus	Moraceae	Bark and fruit	76.90 µg/ml	Nair et al., 2013
Artocarpus heterophyllus	Moraceae	Leaf	70.58 ± 9.66 µg/m	Mahmoodally et al., 2016
Artocarpus heterophyllus	Moraceae	Leaf	76.90µg/ml	Nair et al., 201
Asystasia gangetica	Acanthaceae	Leaf	325 µg/ml	Suvarchala et al., 2012
Camellia sinesis	Theaceae	Leaf	2.47 ± 0.30 µg/ml	Mahmoodally et al., 2016
Capparis spinosa	Capparaceae	Leaves and bud powder	21.09 ± 0.48 mmol Acarbose equivalent/gram	Mollica et al., 2018
Cinnamomum zeylanicum	Lauraceae	Bark and fruit	140.01 µg/ml	Nair et al., 2013
Cinnamomum zeylanicum	Lauraceae	Bark	2.3 ± 0.5 µg/ml	Ali-Shtayeh et al., 2012
Crataeva magna	Capparaceae	Bark	137.4 µg/ml	Loganayaki and Manian, 2012
Cyperus rotunda	Cyperaceae	Tubers	3.98 ± 0.55 µg/ml	Bachawat et al., 2011
Echeveria craigiana	Crassulaceae	Leaf	56.67 ± 4 µg/ml	Lopez-angulo et al., 2014
Echeveria kimnachi	Crassulaceae	Leaf	50.57 ± 1.48 µg/ml	Lopez-angulo et al., 2014

Ficus racemosa	Moraceae	Fruit	278.6 ± 49.1 µg/ml	Ocvirk et al., 2013
Fucus vesiculosus	Fucaceae	Whole plant	0.32 µg/ml	Loradn et al., 2012
Glycine max	Fabeaceae	Beans	75.41 ± 3.11 µg/ml	Tan et al., 2017
Gossypium herbaceum	Malvaceae	Leaf	4.00 mg/ml	Olabiyi et al., 2016
Khaya senegalensis	Meliaceae	Root	5.15 ± 1.10 µg/ml	Ibrahim et al., 2014
Lageneria siceraria	Cucurbitaceae	Leafy shoots	61.25 ± 0.57% (at 1.25 mg/ml concentration)	Nag et al., 2015
Luffa acutangula	Cucurbitaceae	Fruit	135 µg/ml	Indrianingsih et al., 2015
Mallotus japonicus	Cucurbitaceae	Leafy shoots	8.4 µg/ml	Indrianingsih et al., 2015
Momordica charantia	Cucurbitaceae	Leafy shoots	66.64 ± 2.94% (at 1.25 mg/ml concentration)	Nag et al., 2015
Momordica charantia	Cucurbitaceae	Fruit	72.12 ± 6.97 µg/ml	Shaheen et al., 2017
Myrcia guianensis	Myrtaceae	Leaves	7.80 ± 0.92 µg/m	De Cássia Lemos Lima et al., 2018
Myrcia rubella	Myrtaceae	Leaves	4.36 ± 0.08 µg/ml	De Cássia Lemos Lima et al., 2018
Myrcia torta	Myrtaceae	Leaves	5.31 ± 0.28 µg/ml	De Cássia Lemos Lima et al., 2018
Myrcia variabilis	Myrtaceae	Leaves	3.17 ± 0.25 µg/ml	De Cássia Lemos Lima et al., 2018

Myrcia vestita	Myrtaceae	Leaves	13.60 ± 0.96 µg/ml	De Cássia Lemos Lima et al., 2018
Myrcia virgate	Myrtaceae	Leaves	6.29 ± 0.65 µg/ml	De Cássia Lemos Lima et al., 2018
Myristica fragans	Myrtaceae	Fruit	0.85 mg/ml	Patil et al., 2011
Nerium indicum	Apocynaceae	Leaf	> 200 µg/ml	Barkaoui et al., 2017
Passiflora subpeltata	Passifloraceae	Fruit pulp	32.63 µg/ml	Shanmugam et al., 2018
Phaleria macrocarpa	Thymelaceae	Fruit	2.40 ± 0.23 µg/ml	Winarno, 2010
Phaseolus vulgaris	Fabeaceae	Beans	64.12 ± 2.12 µg/ml	Tan et al., 2017
Phyllanthus nirruri	Phyllanthaceae	Aerial parts	3.7 ± 1.1 µg/ml	Beidokhti et al., 2017
Piper betel	Piperaceae	Bark,fruit	96.56 µg/ml	Nair et al., 2013
Plectranthus esculenta	Lamiaceae	Tubers	128.89 ± 4.85 µg/ml	Eleazu et al., 2016
Plumbago zeylanica	Plumbaginaceae	Root	3.46 ± 0.53 µg/ml	Bachawat et al., 2011
Podocarpus macrophyllus	Podocarpaceae	Leaf	45.2 µg/ml	Indrianingsih et al., 2015
Punica grantum	Punicaceae	Leaf	94.25 µg/ml	Wu et al., 2009
Quercus dentate	Fabeaceae	Leaf	42.2 µg/ml	Indrianingsih et al., 2015
Quercus gilva	Fabeaceae	Leaf	110.0 µg/ml	Indrianingsih et al., 2015
Quercus glauca	Fabeaceae	Leaf	44.7 µg/ml	Indrianingsih et al., 2015
Quercus phillyraeoides	Fabeaceae	Leaf	9.8 µg/ml	Indrianingsih et al., 2015

Raphia farinifera	Palmaceae	Leaves	99.96 ± 4.31 µg/ml	Dada et al., 2017
Rubia cordifolia	Rubeaceae	Roots	253.42 ± 4.88 µg/ml	Bachawat et al., 2011
Santalum spicatum	Santalaceae	Bark	0.9 µg/ml	Gulati et al., 2012
Saussurea lapp	Asteraceae	Rhizome	401.76 ± 5.17 µg/ml	Bachawat et al., 2011
Scabiosa arenaria	Dipsacaceae	Flowers and fruits	0.11-0.22 mg/ml	Hlila et al., 2015
Sechium edule	Cucurbitaceae	Leafy shoots	51.49 ± 2.13% (at 1.25 mg/ml concentration)	Nag et al., 2015
Solanum surrattense	Solanaceae	Leaf	43.66 µg/ml	Manjusha et al., 2013
Swertia chirata	Gentianaceae	Leafy shoots	4.72 ± 0.02 µg/ml	Nag et al., 2015
Symplocos racemosa	Symplocaceae	Bark	8.16 ± 0.28 µg/ml	Bachawat et al., 2011
Syzygium aromaticum	Myrtaceae	Flower	0.8 ± 0.4 µg/ml	Ali-Shtayeh et al., 2012
Terminalia arjuna	Combretaceae	Bark	0.69 ± 0.08 µg/ml	Bachawat et al., 2011
Trichosanthes cucumerina	Cucurbitaceae	Leafy shoots	61.91 ± 0.96% (at 1.25 mg/ml concentration)	Nag et al., 2015
Trigonella foenumgraecum	Liguminosaeae	Seed	14,4 ± 1.6 µg/ml	Salehi et al., 2013
Xanthium strumarium	Compositae	Fruits	15.25 µg/ml	Ingwale et al., 2018
Xylosoma congestum	Salicaceae	Leaf	182.3 µg/ml	Indrianingsih et al., 2015
Zea mays	Poaceae	Flower	0.93 mg/ml	Ezuruike and Prieto, 2014

Table 1.7. List of α-glucosidae inhibitors obtained from medicinal plants

Compound name	IC50 value	Reference
Betulin	10.02 ± 1.24 µg/ml	Somtimuang et al., 2018
Betulinic acid	12.86 ± 0.97 µg/ml	Somtimuang et al., 2018
Chlorogenic acid	2.18-2.99 mM	Ishikawa et al., 2007
Chrysoeriol	155.9 ± 3.2 µM	Choi et al. 2018
Daphnoretin	86.0 µg/ml Z	Zhao et al., 2015
Edgeworin	18.7 µg/ml	Zhao et al., 2015
Edgeworoside A	780.0 µg/ml	Zhao et al., 2015
Edgeworoside C	760 µg/ml	Zhao et al., 2015
Edgeworthin	49.6 µg/ml	Zhao et al., 2015
Gymnemioc acid derivative	145-310 µg/ml	Alkefai et al. 2018
Hespiritin	0.38 ± 0.06 mM	Gong et al., 2017
Hyperin	0.5 mM	Zhou et al., 2012
Kaemferol	109 µg/ml	Chen and Gou, 2017
Linoleic acid	73.8 µM	Teng and Chen, 2016
Lionolenic acid	17.9 µM	Teng and Chen, 2016
Lupeol	3.16 ± 1.34 µg/ml	Somtimuang et al., 2018
Lutelin	39.8 ± 4.2 µM	Collado-González et al., 2017
Luteolin	39.8 ± 4.2 µM	Choi et al. 2018
Methyl linoleate	51.8 µM	Teng and Chen, 2016
Methyl linoleneate	47.5 µM	Teng and Chen, 2016
Methyl oleate	20.1 µM	Teng and Chen, 2016
Methyl stearate	24.8 µM	Teng and Chen, 2016
Methyl-3,4-di-O-caffeoylquinate 1	10.7 µM	Thien et al., 2017
Methyl-3,5-di-O-caffeoylquinate	52.1 µM	Thien et al., 2017
Oleic acid	64.2 µM	Teng and Chen, 2016
Palmitic acid	21.3 µM	Teng and Chen, 2016
Piceatanol	0.060 mg/ml	Zhang et al. 2017

Quercetin	4.02 ± 1.09-66.8 µg/ml	Chen and Gou, 2017; Muccilli et al., 2017
Quercitrin	0.4 mM	Zhou et al., 2012
Resveratrol	0.091 mg/ml	Zhang et al., 2017
Rutin	26.31 ± 0.02 µg/ml Ş	Şöhretoğlu et al., 2018a
Scopoletin	10.14 ± 1.96 µg/ml	Somtimuang Et al., 2018
Stearic acid	22.2 µM	Teng and Chen, 2016
Tannic acid	0.44 µg/ml	Zhao et al., 2015
Taraxiroside F	165.1 ± 0.6 µM	Choi et al. 2018
trans-Desoxy-rhapontigenin	0.042 mg/ml	Zhang Et al., 2017
trans-Pinostilbene	0.028 mg/ml	Zhang Et al., 2017
Umberliferone	107 µg/ml	Zhao Et al., 2015
α-amyrin	3.71 ± 2.10 µg/ml	Somtimuang Et al., 2018
β-amyrin	6.02 ± 0.91 µg/ml	Somtimuang Et al., 2018
1,5 dicaffeoylquinic acid	0.6 mM	Pimple Et al., 2011
3'-Hydroxy-pterostilbene	0.230 mg/ml	Zhang et al. 2017

1.4.3. Lead compounds with antidiabetic potential

Natural goods are a fantastic source of beneficial compounds with positive impacts on health (Papoutsis et al., 2021). In reality, indigenous knowledge has shown to be a useful tool in the creation of cutting-edge plant-based treatments (Seetaloo et al., 2018). Different phytochemicals with potential anti-diabetic effects have been identified and described in the last several years (Tupas et al., 2020).Several herbs, including those found in fruit, vegetables, and fungi (such as mushrooms), are a rich source of bioactive substances, including alkaloids, anthocyanins, capsaicinoids, carotenoids, fatty acids, flavonoids, phenolic acids, proteins, saponins, and terpenes with established α-amylase and α-glucosidase inhibitory activities (Papoutsis et al., 2021). Moreover, one of the prescription drugs now used to treat type II diabetes is metformin, which was biosynthesized and developed based on a biguanide natural component in French lilac (*Galega officinalis* L.) (Seetaloo et al., 2018).

Table 1.8. List of anti-diabetic lead molecules and their possible mode of action

Compound	Plant name	Plant parts	Mode of action	Reference
Acarbose	Actinoplanes sp. SE50/110	Strain	Potent inhibitor of digestive enzymnes, e.g. α-amylase, α-glucosidase, sucrose and maltase	Naik and Kokil, 2013
Anacardic acid	Anacardium occidentale	Fruit	Regulate β-cell function	Sokeng et al., 2007
Andrographa lide	Andrographis paniculata	Leaf	↑ Glucose utilization	Yu et al., 2004
Berberine	Berberis vulgaris	Bark	↓ Lipid peroxidation ↑Regeneration of pancreatic β-cell ↓Hyperglycemia ↑ Insulin resistance	Chen et al., 2010, Joeng et al., 2009, Kim et al., 2009, Lee et al., 2006
Butylisobutylphthalate	Laminaria japonica	Rhizoid	Inhibition of α-glucosidase activity	Akar et al., 2011
Capsaicin	Capsicum frutescens	Fruit	Regulation of insulin resistance Presearvation of pancreatin β-cells	Islam & Choi, 2008; Gram et al., 2007
Catechin	Withania somnifera	Root	Stimulation of glucagon uptake	Zanwar et al., 2014
Cinnamaldehyde	Cinnamomum zeylanicum	Bark	↓ insulin resistance	Qin et al., 2012; Park et al., 2011
Conophylline	Ervatamia microphylla	Leaf	Decreasing the fibrosis of pancreatic β-cell	Kawakami et al., 2010; Saito et al.,2012
Curcumin	Curcuma longa	Rhizome	Regulation of β-cell function and insulin resistance	Madkor et al., 2011; Meng et al., 2013; Lekshmi et al., 2012

Diphlorethoh ydroxycarma lol	Ishige okamurae	Seaweed	↓ insulin resistance Regulation of the hepatic glucose metabolic enzymes	Heo et al., 2009
Ellagitanin	Lagerstroemia speciosa	Leaf	↓ Glucogenesis	Stohs et al., 2012
Epigallocate chin-3-gallate	Camellia sinensis	Leaf	↓Glucogenesis ↓Insulin tolerance ↑Insulin scretion	Ortsäter et al., 2012; Wolfram et al., 2006;
Eugenol	Ocimum sanctum	Leaf	Reducing alkalin phosphatase, aspartate transminsae, alanine transaminase, lactate dehydrogenase cholesterol and triglyceride	Soto et al., 2004
Ferulic acid	Spinacia oleracea	Leaf	Inhibition of lipid peroxidation	Srinivasan et al., 2007
Genistein	Glycine max	Seed	Im[proves glucose and lipid metabolism	Tie et al., 2013; Fu et al., 2010
Gingerol	Zingiber officinale	Rhizome	↑ Insulin sensitivity	Li et al., 2012; Rani et al., 2011; Fritsche et al., 2010
Gymnemic acid	Gymnema sylvestre	Leaf	Inhibiting the sugar molecules uptake by the intestine	Baskaran et al., 1990
Kaempferitri n	Bauhinia forficata	Leaf	Stimulating the metabolism of peripheral glucose	Kavishankar et al., 2011
Kaempferol	Ginkgo biloba	Leaf	Promotes glucose metabolism in skeletal	Alkhalidy et al., 2018

			muscle, inhibits glucogenesis	
Karanjin	Pongamia pinnata	Fruit	Downregulate the IR cascade	Tamrakar et al., 2008
Kinsenoside	Anoectochilus roxburghi	Whole Plant	Repairing and regeneration of pancreatic β-cell	Zhang et al., 2007
Lepidine	Lepidium sativum	Seed	Potentiatial of insulin secretion	Gaikwad et al., 2014
Narigenin	Vitis vinifera	Fruit	GLUT-4 stimulated glucose uptake, ↑ AMPK phosphorylation	Ghosh et al., 2017
Nymphayol	Nymphaea stellata	Flower	Improving the generation of pancreatic β-cell	Guo et al., 2004; SubashBabu et al., 2009
Pelargonidin	Ficus benghalensis	Bark	Stimulating pancreatic βcells for secreting insulin	Zhang & Muller, 2000
Penta-Ogalloylglucopyranose	Lagerstroemia speciosa	Leaf	Antiadipogenic	Klein et al., 2007
Picrosides	Picrorhiza kurroa	Leaf	- Defending pancreatic β-cell - reducing serum lipid peroxides and blood urea nitrogen concentrations - Diabetes complications can be delayed or avoided.	Thakkar & Patel, 2010
Pongamol	Paullinia pinnata	Fruit	Downregulate the IR cascade	Tamrakar et al., 2008
Rosmarinic acid	Rosmarinus officinalis	Aerial parts	Inhibits sugar digestive enzymes	Ngo et al., 2018

Rutin	Aspalathus linearis	Stem	↑ insulin secretion and glucose uptake	Kawano et al., 2009; Lee et al., 2012
Silymarin	Silybum marianum	Seed	Improving the function of pancreatic β-cell	Rui, 1991; Soto et al., 2004
Tecomine	Tecoma stans		Stimulates glucose uptake in insulin-senitive and insulinresistant murine and human tissue	Al-Azzawi et al., 2012
Trigonelline	Trigonella foenumgraecum	Seed	Activation of enzyme related to glucose metabolism	Zhou et al., 2012
Vanillin	Gastrodia elata	Tuber	↓ insulin resistance	Subash Babu et al, 2007
3,5-Odicaffeoylqui nic acid	Ilex paraguariensis	Leaf	↑Serum GLP-1 level	Hussein et al., 2011; Chang et al., 2007
4- Hydroxyisole ucine	Trigonella foenumgraecum	Seed	Stimulation of insulin on pancreatic ilets	AvalsoSoriano et al., 2016

1.4.4 Importance of Cucurbitaceae family of plants as anti-diabetic agents

Various gourds and pumpkins may be found among the therapeutic plants of the Cucurbitaceae family. Typically, these plants are eaten as vegetables. These plants have many phenolic compounds, such as caffeic acid, chlorogenic acid, luteolin, apigenin, EGCG (epigallocatechin-3-gallate), gallic acid, cucurbitacins, quercetin, quercitrin, isoquercetin, ferulic acid, etc., that are present in their fresh fruits, leaves, tendrils, seeds, fruit and seed coatings and many of these substances, including luteolin, caffeic acid, and chlorogenic acid (Longvah et al, 2017), exhibit strong alpha-glucosidase inhibitory potential. Additionally, it has been demonstrated that several members of the cucurbitaceae family of plants have strong alpha-glucosidase inhibitory properties. Studies in both animal and human models have demonstrated that several types of cucurbits exhibit anti-diabetic effects comparable to those of frequently prescribed anti-diabetic medications, including hypoglycaemic effects and

stimulation of beta cell regeneration. It has also been demonstrated that a polysaccharide is at least one of the bioactive components that stimulates these effects. (Simpson et al, 2014). The two most well-known plants that have been proven to exhibit Alpha-glucosidase inhibitory capabilities are Coccinia indica (Tela-kucha) and Momordica charantia (Karela, Bitter Melon). For the first time, total phenolic contents (TPC) and antioxidant and -glucosidase activity in consecutive extracts of dried pulps from seven cucurbit fruit vegetables were measured. The chloroform extracts of Luffa acutangula and Benincasa hispida, respectively, had the highest levels of TPC and metal chelating activity (28.04 0.37 mg GAE/g extract and 0.44 0.03 mg/mL, EC50, respectively) (Ishikawa et al., 2007). The maximum 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was demonstrated by the ethyl acetate extract of Sechium edule (951.73 29.14 mm TE/g extract). Specified Cucurbitaceae seeds have anti-hyperglycemic globulins with a high amount of action. (Teugwa et al, 2013). possess very active anti-hyperglycemic globulins. The aqueous, glycosidic, and saponin extracts of Citrullus colocynthis (L.) Schrad., sometimes known as the bitter apple, were found to have anti-diabetic efficacy when administered orally to healthy rabbits. In both healthy and diabetic rats caused by streptozotocin (STZ), aqueous seed extract has been shown to have blood glucose reducing properties. It has been discovered that this plant extract has insulinotropic properties. Alcoholic leaf extract of Coccinia indica Wight & Arn has been observed to have hypoglycemic action (Vine gourd). Through inhibition of the essential gluconeogenic enzymes glucose-6-phosphatase and fructose-1, 6-bisphosphatase, it inhibits the production of glucose. After receiving therapy for 15 days, Momordica cymbalaria Fenzl ex Naudin fruit powder reduced blood sugar levels in fasting, alloxan-induced diabetic mice (Mukherjee et al, 2006). Additionally, cucumber (Cucumis sativus L.) exhibits strong anti-diabetic properties. It has been discovered to lessen the hepatic lipid peroxidation and serum glucose associated with diabetes. Additionally, it has been discovered to significantly reduce the area under the glucose tolerance curve and the hyperglycemic peak (Mukherjee et al, 2013). Alkaloids, palmitic, oleic, linoleic, and flavonoid acids are only a few of the many phyto-constituents that pumpkin possesses. Due to its diverse chemical composition, pumpkin has powerful anti-diabetic properties (Adams et al, 2011). The Momordica charantia methanol and ethyl acetate extracts showed the strongest reducing and anti-glucosidase activity. The TPC and DPPH values of S. edule showed the strongest connection (r2= 0.99). Although the methanol extract of Lagenaria siceraria included a significant amount of caffeic acid, isoquercetin was discovered to be the primary component that contributed to the activity. Gallic acid has been discovered to be the most effective

antioxidant component in the ethyl acetate extract of Sechium edule. (Sulaiman et al, 2013). This fruit family has been chosen to test the ability of the plants to inhibit the enzymes alpha-glucosidase and alpha-amylase as well as to determine the effectiveness of the plants *in-vivo* anti-diabetic effects. We will learn more about the effectiveness and safety of these plants as a result.

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Chapter 2

Scope, objective and plan of work

2.1. Scope and rationale of the study

2.2. Objective of the study

2.3. Plan of work

2.1. Scope and rationale of the study

Since ancient times, medicinal food plants have been used to prevent and treat diseases. At the moment, they are also being considered as a possible therapeutic development possibility (Mukherjee et al., 2015). According to several studies, eating enough fruits and vegetables can help maintain a healthy lifestyle and lower the possibility of metabolic disorders such as diabetes, hypertension, dyslipidaemia and obesity (Mihaylova et al., 2021). High fasting and postprandial blood glucose levels are a hallmark of the chronic metabolic disease known as diabetes (Modak et al., 2007). Approximately, 800 plants and their bioactive components may have anti-diabetic potential, according to ethno-pharmacological studies (Arulselvan et al., 2014). Since some herbs have therapeutic potential, the WHO additionally supports their use in the management of diabetes (Somtimuang et al., 2018). Several herbs, as well as those found in fruits, vegetables, and fungi (such as mushrooms) are a rich source of bioactive compounds, including alkaloids, carotenoids, fatty acids, flavonoids, phenolic acids, proteins, saponins, and terpenes which have been shown to have anti-diabetes potential (Papoutsis et al., 2021).

The use of natural medication grows worldwide and most people believe it to be safe (WHO, 2004; Zhang et al., 2015). Each herbal medication is an exacerbated combination of several active ingredients and they all have the potential to be contaminated, falsified or even include hazardous substances (Wachtel-Galor & Benzie, 2011; Mukherjee, 2019). Various bioactive substances found in medicinal plants may enhance interactions with conventional medications (Zhang et al., 2015). However, the safety of herbal medications is a concern for many doctors as well as regular people. Currently, there are many reported side effects from using herbs and natural remedies and these side effects are usually brought on by poor quality products or improper care (WHO, 2004). As herbal products gain popularity around the world, it is important to assess the quality and safety of the herbs taking into account the element of bioactive components variability (Xie et al., 2009; Mukherjee et al., 2019). However, the standardisation and chemo profile is essential for determining the most effective amount of bioactive components for herbs and herbal products (Mukherjee et al., 2015; Rivera-Mondragon et al., 2019). For qualitative features, many techniques including UV and IR have been used extensively. Whereas, LC-MS/MS, HPLC, GC-MS and HPTLC are utilised to quantitatively determine medicinal plants for quality control analysis (Balekundri & Munnur, 2020). The identification of the bioactive compounds discovered in

food plants as well as the validation of the standards for quality, efficacy, and safety are therefore absolutely necessary.

Natural therapies, such as herbal preparations and botanicals have been the main methods of treating diabetes for millennia despite the development of pharmaceutical agents for the condition. These methods have their own ideologies and practices (Xu et al., 2018). Furthermore, Herbal remedies are a common type of complementary and alternative medicine (CAM) that diabetics use (Seetaloo et al., 2018). The plant species include a number of active compounds that have anti-diabetic benefits and have very few, if any, detrimental side effects that have been observed. Numerous herbal extracts and phyto-molecules have demonstrated potent hypoglycaemic properties (Shori, 2015). Through tradition, knowledge has been passed down over time, and it is now fostering the development of phytochemicals with medicinal potential (Franco et al., 2018). Additionally, the WHO has endorsed the use of such herbs for the treatment of diabetes due to their potential for healing (Somtimuang et al., 2018). Approximately 800 plants and their bioactive constituents may have anti-diabetic potential, according to ethno-pharmacological studies (Arulselvan et al., 2014). For example, Blood sugar levels are traditionally lowered by consuming juice from the bitter gourd (*Momordica charantia* L) fruit and leaves in a number of nations, including the Indian subcontinent and other African nations. Because they have few undesirable side effects, herbs and food plants can therefore be used instead of or in addition to conventional treatments (Seetaloo et al., 2018).

In the Cucurbitaceae family, 125 genera and 960 species of vegetable and fruit crops are included (Mukherjee, 2019). This family of food plants has widespread global consumption. It is well known that these food plants have a significant role in Indian traditional medicine, including Ayurveda and other healthcare systems (Renner and Pandey, 2013). According to ethno-botanical research, the ethnic communities of India and Bangladesh recommend Cucurbitaceae food plants and their preparations for the treatment of several kinds of diseases (Rahmatullah et al., 2012). These edible plants have a variety of purported therapeutic uses viz. *Cucumis melo* L. (Melon), *Cucumis sativus* L. (Cucumber), *Lagenaria siceraria* (Bottle gourd), Benincasa hispida (Wax Gourd), *Luffa acutangula* (Ridge gourd), etc (Dhiman et al., 2012; Saboo et al., 2013; Avinash and Rai, 2017). Traditionally, *Solena heterophylla* fruits are widely available in North East India, where the fruits are known as “Golkakri” in Sikkim. The leaves of these fruits are applied to irritated skin, and the root juice has historically been used to treat dysuria and spermatorrhoea, as well as the common cold, infant pneumonia,

throat discomfort, and fever. This whole herb is used as a folk remedy for diabetes by the tribal doctors of Sikkim in India (Janbaz et al., 2015). Furthermore, tribal people with diabetes, high blood pressure, arteriosclerosis, circulatory issues or high blood cholesterol are advised to use the fruit juice or infusion of *Cyclanthera pedata* as a treatment. Boiling fruit and leaves in olive oil is applied externally as a topical analgesic and anti-inflammatory. Intestinal parasites can be treated using dried and powdered seeds. For the treatment of diabetes, leaves are made as a decoction since they are thought to be hypoglycemic (Zuccolo et al., 2023).

An important plant family in terms of both medicine and commerce is the "Cucurbitaceae" (Bajcsik et al., 2017). There are 12 different Cucurbitacins (A-T) in this family, which are well-known for their bitterness and toxicity (Chen et al., 2005; Zhang et al., 2019). Therefore, some unknown genetic factors are responsible for the variety of Cucurbitacins found in stronger cucurbits (Zhou et al., 2016). A class of tetracyclic triterpenes with high oxygen content is known as Cucurbitacins (Bajcsik et al., 2017). In the traditional system, the cucurbitacin enriched medications are used to treat conditions like diuresis, emesis, and malaria (Zhang et al., 2019). Cucurbitacins have been studied both *in-vivo* and *in-vitro*, and they show a variety of pharmacological activity depending on the cells they interact with including anticancer, hepatoprotective, antibacterial, cardiac and anti-diabetic effects (Alghasham, 2013).

Among others, cucurbitacin B (Figure 1.6) and cucurbitacin B tetracyclic triterpenoids are the most common curcurbitacins identified in the food plants of Cucurbitaceae family. The natural substance cucurbitacin B (CuB) is widely dispersed. CuB has been identified in a wide range of plants, including *Cucumis melo*, *Cucurbita andreana*, *Ecballium elaterium*, *Wilbrandia ebracteata*, and *Trichosanthes cucumerina*. It is primarily found in plants in the Cucurbitaceae and Cruciferae families (Dai et al., 2023, Cai et al., 2015, Chen et al., 2005, Garg et al., 2018).

Various pharmacological properties of CuB, including anti-inflammatory, antioxidant, antiviral, hypoglycemic, hepatoprotective, neuroprotective, and anti-cancer effects, have been documented in studies (Garg et al., 2018, Yesilada et al., 1988, Lin et al., 2019, Park et al., 2015, Yang et al., 2020, Kim et al., 2018, Lu et al., 2012, Chen et al., 2008) which have a major role in the diagnosis, treatment, and prevention of a number of illnesses, including cancer, diabetes, neurodegenerative diseases, and inflammatory diseases. Researchers have

focused more on CuB than other classes of cucurbitacin since it is the most prevalent and active type of cucurbitacin (Sallam et al., 2018, Shahiduzzaman et al., 2020).

The formation and presence of plant bioactive compounds, sometimes referred to as marker compounds, are important factors in determining or validating the pertinent relationship of a specific plant (Mukherjee, 2019). It is apparent that genotypic variances, external growing factors (climate, temperature, soil, fertilisers, and irrigation), and plant growth conditions cause differences in the content of phyto-markers within species, groups and variants (Ibrahim et al., 2019; Zhang et al., 2019; Devendra et al., 2011; Rehm et al, 1957). For quality control of these fruits for safe therapeutic application, it is crucial to comprehend the variation of cucurbitacin B content from different types of "Cucurbitaceae" food plants.

The purpose of this study was to evaluate marker profiling based on the aforementioned literature reports and ethno-pharmacological relevance (HPTLC and LC-QTOF-MS) and anti-diabetic evaluation of the selected food plants *Solena heterophylla , Cyclanthera pedata* fruits which explore the molecular mechanism through modulation of oxidative stress and glycosylated hemoglobin (HbA1C). Furthermore, the anti-diabetic activity of the selected fruits was performed through *in-vitro* carbohydrate metabolizing enzymes (α-amylase and α-glucosidase) assay and *in-vivo* study of biochemical, tissue antioxidant and histo-pathological screening on low dose streptozocin and high fat diet induced diabetic rats and combination synergy through high fat diet induced diabetic wound healing mice model.

2.2. Objective of the study

The current study aimed to assess phytomarkers and metabolite profiles of anti-diabetic assessment via interactions between the α-amylase and α-glucosidase enzymes of several medicinal plants from the Cucurbitaceae family. *In-vivo* models were also used to assess the anti-diabetic potential of the chosen fruit extracts. The goal of the study was to determine which fruits were most effective at preventing diabetes when used as a treatment. The scientific community may find this study helpful in determining the importance and efficacy of the variety of medicinal plants selected. The schematic diagram of the workflow is displayed in the **Fig 2.1.**

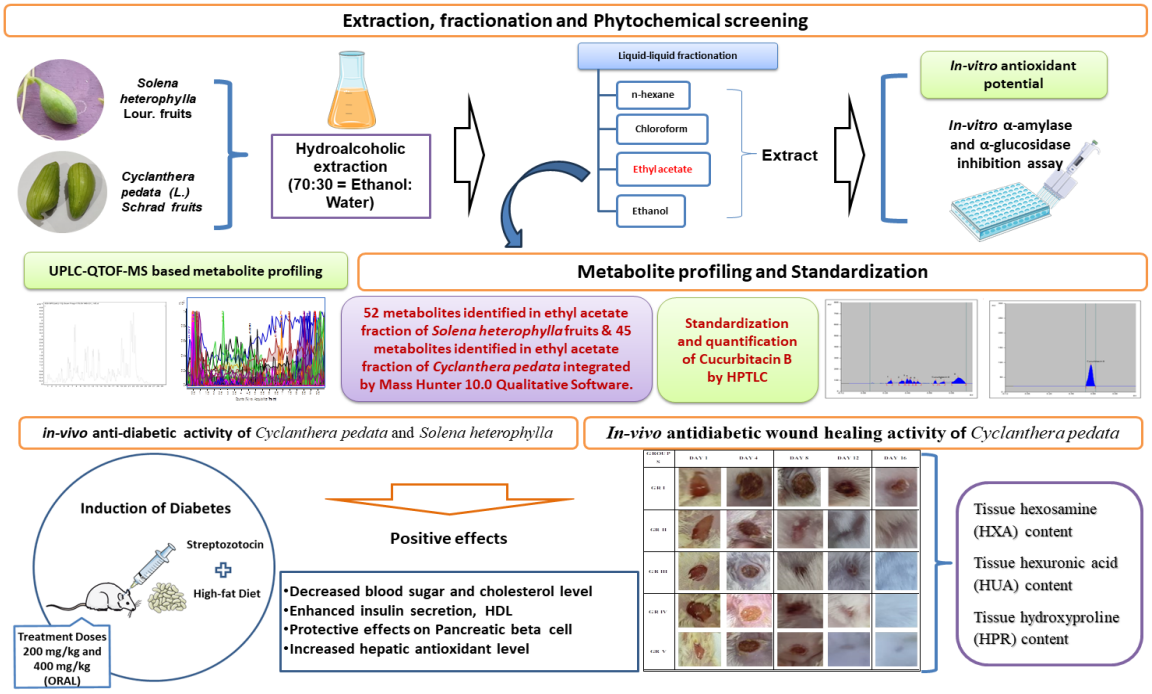

Fig 2.1. Schematic diagram of the workflow

2.3. Plan of work

In this study, the specific plan of work was as follows:

- Literature review and plant profile of the selected food plants
- Selection of the food plants on the basis of traditional uses
- Collection and authentication of the food plants and their fruits
- Extraction of the selected fruits with suitable solvent
- Metabolite profiling, marker analysis and standardization of the selected food plant extracts
    - ❖ Liquid Chromatography-Quadrupole Time of Fly-Mass Spectrometry (LC-QTOF-MS)
    - ❖ High Performance Thin Layer Chromatography (HPTLC)
- Evaluation of anti-diabetic potential of the selected food plant extracts by using
    - ❖ *In-vitro* carbohydrate metabolizing enzymes assay

- α-amylase enzyme inhibition assay
- α-glucosidase enzyme inhibition assay

- Evaluation of *in-vivo* anti-diabetic study of the plant fruit extracts
  - Serum biochemical parameters
  - Tissue antioxidant parameters
  - Histopathological analysis
- Formulation development
  - Preparation of the formulation
  - Acute skin irritation test
- *In-vivo* wound healing study

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Chapter 3

Plant profile, collection, authentication and extraction

3.1. *Solena heterophylla* Lour.

3.1.1. Taxonomy

3.1.2. Plant description

3.1.3. Traditional use of *S. heterophylla*

3.1.4. Pharmacological activities of *S. heterophylla*

3.1.5. Collection and authentication of plant materials

3.1.6. Extraction and fractionation of plant material

3.2. *Cyclanthera pedata* (L.) Schrad

3.2.1. Taxonomy

3.2.2. Plant description

3.2.3. Traditional use of *C. pedata*

3.2.4. Pharmacological activities of *C. pedata*

3.2.5. Collection and authentication of plant materials

3.2.6. Extraction and fractionation of plant material

3.1. *Solena heterophylla* Lour.

3.1.1. Taxonomy

Scientific classification	**Vernacular names**
Kingdom: Plantae	Sanskrit : Amlavetasa
Division : Magnoliophyta	Hindi : Amantamul
Class : Magnoliopsida	English : Creeping cucumber
Order : Cucurbitales	Mizoram : Nauawimuhrui, Zawng-awmpawng
Family : Cucurbitaceae	Manipuri: Lam sebot
Genus : Solena	Marathi : Jalnaveri
Species : *Solena heterophylla*	Sikkim: Golkakri

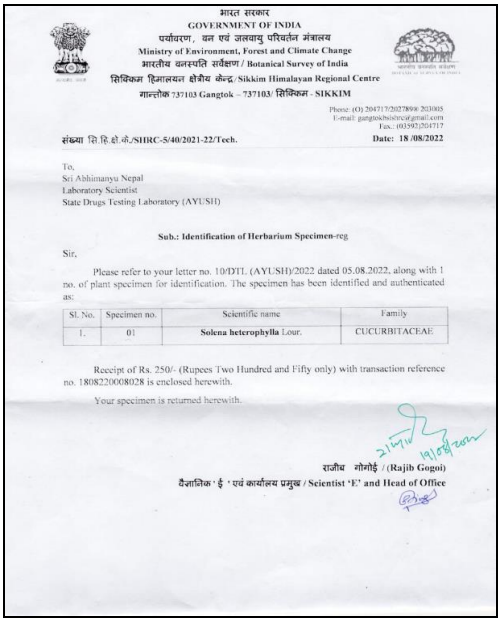
3.1.2. Plant description

Solena heterophylla fruits are widely available in North East India, where the fruits are known as "Golkakri" in Sikkim. It is a climber with stem and branches hairless. Leaf-stalks are slender, 4-10 mm. Leaves are very variable, ovate, oblong, ovate-triangular, or arrow-shaped, undivided or 3-5-lobed, leathery, 8-12 x 1-5 cm, below densely bristly or almost hairless, above densely bristly or scabrous, base heart-shaped, margin entire or toothed, tip blunt or tapering. Tendrils are slender. Mainly, male flowers are borne in umbels, on very short flower-cluster-stalks, 10-20 flowered. Flower-stalks are 2-8 mm; calyx tube 3-5 mm, about 3 mm in diameter; segments slender pointed, 0.2-0.4 mm. Flowers are yellow or yellow-white; petals triangular, 1-1.5 mm, tip blunt or pointed; filaments thread-like, about 3 mm. Female flowers are usually solitary; flower-stalk 2-10 mm, finely velvet-hairy; calyx and flower as in male flowers. Fruit is red-brown, broadly ovoid, oblong, or nearly spherical, 2-6 x 2-5 cm. Himalayan Creeping Cucumber is a found in mixed forests, thickets, grasslands, roadsides, mountain slopes, at altitudes of 600-2600 m, in NE Afghanistan,

Himalayas to Bhutan and SE Asia. It is also widely found in rest of India (Anonymous, 2019).

Fig 3.1. Fruit part of *S. heterophylla*

Fig 3.2. Flowering plant of *S. heterophylla*



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संख्या बि.हि.क्षे.के./SHRC-5/40/2021-22/Tech. Date: 18/08/2022

To,
Sri Abhimanyu Nepal
Laboratory Scientist
State Drugs Testing Laboratory (AYUSH)

Sub.: Identification of Herbarium Specimen-reg.

Sir,

Please refer to your letter no. 10/DTL (AYUSH)/2022 dated 05.08.2022, along with 1 no. of plant specimen for identification. The specimen has been identified and authenticated as:

Sl. No.	Specimen no.	Scientific name	Family
1.	01	**Solena heterophylla** Lour.	CUCURBITACEAE

Receipt of Rs. 250/- (Rupees Two Hundred and Fifty only) with transaction reference no. 180822000028 is enclosed herewith.

Your specimen is returned herewith.

राजीव गोगोई / (Rajib Gogoi)
वैज्ञानिक 'ई' एवं कार्यालय प्रमुख / Scientist 'E' and Head of Office

Fig 3.3. Identification certificate of herbarium specimen

3.1.3. Traditional use of *S. heterophylla*

The Hani people in Yunnan, thousands of kilometres distant, utilise a decoction of *S. heterophylla* tuber to relieve stomach aches. The Sherpas just discovered the use of *S. heterophylla* as food (Ding et al., 2021). The leaves are applied to irritated skin, while the root juice has been used to treat dysuria and spermatorrhoea. This plant's many components are used to treat rheumatism, respiratory issues and toothaches, anti-malarial, anti-diabetic, analgesic, sedative, and purgative effects. The fruits have historically been used to cure

common colds, child pneumonia, throat soreness, and fever (Janbaz et al., 2015).The leaf juice is applied to treat wounds by some native rural folks (Handoo et al., 2006).The roots of the plant are boiled with water and made into a decoction which is consumed to treat cough, fever and headache in certain regions of Vietnam (Nguyen et al., 2020).

3.1.4. Pharmacological activities of *S. heterophylla*

- In the Indian system of medicine, various parts of this plant possess anti-malarial, anti-diabetic, analgesic, sedative and purgative properties and used to treat toothache, rheumatism and respiratory disorders (Bhattarai et al., 1989; Bhattari et al., 1993).
- In the report, the fruits have traditionally been used in the management of common cold, child pneumonia, throat pain and fever; the leaves are applied over inflamed skin, whereas root juice has been used to treat dysuria and spermatorrhoea (Khare et al., 2007; Rajbhandari et al., 2001).
- In other report, scientific investigations on plant extract revealed its hepatoprotective potential, while coumarin and flavonoids isolated from plant were found to inhibit platelet aggregation (Iman et al, 2006; Kunwar et al, 2006; Kunwar et al, 2010).
- In the scientific report shows that recent study has reported *in-vitro* and *in-vivo* antioxidant activity of methanolic extract of *Solena heterophylla* plant (Venkateshwarlu et al, 2011).
- Other report shows that *S. heterophylla* has traditionally been used for the management of gastrointestinal, respiratory and cardiovascular ailments (Pant et al., 2010).

3.1.5. Collection and authentication of plant materials

The fruits of this plant are *Solena heterophylla* Lour. were collected in July 2020 from South Sikkim, Bermiok, India. The Botanical Survey of India (Ministry of Environment, Forest, and Climate Change; Govt of India), Gangtok, Sikkim, India verified and validated the plant. The voucher specimen (SHRC-5/40/2021-22/Tech) was preserved at the laboratory for future reference.

3.1.6. Extraction and fractionation of plant material

Solena heterophylla fresh fruits were washed then shade dried and mechanically crushed and stored in an airtight container. Crushed dried fruit (500 g) was extracted with the maceration extraction method in 70:30 aqueous ethanol for 3-5 days at room temperature. Then liquid-

liquid fractionation was performed using 800 mL each of ethanol, n-hexane, chloroform, ethyl acetate, and each of their polar counterparts. Rotary evaporator (Eyela Rotary Evaporator, Japan) was used for the evaporation of the fractions and percentage yield of each fraction i.e. hexane (HFSH), chloroform (CFSH), ethyl acetate (EASH) and ethanol (EFSH) fractions were, respectively 2.8, 10.3, 12.6 and 13.29% (w/w) respectively. Before analysis, the samples were kept at 4°C in borosilicate glass vials.

3.2. *Cyclanthera pedata* (L.) Schrad

3.2.1. Taxonomy

Scientific classification	**Vernacular names**
Kingdom : Plantae	Hindi : Meetha karela
Division : Magnoliophyta	English : Stuffing Cucumber
Class : Dicotyledonae	Spanish : Caigua
Order : Violales	German : Korila
Family : Cucurbitaceae	French : Achocha
Genus : Cyclanthera	Nepali : Barela
Species : *Cyclanthera pedata*	

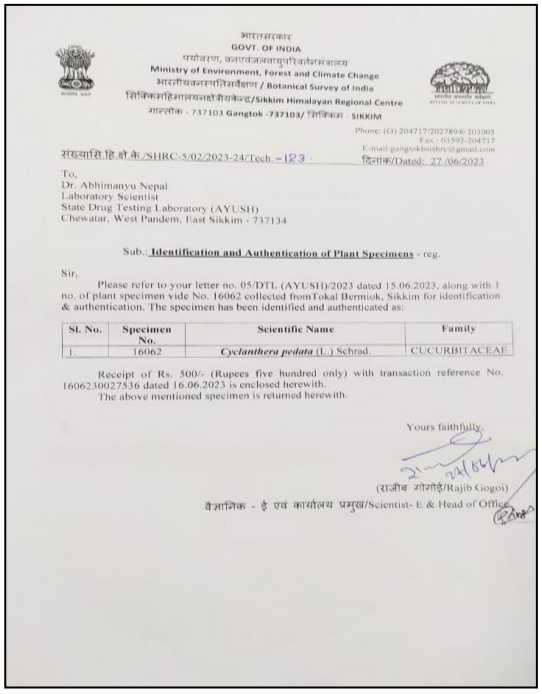
3.2.2. Plant description

Cyclanthera pedata is a climbing plant which fruits are also known as “Chuchay Karela” in north east region of India. These plants have thin stems and leaves that can grow up to 24 cm long and have either a palmate or pedate shape. The vine can reach a maximum length of 12 meters. The tiny blooms are carried in racemes and can be either white or greenish. The fruit is light yellow-green with somewhat darker veins, measuring 5–15 cm in length and 3–8 cm in width. It is irregularly ovoid and pointy at the ends. The fruit flesh is only 0.5 cm thick, soft and spongy rather than crunchy, and is reported to taste like cucumbers. The seeds are 12 x 7 x 3 mm and have a matt black colour. It is reported that the seeds are edible (Anonymous

2008). "Caigua" fruits can be eaten both raw and cooked. The older, riper fruits can be stuffed with meat or fish and eaten raw, such as in salads. Young leaves and sprouts can both be eaten (Monigatti et al, 2013).

3.4. Fruit part of *C. pedata*

3.5. Flowering plant of *C. pedata*



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संख्यासि हि बो के./SHRC-5/02/2023-24/Tech. –129 · दिनांक/Dated: 27 /06/2023

To,
Dr. Abhimanyu Nepal
Laboratory Scientist
State Drug Testing Laboratory (AYUSH)
Chewatar, West Pandem, East Sikkim - 737134

Sub.: **Identification and Authentication of Plant Specimens** - reg.

Sir,

Please refer to your letter no. 05/DTL (AYUSH)/2023 dated 15.06.2023, along with 1 no. of plant specimen vide No. 16062 collected fromTokal Bermiok, Sikkim for identification & authentication. The specimen has been identified and authenticated as:

Sl. No.	Specimen No.	Scientific Name	Family
1.	16062	*Cyclanthera pedata* (L.) Schrad.	CUCURBITACEAE

Receipt of Rs. 500/- (Rupees five hundred only) with transaction reference No. 1606230027536 dated 16.06.2023 is enclosed herewith.
The above mentioned specimen is returned herewith.

Yours faithfully,

(राजीब गोगोई/Rajib Gogoi)
वैज्ञानिक - ई एवं कार्यालय प्रमुख/Scientist- E & Head of Office

3.6. Identification certificate of herbarium specimen

3.2.3. Traditional use of *C. pedata*

Cyclanthera pedata is suggested that those with high blood pressure, arteriosclerosis, diabetes, circulatory issues and high blood cholesterol use fruit juice or infusion as a treatment. Boiling fruit and leaves in olive oil can be applied topically as a topical analgesic

and anti-inflammatory. Intestinal parasites can be treated with seeds that have been dried and ground into powder. For the treatment of diabetes, leaves are prepared as a decoction because they are hypoglycaemic (Antih et al., 2016). "Caigua" has recently drawn interest from the pharmaceutical industry (Frigerio et al. 2021). Various effects have been ascribed to this plant, with the most intriguing being its ability to reduce hypertension (Ranilla et al. 2010) and have hypoglycaemia and hypercholesterolemia (Egg 1999; Gonzales et al. 1995; Ranilla et al. 2010) qualities in addition to analgesic and antioxidant properties (Vásquez 2003; Garcìa et al. 2010).

3.2.4. Pharmacological activities of *C. pedata*

- Anti-inflammatory property was evaluated from the ethanolic extract of *C. pedata* fruit by *in-vitro* lipoxygenase enzyme assay (Rivas et al., 2013).
- The antioxidant potential of the *C. pedata* fruits and leaves were analysed through HPTLC, HPTLC-MS and HPTLC-DPPH approach (Orsini et al., 2019).
- By using affinity chromatography on immobilised chymotrypsin in the presence of 5 M NaCl and preparative native PAGE at pH 8.9, seven novel trypsin inhibitors, CyPTI I - VII, were isolated from mature seeds of *Cyclanthera pedata* (Kowalska et al., 2006).
- The *C. pedata* has shown anti-hypertensive effect by inhibiting the hypertension associated Angiotensinogen-I converting enzyme by Angiotensin I-converting enzyme (ACE) inhibition assay (Ranilla et al ., 2010).
- The *C. pedata* has proved to be beneficial in treating hypertriglyceridemia in adult women (Torres et al., 2013).
- Serum lipoprotein levels in post-menopausal women have been found to be alleviated by consumption of the *C. pedata* fruit (Gonzales et al., 1995).

3.2.5. Collection and authentication of plant materials

Cyclanthera pedata, the plant fruit, was harvested in January 2023 in South Sikkim, Bermiok, India. The plant was confirmed and approved by the Botanical Survey of India (Ministry of Environment, Forest, and Climate Change, Government of India), located in Gangtok, Sikkim, India. For future use as a guide, the voucher specimen (SHRC-5/02/2023-24/Tech-123) was kept in the lab.

3.2.6. Extraction and fractionation of plant material

Fresh fruits of *Cyclanthera pedata* were cleaned, dried in the shade, mechanically crushed, and then sealed in an airtight vessel. For three to five days at room temperature, 500 gm of crushed dried fruit were extracted using the maceration extraction technique in 70:30 aqueous ethanol. Then, 800 ml of ethanol, n-hexane, chloroform, ethyl acetate, and each of their polar equivalents were used for liquid-liquid fractionation. Hexane (HFCP), chloroform (CFCP), ethyl acetate (EACP), and ethanol (EFCP) were the fractions that were evaporated, along with the % yield of each component, using a rotary evaporator (Eyela Rotary Evaporator, Japan). The corresponding fractions were 2.5, 9.3, 11.5, 12.17% (w/w). The samples were stored in borosilicate glass vials at 4°C before to analysis.

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Chapter 4

In-vitro antidiabetic activity & free radical scavenging assay

4.1. Introduction

4.2. Material and Methods

4.2.1 Drugs and chemicals

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4.1. Introduction

Free radicals are ubiquitous in our body and are generated by normal physiological processes, including aerobic metabolism and inflammatory responses, to eliminate invading pathogenic microorganisms (Hussain et al., 2003). Naturally occurring in the body, free radicals are crucial to many regular cellular functions. Free radicals also may damage all the primary cell components, including DNA, proteins, and cell membranes if found in high adequate concentrations to be toxic to the body. The damage to cells caused by free radicals, especially the damage to DNA, may play a vital role in the development of cancer and other health disorders like diabetes, liver damage, nephrotoxicity, inflammation, neurological, cardiovascular disorders and aging (Valko et al., 2007; Diplock et al., 1998).

Oxidation is transferring electrons from one atom to another. It is an essential part of aerobic life and human metabolism because oxygen is the final electron acceptor in the electron flow system that generates ATP, which is the energy source for aerobic life. Free radicals are two types: known as "reactive oxygen species" (ROS) and "reactive nitrogen species" (RNS) (Bala et al., 2009). They contents superoxide (O^{2-}), peroxyl (ROO), alkoxyl (RO), hydroxyl (HO), and nitric oxide (NO). The hydroxyl (half-life of 10^{-9} sec) and the alkoxyl free radicals are very reactive and rapidly attack the molecules in nearby cells. On the other hand, the superoxide anion, lipid hydro peroxides and nitric oxide are less reactive (Ames et al., 1993). In addition to these ROS radicals, in living organisms there are other ROS nonradicals, such as the singlet oxygen ($^1O2$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). It has been determined that ROS function significantly in living things. Some are positive and are related to their involvement in energy production, phagocytosis and regulation of cell growth, intercellular signalling and synthesis of biologically important compounds (Halliwel, 1987). Reactive oxygen species have the potential to cause significant harm, as they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates, and DNA. This may cause oxidations that damage membranes, adapt proteins, including enzymes, and damage DNA. These oxidations have been associated with cancer, atherosclerosis, aging, immunosuppression, inflammation, ischemic heart disease, diabetes, hair loss, and neurodegenerative diseases like Alzheimer's and Parkinson's disease (Blake et al., 1995).

Mammalian cells have complex defense systems for detoxifying radicals. Important metabolic processes include the dismutase (SOD)-catalyzed breakdown of superoxide into hydrogen peroxide (H2O2) and oxygen, as well as the catalase (CAT) and glutathione peroxidase (GPX)-catalyzed conversion of H2O2 into water and oxygen, which eliminates

toxic peroxides. Several small-molecule antioxidants, besides antioxidant enzymes, are essential components of the antioxidant defense systems that can prevent or repair damage caused by oxygen to the body's cells by delaying or inhibiting the oxidation of lipids or other molecules by preventing the start or propagation of an oxidative chain reaction (Velioglu et al., 1998; Hall et al., 1997).

Antioxidants are classified into two main categories: synthetic and natural. Ascorbic acid and carotenoids are examples of natural antioxidants, while synthetic antioxidants are typically compounds with phenolic structures of varying degrees of alkyl substitution. Phenolic compounds include tocopherols, flavonoids and phenolic acids, nitrogen compounds include alkaloids, chlorophyll derivatives, amino acids, amines and carotenoids (Hudson et al., 1990; Hall et al., 1997). Antioxidants, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are synthetic compounds that have been utilized since the turn of the century. However, because of these compounds' carcinogenicity, limitations are placed on their use (Karmakar et al., 2011). Natural antioxidants have seen a significant rise in popularity. Procyanidins, anthocyanins, flavonoids, catechins, phenolic acids, procyanidin diterpene, vitamin E and vitamin C are examples of plant-derived antioxidants that are being proposed as significant dietary components more and more. Some suggest that daily fruit and vegetable consumption be increased, as these foods are high in nutrients that reduce the risk of chronic health issues linked to the aforementioned disorders (Klipstein-Grobusch et al., 2000).

Diabetes, excess weight, and dental disorders a disturbance of the absorption of carbohydrates is linked to serious health issues. The growing global population is at risk due to these serious health issues (Michelle de Sales et al., 2012). Carbohydrates are the usual source of simple sugars, or monosaccharides, which are absorbed by the intestine. Antidiabetic medication involves reducing the amount of glucose produced and absorbed by the gastrointestinal tract. After a mixed carbohydrate diet, the enzymes α-amylase and α-glucosidase primarily aid in the digestion of carbohydrates, leading to an increase in postprandial blood glucose. Blood glucose levels can be regulated by inhibiting the α-amylase and α-glucosidase enzymes (Thilagam et al., 2013). The primary sources of the enzyme amylase are saliva and pancreatic juice. When calcium is present, the calcium metalloenzyme α-amylases function. The primary hydrolyzing agent for α-1,4 glycosidic bonds found in starch, amylopectin, amylase, glycogen, and other malto dextrins is α-amylase, the most significant digestive enzyme. It is also in charge of starch digestion

(Agrawal et al., 2016). The α-glucosidase enzyme cannot hydrolyze maltase, sucrose, and other disaccharides found on the brush border of the small intestine. This enzyme prevents disaccharides from being converted to monosaccharides. Anti-diabetic medication α-glucosidase is effective because it delays the absorption of carbohydrates, hence improving postprandial hyperglycemia (Sugihara et al., 2014). α-glucosidase and α-amylases, which are involved in the metabolism and digestion of carbohydrates, considerably lowered blood sugar levels. In patients with type 2 diabetes, it may be one of the most crucial strategies for controlling their postprandial blood glucose levels (Subramaniam et al., 2008).

The goal of this chapter was to assess the fruits of the selected plants that were chosen for their antioxidant potential and *in-vitro* antidiabetic activities using various *in-vitro* models.

4.2. Material and Methods

4.2.1. Drugs and chemicals

1, 1-Diphenyl-2-picrylhydrazyl (DPPH), α-glucosidase (50 µl, 0.5 U/ml) and 0.2 M K3PO4 buffer (pH 6.8, 50 µl), Acarbose, α amylase, PNPG, starch, Na2CO3, Iodine solution were procured from Sigma Chemicals, USA. Sisco Research Laboratories Pvt. Ltd., Mumbai, India is the source of the following products: nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), sodium nitroprusside, napthyl ethylene diamine dihydrochloride, ascorbic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylene diamine tetra acetic acid (EDTA), sodium hydroxide (NaOH), hydrogen peroxide (H2O2), butylated hydroxy anisole (BHA), deoxyribose, potassium ferricyanide [K3Fe(CN)6], butylated hydroxy anisole (BHA), deoxyribose, and Folin-Ciocalteu's phenol reagent (FCR). High analytical grade chemicals were utilized for all other compounds.

4.2.2. Equipment used

Digital balance (Shimadzu), 96-well microwell assay plate (Tarsons), Micropipette & microtips (Eppendorf and Accupipet), Membrane filters 0.45µ & 0.22µ (Millipore), Spectrophotometer (Spectramax ID3).

4.2.3. Test sample preparation

Different concentrations of test samples were prepared from stock solution 1mg/ml and standard (acarbose) was prepared in phosphate buffer (100 mM, pH: 6.8).

4.2.4. α-amylase inhibition assay

α-amylase inhibition assay was conducted according to the stated protocol by Jana et al., 2024. In pre-labelled test tubes, different concentrations of *S. heterophylla* and *C. pedata* partitioned fractions and standard marker acarbose (50, 100, 300 and 500 g/ml) were made. Each test tube received 20µl of α-amylase which was then added and 10 minutes of incubation at 37 °C. Each test tube received 200µl of 1% starch solution after the initial incubation and the combination was then again set for 1 hour at 37°C. Each test tube was filled with 10 ml of distilled water and 200µl of 1% iodine solution. At 565 nm, the mixture absorbance was measured. The samples, substrate and α-amylase blank were performed under identical circumstances. Each experiment was carried out three times. The value of the IC50 was calculated by regression analysis.

4.2.5. α-glucosidase inhibition assay

The test samples were combined with 50 µL of α-glucosidase (0.5 U/ml), 50 L of 0.2 M K3PO4 buffer (pH 6.8) and 50µL of the buffer. After a 15-minute pre-incubation period at 37°C, 3mM PNPG (100µl) was added. After being allowed to continue for 10 minutes at 37°C, the enzymatic process was stopped by adding 750µL of 0.1 M Na2CO3. A spectrophotometer was used to detect the 4-nitrophenol absorption at 405 nm. As a control, a solution without the samples were employed and as a blank solution without the substrate. As a standard reference, the anti-diabetic medication acarbose was also measured (Jana et al., 2024).

Inhibitory activity (%) = $(1 - A_S/A_C) \times 100$
Where,
A_S= absorbance of the sample
A_C = absorbance of control

4.3. Free radical scavenging assay

4.3.1. DPPH radical scavenging activity

Based on a technique developed from Jana et al., 2024, the DPPH free radical scavenging assay was used to evaluate the antioxidant capacities of *S. heterophylla* and *C. pedata* fruit fractions. In this test, a 96-well microplate, the sample and solution of DPPH (0.2 mg/ml) were combined and left to stand for 10 minutes at room temperature and in the dark. The reduced absorbance of the sample solution at 517 nm was discovered using a

spectrophotometer. As a reference marker, ascorbic acid was employed. The free radical scavenging potential was represented in terms of IC50 value (µg/ml).

Percentage inhibition = [(C - T) / C] × 100

Where,
C = Absorbance of the control and
T = Absorbance of the test

4.4. Statistical Analysis

All the values were expressed as the mean ± SD. Statistically significant (p) value was calculated by using one way ANOVA followed Dunnett's multiple comparison test using Graph pad prism 8.1.1 (Graph Pad Software, San Diego USA), where $p< 0.05$ is considered as statistically significant.

4.5. Results

Table 4.1. IC_{50} values of *in-vitro* antidiabetic assay of selected plant fruit extracts

Type of Extract	α-amylase inhibitory activity IC_{50}(mg/ml)	α-glucosidase inhibitory activity IC_{50}(mg/ml)
EACP	0.531 ± 0.32	0.116 ± 0.21
CFCP	0.864 ± 0.58	0.142 ± 0.36
HFCP	1.052 ± 0.57	0.157 ± 0.62
EFCP	0.729 ± 0.19	0.119 ± 0.82
EASH	0.578 ± 0.51	0.118 ± 0.34
HFSH	1.130 ± 0.25	0.161 ± 0.41
CFSH	0.948 ± 0.63	0.150 ± 0.27
EFSH	0.778 ± 0.16	0.122 ± 0.75
Acarbose	0.374± 0.73	0.055 ± 0.52

Table 4.2. IC_{50} values of antioxidant assay of selected plant fruit extracts

Type of Extract	DPPH IC_{50} (mg/ml)
EASH	0.102 ± 0.19
EACP	0.97 ± 0.26
Ascorbic acid	0.72 ± 0.15

Each value expressed as mean± standard deviation (n = 3)

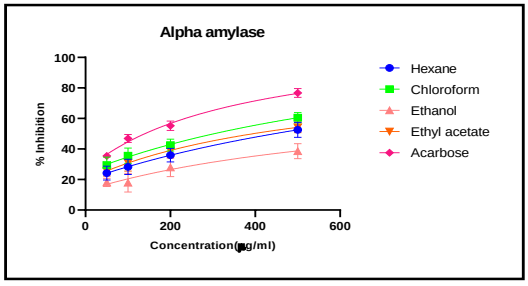

Fig 4.1. % Inhibition of alpha amylase shown by different fractions of *C.pedata* and Standard.

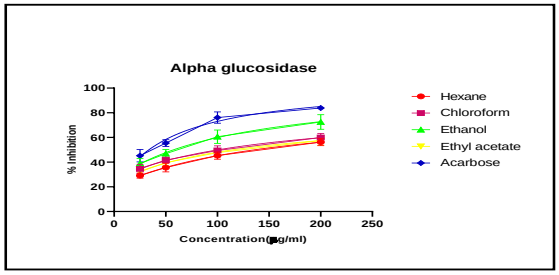

Fig 4.2. % inhibition of alpha glucosidase shown by different fractionss of *C.pedata* and Standard.

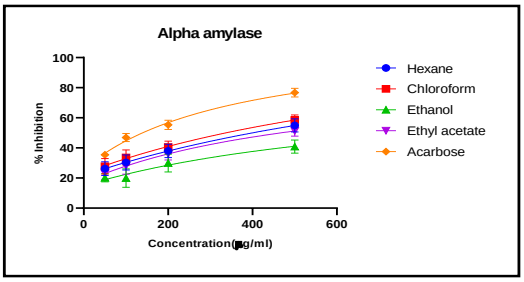

Fig 4.3. % Inhibition of alpha amylase shown by different fractions of *S.heterophylla* and Standard.

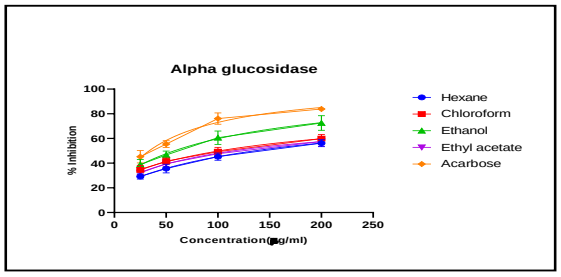

Fig 4.4. % Inhibition of alpha glucosidase shown by different fractionss of S.heterophylla and Standard.

In-vitro antidiabetic Activity

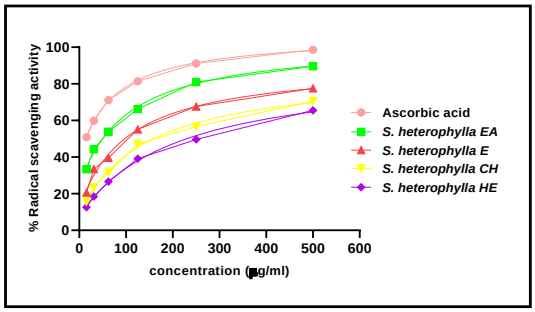

Fig 4.5. 1,1-diphenyl-2-picrylhydrazil (DPPH) scavenging activity of different fractions of *S.heterophylla* and standard.

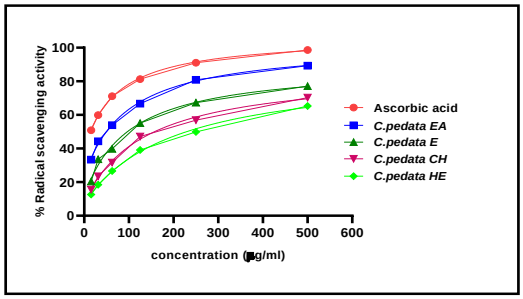

Fig 4.6. 1, 1-diphenyl-2-picrylhydrazil (DPPH) scavenging activity of different fractions of *C. pedata* and standard.

Antioxidant Activity

4.6. Discussion

Numerous nutritional supplements that contain flavones, polyphenols, or vitamins are also important in this regard. Since they have an antioxidant effect by neutralizing lipid free radicals or stopping the breakdown of hydroperoxides into free radicals, phenolic compounds are crucial components of plants (Saumanya et al., 2014).

Scavenging free radicals, inhibiting the enzymes that produce free radicals, boosting the antioxidant system, or focusing on the signalling pathways and molecular expression of the inflammatory cascade are some of the therapeutic strategies that can reduce oxidative stress. Many synthetic medications have negative side effects despite providing oxidative damage protection. Using food-based natural antioxidants as conventional medicines is an alternate way to solve this problem (Karmakar et al., 2011).

DPPH is a stable free radical that can turn into the stable diamagnetic compound hydrazine by accepting an electron or hydrogen radical. A drop in absorbance at 517 nm shows that the DPPH radical has reacted with appropriate reducing agents, which causes electrons to pair off and the solution to turn yellow stoichiometrically with the number of electrons taken up (Rajani and Ashok, 2009; Bajpai et al., 2014).

Pancreatic α-amylase and α-glucosidase are essential enzymes for the metabolism of carbohydrates in the small intestine, as they transform ingested polysaccharides into monosaccharides. Because of this enzyme's action, the small intestine absorbs produced glucose from polysaccharides, raising blood glucose levels after meals (Ramachandram et al; 2013).

Various natural sources have been studied to inhibit the gut ability to produce glucose from carbs or absorb it from the intestines (Mastsui et al., 2001).

The enzymes such as α-amylase and α-glucosidase, which convert dietary carbs to glucose, are what cause postprandial hyperglycemia. Thus, a decrease in postprandial hyperglycemia in diabetes may result from the inhibitory activity of the ethyl acetate fractions of *S. heterophylla* and *C. pedata* on these enzymes. Some suggest that postprandial hyperglycemia is a separate risk factor for several illnesses. As a result, managing postprandial hyperglycemia is crucial for both treating diabetes and avoiding its consequences. Medication that inhibits the action of these enzymes can control postprandial blood glucose levels,

particularly in those with type 2 diabetes or those who are borderline. Currently, the only drugs in this family that can primarily reduce the activity of these two enzymes are miglitol and acarbose. Bloating and abdominal gas are common side effects of these drugs. New drug formulations or formulations without the previously described side effects will be more complied with by patients with type 2 diabetes (Ramachandram et al; 2013). Based on the results of our current study, EACP has strong pancreatic α-amylase and α-glucosidase inhibition rather than EASH, confirming its ability to regulate postprandial hyperglycemia.

4.7. Conclusion

The current investigation unequivocally shows that compared to *Solena heterophylla* fruit extract, daily ingestion of *Cyclanthera pedata* fruit extract exhibits a substantial hypoglycemic impact (FBG) and also controls the postprandial hyperglycemia. To confirm the molecule causing this activity, however, further research into the secondary metabolites of this fraction responsible for the hypoglycemic effect should be performed.

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Chapter 5

Standardisation and metabolite profiling

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5.2.6. Discussion

5.3. Conclusion

5.1. Introduction

High-performance thin-layer chromatography, or HPTLC, is a very advanced analytical technique used to analyse medicinal plant extracts for fingerprinting and marker analysis. The development of marker profiling as criteria for herbal medications is crucial for medicinal plant quality control and standardization. Plant-based medicines and dietary supplements are carefully monitored for quality control to guarantee the efficacy and safety of herbal medications. Standardization is crucial to getting the best concentration of bioactive components from plants (Houghton and Mukherjee, 2009). Various medicinal plants have been the subject of HPTLC densitometry standardization and marker analysis reports from our laboratory (Gantait et al., 2010; Rai et al., 2006; Kumar et al., 2008; Mukherjee et al., 2008). Cucurbitacin B, a bioactive molecule found in the ethanolic extract of the two chosen plants, was standardized using these supporting processes for bioactive molecule standardization. The crude drug can be considered legitimate if the marker compound is present (Mukherjee et al, 2002). By using metabolomics analysis, HPTLC can help generate a variety of complimentary data types that can be utilised for additional studies like Principal component analysis (PCA) or Tree Clustering Analysis (TCA).

Ultra-performance liquid chromatography or UPLC systems connected to a range of mass detectors, including time-of-flight (TOF) mass detectors, triple quadrupole mass detectors, and single quadrupole mass detectors are used for distinct analytical uses. Before comprehending the uses of LC-MS/MS, it is important to understand the many kinds of mass detectors that are connected to UPLC systems. A single-quadrupole assembly of Q1 scans and filters ionized molecules in a single-quadrupole mass spectrometer by applying scaling voltages across diagonally opposing quadrupole rods. By completing a ramp, one can gain full scan mass spectra, commonly known as multiple ion monitoring (MIM). This makes it possible to assess the components included in the sample quantitatively. With SIM mode, ions filter out by applying a constant voltage, and only ions with a specific m/z ratio may reach the detector. The three quadrupoles that compose the triple quadrupole mass spectrometer are Q1, Q2, and Q3. Mass filter by quadrupoles Q1 and Q3, whereas the intermediate quadrupole (Q2) acts as a collision cell. To ensure that only ions with a particular m/z ratio can reach the detector, the product ions filter by the third quadrupole assembly Q3 (Xue et al., 2021). Time-of-flight (TOF), which is based on subjecting the sample to an electron beam bombardment, causes the sample to fragment, forming smaller

groupings of atoms or ions. In the end, TOF mass spectrometry offers precise and trustworthy chemical fragmentations, which are useful in elucidating the structures of substances. The produced ions then measure the time it takes for each ion to reach the detector (REF.). When compared to single ion monitoring (SIM) using a single quadruple LC-MS, multiple reaction monitoring using LC-MS/MS provides selectivity improvements that are many times greater (Chernushevich et al., 2001; Castro-Perez et al., 2005; Vyas et al., 2010). Many fields, including bio-analytical investigations, therapeutic drug monitoring, clinical monitoring, forensic toxicology, and proteomics, have used the LC-MS/MS because of its superior performance in terms of higher accuracy and repeatability of data (Rathod et al., 2019).

5.2. Metabolite profiling and standardization of selected medicinal plants

5.2.1. Chemicals and reagents

Acetonitrile [≥ 99.97% (LCMS), liquid] and Formic acid [≥ 98% (LCMS), liquid] from Merck (KGaA, Darmstadt, Germany), Ultra-pure water from Milli-Q water filter (Bedford, USA), pH metre (Orion3Star, Thermo-Scientific), Cucurbitacin B [≥95% (HPLC), powder] from Sigma (St. Louis, MO). The chemicals and reagents utilized in the test were of analytical grade and came from Sigma (St. Louis, MO), Merck (Mumbai, India). Additionally, each fruit extract was diluted in LCMS grade ethanol to create a 100 mg/mL solution. Extract solutions were filtered using 0.22 μm Agilent syringe filters before being transferred to high-recovery amber vials (Agilent Technologies, Santa Clara, CA, USA).

5.2.2. LC-QTOF-MS method

Analysis through LC–QTOF–MS was conducted as per the previously reported method **(Jana et al., 2024).** The Agilent 1260 Infinity II LC System and Agilent 6530 LC/Q-TOF equipment were used to identify the phytomolecule in the EASH and EACP. ACN (A) and H2O (B), both consisting of 0.1% formic acid, made up the mobile phases. With a flow rate of 0.5 ml/min, the gradient profile was changed from 0 to 1 minute to 5% B, 35% B at 3 minutes, 50% B at 5 minutes, 80% B at 7 minutes, 90% B for 8 minutes, and 10% B for 9 minutes and 5 μl of 200 μg/ml of the samples were injected. Based on the mass that was connected to the score (0 -100) of the measured mass (m/z) with their theoretical formula in negative and positive ionization mode, compound analysis was operated (Agilent Mass Hunter). Acquisition B.06.01 software (Agilent Technologies, Santa Clara, CA, USA) was

used for metabolite profiling and individual peaks were identified with Agilent Mass Hunter Qualitative Analysis v. 10.0 (Mass Hunter Qual; Agilent Technologies, Santa Clara, CA, USA). The compounds were identified with a custom library and a bespoke library uploaded into the algorithm of Mass Hunter Qual Software (Chanda et.al., 2019; Singha et. al., 2024).

5.2.3. HPTLC analysis study of the selected fruit extracts

5.2.3.1. Reference and sample solutions preparation

The standard Cucurbitacin B (0.1 mg; Sigma ≥95% pure) was diluted in 1 mL ethanol after being weighed in an eppendorf microcentrifuge tube for the HPTLC analysis of *Solena heterophylla* and *Cyclanthera pedata* fruits. To create 10 mg/mL EASH and EACP solution, 10 mg of sample is diluted in 1 mL of ethanol. Standard and sample were vortexed separately until fully dissolved and filtered through a Syringe filter (0.45 μm), Millipore, Burlington, Massachusetts, USA. A calibration curve was built using 5 different volumes of the reference (Cucurbitacin B, 1 mg/mL) in the 2–10 μL range to evaluate the linearity of the response–made references. The HPTLC analysis solutions were appropriately vortexed, filtered and sonicated (Jana et al., 2020).

5.2.3.2. Chromatographic conditions

Cucurbitacin B reference solution (1 mg/mL) was injected (Hamilton) in a 100 μL syringe. Then, 5 different volumes (2, 4, 6, 8, and 10 μL) were applied band-wise to 5 distinct tracks 10 cm x 10 cm silica 60 F_{254} gel plates using a CAMAG Linomat V sample applicator. The 10 mg/mL of EASH and EACP solutions were then used in three different tracks and in three different amounts (10, 12, and 14 μL). The mobile phase contains pet ether, ethyl acetate, formic acid, and acetic acid (5:4:0.5:0.5 v/v/v/v) to produce the plates in a CAMAG twin-trough chamber after they had been dried in warm air (40-45 °C). At 254 nm, compounds were identified and scanned (Jana et al., 2022).

5.2.4. Method validation

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) recommendations defines robustness, precision, and linearity as well as the limits of quantification and detection was followed throughout the validation of the HPTLC method (International Conference on Harmonization, 2005).

5.2.4.1. Specificity

To reduce mistakes brought on by sample contamination, the standardization results were evaluated for specificity by ICH criteria. The standard and test samples were examined to determine the method specificity. By comparing peak areas and retention periods of the standard compound with those of the extract and fractions, multivariate analysis was used to verify the purity of the peaks.

5.2.4.2. Limit of detection (LOD) and limit of quantification (LOQ)

The formulae LOD = 3:1 /S and LOQ = 10:1 /S were used to calculate the LOD and LOQ using a technique based on standard deviation (σ) and the slope (S) of the calibration plot.

5.2.4.3. Accuracy

The accuracy of the approach was measured by the % recovery of markers in the plant extract and fractions. The procedure was investigated using the standard addition approach and the results are reported in percentages of analogous standard deviations (%RSD) from the hypothesized concentrations mean recovery. Before injection, the tests were spiked with three separate reference drugs at established amounts. Analyses were conducted to determine the overall average recovery in an ambient environment. The mean levels of the markers were calculated.

5.2.4.4. Precision

To assess the accuracy of the procedure, six duplicates of the standard material, the fractions, and the extract were injected at three different concentrations. The values of the intra-day and inter-day runs were displayed as %RSD. RSD values and the mean amount were computed. Three concentrations were examined in one day to determine the intra-day assay precision. Additionally, the same concentrations were studied over three consecutive days to assess the intra-day accuracy. To determine the repeatability of the process, injections were carried out six times.

5.2.4.5. Robustness

The proposed robustness of the method was examined by analysing samples under various experimental circumstances. To ascertain their impact on the retention time, the test solutions

were studied using multiple flow rates, mobile phase compositions, detection wavelengths, column temperatures, and columns with the same design but different materials.

5.2.5. Results

5.2.5.1. Bioactive molecule identification using LC-QTOF-MS

Based on their respective retention times, m/z values, error in parts per million (ppm) less than 5, categorization of the suggested compounds, score times, molecular formulas, and molecular weights, evaluation of LC-QTOF-MS was used for identifying 52 key phytoconstituents **(Table 5.1)** found in EASH and 47 key phytoconstituents **(Table 5.2)** found in EACP. The total ion chromatogram of EASH and EACP is shown in **Fig 5.1. &5.2.** To establish their elemental composition, the analysis was done within the mass error window of -346737.478 to 696598.52 ppm and -8.4 to 3.5 ppm.

Fig 5.1.: LC QTOFMS total ion chromatogram of EASH in the negative ionization mode

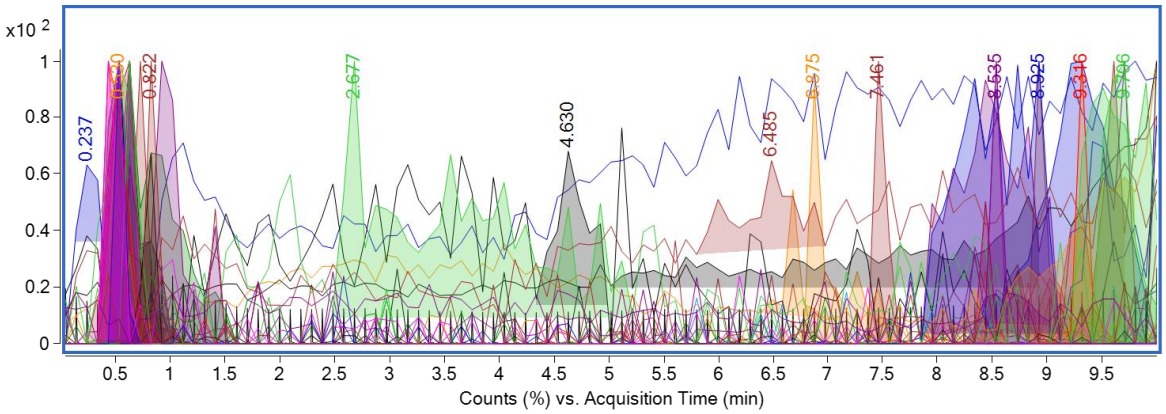


Table 5.1. Compounds identified in ethyl acetate fraction of *Solena heterophylla* fruit by LC QTOFMS

Sl No.	Retention time	Molecular formula	Theoretical mass	Expected mass	Error (ppm)	Score	Compounds	Class
1.	8.827	C19 H17 N O3	307.3	308.128	2694.435405	98.03	N-Acetylanonaine	Isoquinoline alkaloid
2.	8.925	C28 H40 O12	568.5	586.2847	31283.55	85.92	Musabalbisiane C	Diterpenoids
3.	0.53	C14 H15 N	197.28	220.1092	116172.413	85.43	3-Phenyl-4-propylpyridine	Alkaloids
4.	0.627	C18 H26 O10	402.4	425.1419	56515.65	83.51	Benzyl O-[arabinofuranosyl-(1->6)-glucoside]	Glycoside

5.	9.316	C24 H42 O11	506.6	507.2771	1336.557	82.8	4-Megastigmene-6a,9R-diol 9-[apiosyl-(1->6)-glucoside]	Fatty acyl glycosides of mono and disaccharides
6.	0.627	C15 H21 N O	355.4	232.1695	-346737.478	82.09	Rotundine A	Alkaloids
7.	0.822	C20 H32 O4	336.5	337.2382	2193.759	81.28	9alpha-(3-Methylbutanoyloxy)-4S-hydroxy-10(14)-oplopen-3-one	Sesquiterpenoid
8.	0.627	C41 H64 O17	828.937	851.4062	27106.040	80.31	Digitoxigenin 3-[glucosyl-(1->6)-glucosyl-(1->4)-2,6-dideoxyribohexoside]	Cardenolide glycoside
9.	0.432	C24 H32 O12 S	544.57	545.1723	1106.0102	77.51	Estriol 3-sulfate 16-glucuronide	Steroidal glycosides
10.	0.53	C16 H21 N O7	339.34	357.1656	52530.205	77.26	5-Hydroxytryptophol glucuronide	Glycoside
11.	8.437	C27 H36 O3	408.6	409.2744	1650.513	76.84	Apo-10'-violaxanthal	Sesterterpenoid
12.	0.53	C18 H22 N O2	284.373	302.1999	62688.440	76.58	6,7-Dihydro-4-(hydroxymethyl)-2-(p-hydroxyphenethyl)-7-methyl-5H-2-pyrindinium	Alkaloids
13.	6.68	C16 H32 O2	256.42	274.2758	69634.973	74.97	Trimethyltridecanoic acid	Sesquiterpenoids
14.	6.485	C32 H37 N5 O5	571.7	594.27	39478.747	73.78	Endomorphin-2	isoflavonoids
15.	9.608	C29 H48 O	386.65	413.378	39478.7	72.9	Clerosterol	Tetracyclic triterpene
16.	8.437	C24 H42 O11	436.5	507.2782	162149.369	71.38	4-Megastigmene-6a,9R-diol 9-[apiosyl-(1->6)-glucoside]	Fatty acyl glycosides of mono and disaccharides
17.	0.627	C26 H28 O6	506.6	437.1983	-136995.065	69.75	Kanzonol K	6-prenylated isoflavanoids
18.	0.53	C15 H8 O5	268.22	286.073	66561.031	69.67	Coumestrol	Isoflavonoids
19.	0.53	C11 H20 O5	232.2735	250.167	77036.338	69.42	1,2-dibutyrin	Glycerolipids
20.	0.627	C20 H16 O5	342.3	354.135	34574.934	68.75	Sojagol	Isoflavonoids
21.	0.627	C16 H10 N2 O3	278.2622	296.105	64122.255	68.22	Dehydroxymethylflazine	Harmala alkaloids
22.	0.627	C25 H31 N O3	393.5	416.219	57735.705	66.14	Acidissiminol	Aromatic monoterpenoids

23.	0.53	C15 H10 N2 O2	250.25	268.11	71368.631	65.85	2-Methoxycanthin-6-one	Alkaloid
24.	0.725	C16 H10 O5	282.24	283.059	2901.785	65.6	Pseudobaptigenin	Isoflavonoid
25.	0.237	C32 H42 O9	570.7	593.269	39546.1713	64.23	Ganoderic acid F	Triterpenoid
26.	2.677	C7 H8 O2	124.1372	142.088	144604.518	62.78	4-Methyl-1,2-benzenediol	Catechol
27.	0.627	C11 H12 O2	176.21	194.118	101628.738	62.44	Ethyl cinnamate	Cinammic acid and derivatives
28.	4.63	C15 H24 O3	252.349	275.165	90414.465	61.98	Bisacurone C	sesquiterpenoids
29.	0.627	C19 H21 N O7 S	407.44	425.142	43446.887	61.71	Erysothiopine	Erythrina alkaloids
30.	0.627	C27 H36 O6	460.6	457.262	-7247.069	61.61	Lucidenic acid F	triterpenoids
31.	6.875	C15 H16 O3	244.28	245.119	3434.583	60.66	3-(1,1-Dimethylallyl)herniarin	coumarins and derivatives
32.	0.627	C21 H36 O9	432.5	433.245	1722.543	60.62	Glucosyl (2E,6E,10x)-10,11-dihydroxy-2,6-farnesadienoate	terpene glycosides
33.	9.316	C37 H64 O4	260.24	573.492	54426.50	60.53	Coronin	acetogenins
34.	0.432	C10 H13 N3	175.23	176.116	5056.2	60.38	Debrisoquine	Tetrahydroisoquinoline alkaloids
35.	0.53	C30 H39 Cl O7	547.1	547.251	276.000731	59.93	Physagulin B	Withanolide glycosides
36.	8.535	C15 H22 O2	234.33	235.171	3588.9557	59.76	Fukinanolide	Terpene lactone
37.	0.53	C16 H13 N O6	315.28	316.085	2553.285	59.65	Avenanthramide K	phenolic alkaloids
38.	9.706	C27 H48 O3	420.668	443.353	53926.136	59.24	(3alpha,5alpha,22R,23R)-Cholestane-3,22,23-triol	vitamin e compound
39.	0.53	C14 H25 N O10	367.35	368.155	2191.370	58.86	2-Acetamido-2-deoxy-6-O-alpha-L-fucopyranosyl-D-glucose	fatty acyl glycoside

40.	8.925	C36 H62 O11	670.9	688.459	26172.305	58.76	Ginsenoside Rh6	triterpene saponin
41.	7.461	C30 H27 O14	611.5	634.131	37008.994	58.59	Cyanidin 3-(caffeoylglucoside)	anthocyanidin glycoside
42.	9.706	C30 H46 O4	324.4	493.332	520752.157	58.59	Glabranin A	flavonoid
43.	0.53	C15 H10 O3	238.24	261.053	95756.380	58.47	2-Hydroxymethyl anthraquinone	Anthraquinones glycoside
44.	0.53	C16 H30 O9	366.40	389.1824	62179.039	58.32	(2R,6x)-7-Methyl-3-methylene-1,2,6,7-octanetetrol 2-glucoside	Fatty acyl glycosides
45.	0.53	C21 H24 O9	258.27	438.1805	696598.520	57.91	2',4',6'-Trihydroxydihydrochalcone 4'-glucoside	flavonoids
46.	9.901	C29 H48 O2	414.7	429.3702	35375.452	57.44	4-hydroxymethyl-4-methyl-5-cholesta-8,24-dien-3-ol	triterpenoid
47.	0.53	C32 H46 O8	558.7	581.3048	40459.638	55.98	Cucurbitacin B	tetracyclic triterpenoids
48.	0.53	C26 H32 O12	536.5	554.2225	33033.550	55.13	8-Hydroxypinoresinol 4-glucoside	Lignan glycosides
49.	0.92	C11 H13 N O4	223.22	224.0926	3909.147	53.92	Salsolinol-1-carboxylate	Alkaloids
50.	0.53	C23 H28 O3	352.5	375.1913	64372.482	51.66	Methyl (9Z)-8'-oxo-6,8'-diapo-6-carotenoate	diterpenoid
51.	0.53	C36 H58 O9	634.8	635.4178	973.219	51.11	Momordicin II	Steroidal gycoside
52.	8.437	C26 H32 O3	392.530	393.2445	1820.243	50.95	Methyl (9Z)-6'-oxo-6,5'-diapo-6-carotenoate	diterpenoid

Fig 5.2.: LC QTOFMS total ion chromatogram of EACP in the negative ionization mode

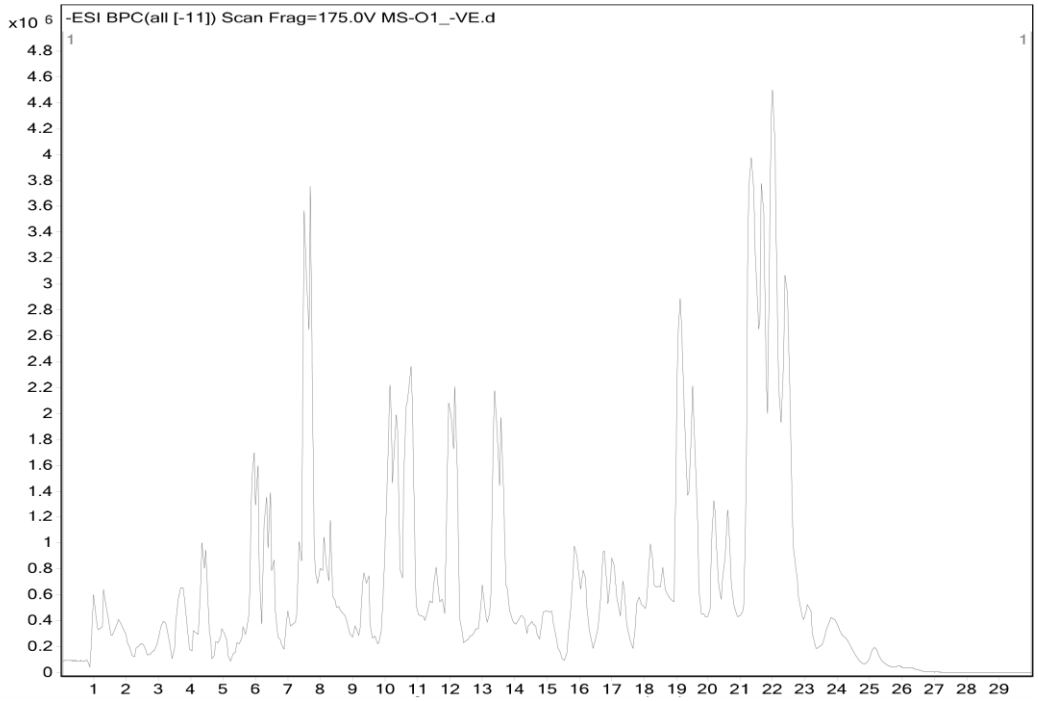


Table 5.2. Compounds identified in ethyl acetate fraction of *Cyclanthera pedata* fruit by LC QTOFMS

Sl. No.	Retention time	Molecular formula	Theoretical mass	Expected mass	Error (ppm)	Score	Compounds	Class
1.	0.53	C32 H46 O8	558.7	581.3048	40459.638	55.98	Cucurbitacin B	tetracyclic triterpenoids
2.	3.71	C20 H28 O8	396.1792	396.4389	-1.95	94.37	Pteroside P	o-glycosyl compound
3.	4.142	C21 H32 O10	444.2002	444.4684	-1.39	86.13	Cynaroside A	lipid
4.	4.398	C20 H30 O8	398.1942	398.4348	-0.24	91.84	Ptaquiloside	glycosides
5.	4.538	C19 H26 O8	382.1644	382.4587	-4.27	91.49	7alpha,8alpha-Dihydroxycalonectrin	lipids
6.	5.025	C16 H22 N4 O3 S	350.1401	396.3729	3.4	90.68	Cafenstrole	triazoles
7.	5.047	C20 H30 O7	382.2009	382.4304	-4.46	91.65	Cinncassiol A	lipids
8.	5.464	C24 H40 O12	520.2542	520.9409	-4.3	85.55	7,8-Dihydrovomifoliol 9-[apiosyl-(1->6)-glucoside]	glycoside
9.	5.521	C20 H26 O7	378.1701	378.509	-5.96	75.42	Niveusin C	Lipids
10.	5.624	C26 H40 O12	544.2537	544.583	-3.13	82.78	Cinncassiol A 19-glucoside	Lipids

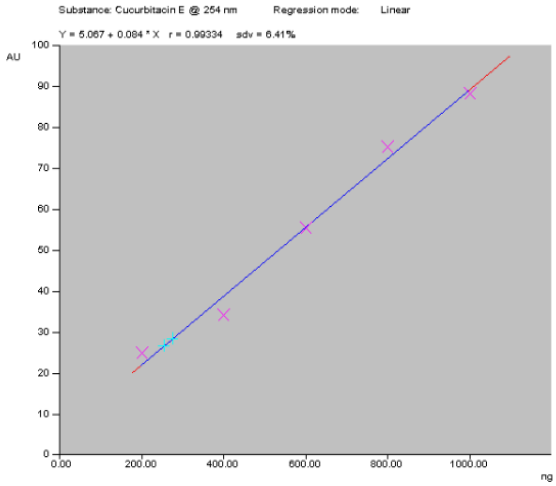
11.	5.989	C19 H30 O5	338.2114	338.4079	-6.14	85.9	[6]-Gingerdiol 5-acetate	Phenol
12.	6.378	C20 H28 O8	396.1811	396.42	-6.89	83.38	4,5-Dihydroniveusin A	Lipids
13.	6.538	C21 H20 O10	432.1089	432.3541	-7.63	78.08	Genistein 8-C-glucoside	flavonoids
14.	7.071	C22 H20 O11	460.1039	460.3677	-7.18	79.07	Oroxindin	Phenol
15.	7.611	C19 H22 N4	306.186	366.4091	-5	87.05	Corrinoid	Tetrapyrroles
16.	8.031	C18 H22 O8 S	398.1042	444.3666	-1.54	91.61	Zearalenone 4-sulfate	Macrolide
17.	8.235	C10 H20 N6 O12	416.1144	416.3622	-1.21	85.87	Tenitramine	alkylamine
18.	9.411	C35 H42 O22	814.2154	860.7264	1.7	92.98	Kaempferol 3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside	Flavonoid
19.	10.205	C35 H42 O21	798.2207	844.7267	1.49	96.5	Rindoside	Glycosides
20.	10.207	C18 H32 O5	328.2278	328.4407	-8.46	76.4	Corchorifatty acid F	Lipids
21.	10.277	C20 H24 O10	424.1403	424.3839	-7.85	77.49	Smyrindioloside	Phenols
22.	10.75	C18 H34 O5	330.2431	330.4648	-7.53	82.99	9S,12S,13S-trihydroxy-10E-octadecenoic acid	Lipids
23.	11.156	C53 H84 O24	1104.5399	1105.1249	-4.24	84.74	Camellidin II	Lipids
24.	11.585	C18 H34 O5	330.2431	330.4357	-7.48	82.69	9,10-Dihydroxy-12,13-epoxyoctadecanoate	Fatty acid
25.	11.759	C29 H30 O13	586.1723	586.5115	-6.32	79.65	Phyllanthostatin A	Glycoside
26.	12.1	C35 H42 O20	782.225	828.728	2.47	93.59	Kaempferol 3-(2"-rhamnosyl-6"-acetylgalactoside) 7-rhamnoside	Flavonoid
27.	12.826	C24 H30 O11	494.1819	494.4485	-6.22	82.05	Harpagoside	Glycoside
28.	13.742	C25 H32 O11	508.1977	508.5154	-6.37	81.25	gibberellin A3 O-beta-D-glucoside	Carbohydrate
29.	13.952	C18 H34 O4	314.2472	314.4289	-4.89	90.55	Dibutyl decanedioate	Lipids
30.	14.582	C40 H34 N2 O8	670.2292	670.623	3.5	83.8	Fagopyrine	Pyrenes
31.	15.034	C18 H30 O3	294.2215	294.4176	-6.86	86.68	10-Oxo-11-octadecen-13-olide	Fatty acid
32.	16.565	C14 H28 O3	244.2055	244.3345	-6.67	76.65	2-Hydroxymyristic Acid	Fatty acid
33.	16.758	C27 H42 O4	430.3072	430.5521	2.69	91.05	Schidigeragenin C	Lignan glycosides

34.	16.858	C18 H34 O3	298.2524	298.4383	-5.3	90.78	Ricinoleic acid	Fatty acids
35.	17.321	C29 H40 O6	484.2833	484.5183	-1.76	85.48	Stigmatellin Y	Chromone
36.	17.409	C12 H26 O4 S	266.1568	266.352	-6.16	88.83	Lauryl hydrogen sulfate	Alkyl sulfate
37.	18.508	C21 H41 O7 P	436.2621	436.4978	-7.23	79.5	1-Oleoyl Lysophosphatidic Acid	Lipids
38.	18.602	C18 H32 O3	296.2375	296.4285	-7.98	79.06	12-Hydroxy-8,10-octadecadienoic acid	Lipids
39.	18.968	C17 H28 O3 S	312.1782	312.41	-7.41	83.26	N-Undecylbenzenesulfonic acid	Benzenoids
40.	19.12	C16 H32 O3	272.237	272.3964	-6.82	87.44	16-Hydroxy hexadecanoic acid	Lipids
41.	19.592	C18 H34 O3	298.253	298.4467	-7.37	84.74	3-keto stearic acid	Lipids
42.	20.605	C18 H32 O2	280.2422	280.4392	-7.1	83.85	Linalyl caprylate	Lipids
43.	21.541	C18 H30 O3 S	326.1937	326.4484	-6.47	85.85	4-Dodecylbenzenesulfonic acid	Benzene
44.	21.56	C20 H40 O3	328.2997	328.4921	-6.1	87.37	Polyoxyethylene 40 monostearate	Lipids
45.	21.992	C53 H93 N7 O13	1035.6874	1036.312	-4.08	86.17	Surfactin	Lipids

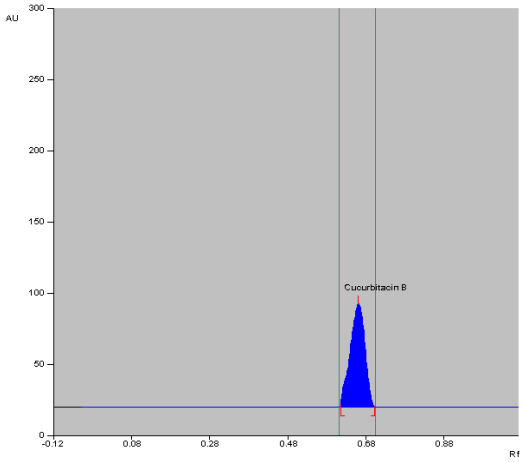
5.2.5.2. HPTLC analysis

The fresh fruit extracts of *Solena heterophylla* Lour. and *Cyclanthera pedata* (L.) Schrad contained significant level of Cucurbitacin B (2.29% and 0.014% w/w respectively), according to the densitometric analysis by HPTLC. The **Fig 5.3.A** displays the HPTLC of the standard cucurbitacin B in the extracts. **Fig 5.3.D and 5.3.E**, show the HPTLC plate visualised at 254 nm. The calibration plot linearity was determined to be 200–1000 ng/spot. The calibration curve revealed a correlation value of > 0.99. This information demonstrates how closely the data follow the best-fit line.

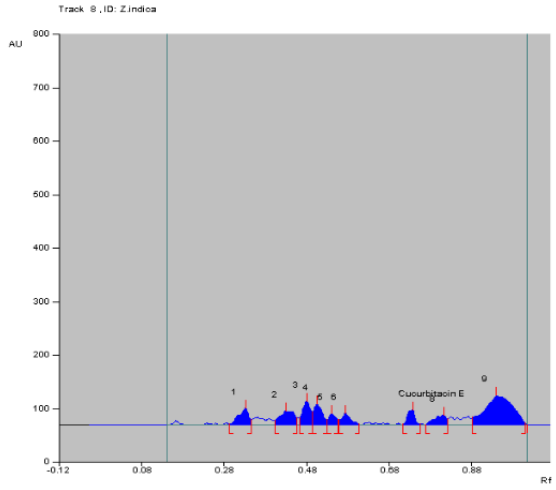
Fig 5.3. HPTLC chromatogram of standard cucurbitacin B and *Solena heterophylla* (EASH) and *Cyclanthera pedata* (EACP) fruit fraction. **A.** HPTLC chromatogram of calibration curve **B.** HPTLC chromatogram of Cucurbitacin B **C.** HPTLC chromatogram of *Solena heterophylla* fruit extract **D.**HPTLC plate photographs of standard cucurbitacin B with sample extract under 254 nm. **E.** HPTLC plate photographs of standard cucurbitacin B with sample extract under 366 nm. **F.** HPTLC plate of *Cyclanthera pedata* fruit extract at 254 nm **G.** HPTLC plate of Solena heterophylla at 366 nm **H.** HPTLC plate of *Cyclanthera pedata* fruit extract at 366 nm



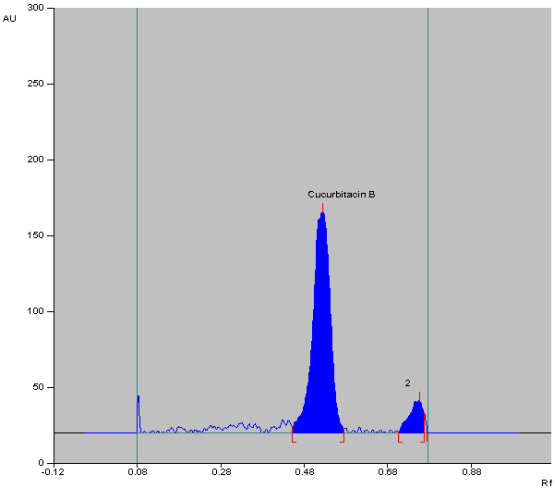
Calibration curve (5.3.A)



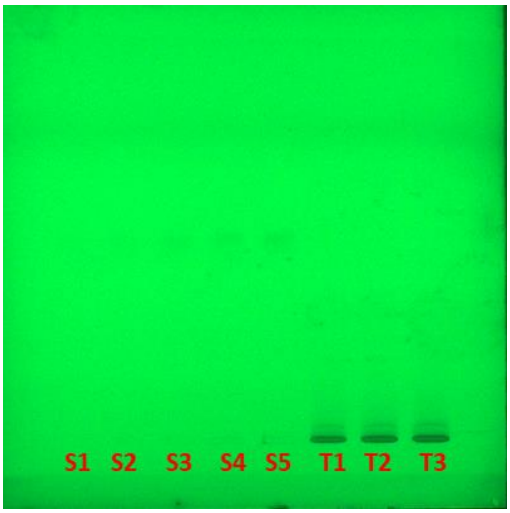
HPTLC chromatogram of Cucurbitacin B (5.3.B)



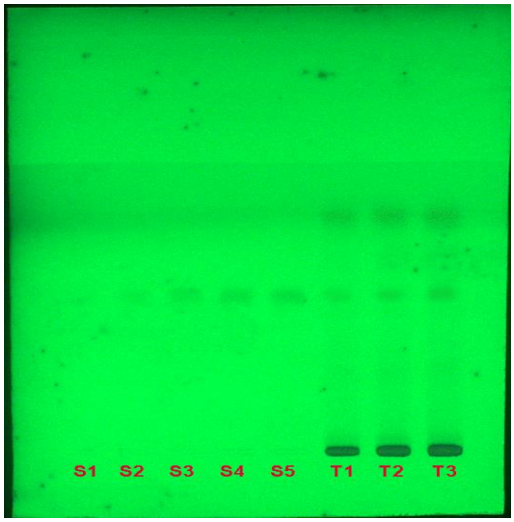
HPTLC chromatogram of *Solena heterophylla* (5.3.C)



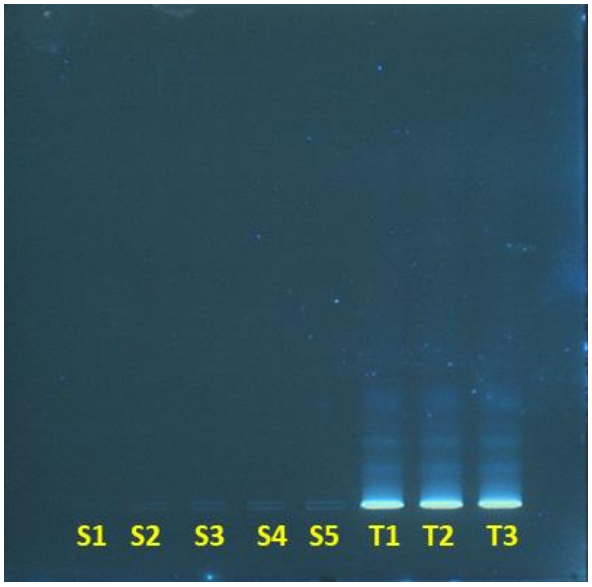
HPTLC chromatogram of *Cyclanthera pedata* (5.3.D)



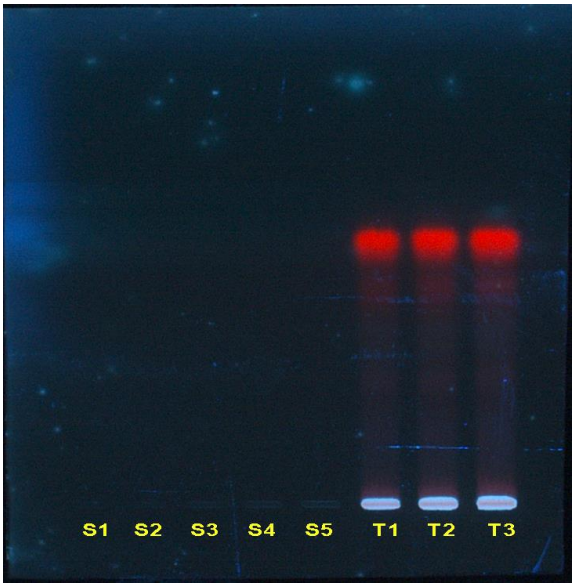
HPTLC plate of *Solena heterophylla* at 254 nm (5.3.E)



HPTLC plate of *Cyclanthera pedata* at 254 nm (5.3.F)



HPTLC plate of *Solena heterophylla* at 366 nm (5.3.G)



HPTLC plate of *Cyclanthera pedata* at 366 nm (5.3.H)

The precision and accuracy of the cucurbitacin B assay were evaluated on the basis of LQC (low-quality control), MQC (medium-quality control), and HQC (high-quality control), Six times each experiment was run, and the percent recovery values ranged from 99.68 - 99.93% for EASH and 99.07 - 99.43% for EACP. The incredible accuracy of the approach was evidenced by the low %RSD values, which ranged from 0.076 - 2.63% and for EASH and 0.088 - 2.41% for EACP. **Table 5.3.** displays the findings of the recovery study. Standard cucurbitacin B was utilized at two different concentrations 200 µg and 400 µg, to gauge the method accuracy. For intra-day and inter-day precision, the average area and RF values for the same were calculated. **Table 5.4.** displays the results of the inquiry on intra-day and inter-day precision. The 6 repetitions for each type of determination produced %RSD values of 0.180 - 0.28% (intra-day) and 0.150 - 0.26% (inter-day), which are incredibly low and show the method accuracy. The LOD ranged between 623.20 ng and 622.12 ng per spot and the LOQ between 1950.12 ng and 1951.32 ng per spot. The approach also qualified for the robustness test.

Table 5.3. Recovery studies of the selected plants

Biomarker	Amount added (ng)	Theoretically expected amount (ng/spot)	Experimentally obtained amount (ng/spot)	*%RSD*	Percentage recovery
Solena heterophylla					
Cucurbitacin B	0	1562.00	1560.83	0.08	99.93
	200	1762.00	1758.62	0.14	99.81
	400	1962.00	1959.67	0.12	99.88
	600	2162.00	2155.06	0.23	99.68
Cyclanthera pedata					
Cucurbitacin B	0	2545.71	2541.7	0.02	99.43
	200	2745.71	2742.18	0.24	99.04
	400	2945.71	2940.95	0.13	99.14
	600	3145.71	3143.85	0.34	99.07

Table 5.4. Intra-day and inter-day precision studies of the selected plants

	Response (Area)		Response (Area)	
	Solena heterophylla			
	Mean	*%RSD*	Mean	*%RSD*
200	9494.69	0.28	9499.13	0.26
400	18981.19	0.18	19321.25	0.15
	Cyclanthera pedata			
200	12874.84	0.24	12871.13	0.28
400	21741.05	0.18	21738.17	0.11

5.2.6. Discussion

The comparative metabolite profile of the two distinct medicinal plants was evaluated in the current study using the LC-Q-ToF-MS technique, and the results were determined to be acceptable. A negative ionization method was utilized to determine the molecular mass of the selected fruit extracts, By using the retention durations, molecular weight (m/z), molecular formula, error (in ppm), published data, and library search, the identity of the compounds was verified. Less than 5 ppm was determined to be the acceptable precision for elemental

composition verification of authenticity. Very precise bioactive ingredient identification in the fruit extract was made possible by the high-resolution Q-ToF-MS, the chemical database, and the library searching method. *Solena heterophylla* yielded isoquinoline alkaloid, diterpenoids, alkaloids, glycoside, fatty acyl glycosides of mono and disaccharides, sesquiterpenoid, cardenolide glycoside, steroidal glycosides, sesterterpenoid, sesquiterpenoids, isoflavonoids, tetracyclic triterpene, 6-prenylated isoflavanoids, glycerolipids, harmala alkaloids, aromatic monoterpenoids, triterpenoid, catechol, cinammic acid and derivatives, erythrina alkaloids, triterpenoids, coumarins and derivatives, terpene glycosides, acetogenins, tetrahydroisoquinoline alkaloids, withanolide glycosides, terpene lactone, phenolic alkaloids, fatty acyl glycoside, triterpene saponin, anthocyanidin glycoside, anthraquinones glycoside, fatty acyl glycosides, flavonoids, tetracyclic triterpenoids, lignan glycosides, diterpenoid, steroidal glycoside class of compounds and *Cyclanthera pedata* yielded alkyl sulfate, alkylamine, benzene, benzenoids, carbohydrate, chromone, fatty acids, flavonoids, glycosides, lignan glycosides, lipids, macrolide, o-glycosyl compound, phenols, pyrenes, tetrapyrroles, triazoles class of compounds.

Previous research has indicated that these compounds may have a positive impact on diabetes management and wound healing. In the realm of diabetes treatment, dietary flavonoids have been found to have a remarkable impact on lowering blood glucose levels. They achieve this by safeguarding pancreatic β-cells, kickstarting insulin signalling, prompting the pancreas to release insulin, curbing glucogenolysis and gluconeogenesis, as well as inhibiting digestive enzymes and carbohydrate metabolising enzymes (Xiao et al., 2022). Flavonoids have been found to have significant effects on various aspects of the body, including the inflammatory process, angiogenesis, re-epithelialization, and oxidative stress. It was demonstrated that they have the ability to influence various types of cells, such as macrophages, fibroblasts, and endothelial cells. This is achieved by regulating the release and expression of important molecules like TGF-β1, VEGF, Ang, Tie, Smad 2 and 3, and IL-10. In addition, they successfully decreased the production of inflammatory cytokines, NFκB, ROS, and the M1 phenotype. Flavonoids have been found to have a positive effect on the regulation of MMPs 2, 8, 9, and 13, as well as the Ras/Raf/MEK/ERK, PI3K/Akt, and NO pathways (Carvalho et al., 2021). Flavonoids and tannins, known as polyphenols, have a crucial impact on carbohydrate metabolism. They act as inhibitors for important enzymes that are responsible for breaking down carbohydrates into glucose, specifically α-glucosidase and α-amylase. According to a recent study by Shahwan et al. (2022), it was found that polyphenols have the

ability to improve glucose uptake in muscles and adipocytes. This is achieved by facilitating the translocation of GLUT4 to the plasma membrane, primarily through the activation of the AMP-activated protein kinase (AMPK) pathway. Polyphenols have been found to possess the ability to promote wound healing through various stages, including inflammation, proliferation, and remodelling. In the inflammation phase, TPP was found to decrease the production of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and prevent the infiltration of neutrophils. Moving on to the proliferation phase, TPP was observed to enhance the expression of growth factor VEGF-A, which plays a role in promoting the division of vascular endothelial cells and inducing angiogenesis. Additionally, TPP was seen to have a positive impact on the wound's appearance and the ratio of type III/I collagens during the remodelling phase, as indicated by Masson-trichrome staining and Sirius red staining assays. Through careful observation of the wound area, it was discovered that TPP and recombinant human epidermal growth factor (rhEGF) had a more significant impact on promoting wound healing compared to povidone-iodine (PVP-I). According to the study conducted by Zhao et al. in 2023, the results indicate that TPP has a significant impact on various stages of wound healing. The mechanisms of TPP differ from those of rhEGF, which suggests that TPP could be a valuable natural agent for wound healing in future clinical applications. Studies have shown that certain plant compounds, such as Cucurbitacin B, can effectively lower sugar levels in tissues, as well as reduce glycogen and triacylglycerol levels (Sang et al., 2021). In a study conducted by Kim et al. (2018), it was found that Cucurbitacin B had a positive effect on reducing hyperglycaemia in diabetic mice. This was achieved by activating intestinal AMPK levels and stimulating the release of plasma GLP-1 and insulin. HPTLC analysis of EASH and EACP showed the presence of Cucurbitacin B in a concentration of 2.29% and 0.014% w/w respectively. The method validation study of HPTLC showed the robustness, accuracy and precision of the method.

5.3. Conclusion

Routine analyses of pharmaceutical and clinical data, analyses of traditional medicines and medicinal plants, analyses of environmental factors, analyses of cosmetics and toxicology, analyses of plants and herbs, and analyses of food and food supplements can all benefit from the HPTLC technique. By looking at the aforementioned uses, HPTLC can be safely utilized in many scientific disciplines because of its various advantages over conventional

chromatographic techniques. EASH and EACP were found to contain cucurbitacin B in significant manner. EASH was found to contain higher concentration of cucurbitacin B.

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Chapter 6

In-vivo antidiabetic activity

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6.1. Introduction

Herbal plants are the most costly gift that nature has given to humans. An isolated plant species combination of secondary products is taxonomically unique, giving the plant its specific therapeutic activity. Plant extracts phytochemicals identify the biochemical pathways, making the medication safer. The adverse effects of synthetic pharmaceuticals are growing, which makes plant-based herbal medications more appealing. Studies on the phytochemical components of medicinal plants and their pharmacological effects have received a lot of attention lately (Rajeswari et al., 2013).

The metabolic disease referred to as a loss of glucose homeostasis characterizes diabetes mellitus. Abnormalities also accompany it in the metabolism of protein, fat, and carbohydrates, which can lead to deficiencies in neither insulin action nor secretion (Imam 2021). The global epidemic of the twenty-first century is among the most difficult to contain. Among the top ten causes of adult mortality worldwide in 2017, diabetes accounted for four million fatalities. Over 425 million people globally were estimated to have diabetes in 2017, and by 2035 that number is expected to climb to 592 million, according to the International Diabetes Federation (Saeedi et al., 2019, Joshi 2015). Currently, diabetes affects 62 million individuals in India. By 2030, the population is expected to reach 101.2 million, the biggest absolute amount in any country. As sedentary habits and eating patterns change, the disease's current burden is fast increasing among the middle-class population in India (Mitra 2019).

Marketed oral hypoglycemic medications have a range of side effects, such as gastrointestinal disturbances from glucosidase inhibitors, meglitinides, sulfonylureas, and congestive heart failure from glitazone. Sulfonylureas frequently cause weight gain and cardiac issues as side effects. Thus, there is a need to find therapeutic agents that are less harmful. A multi-target therapeutic drug is required to treat type II diabetes and its related pathophysiology, given the various pathophysiological factors. Plant extracts are complex mixtures of chemicals that have shown a variety of medicinal uses. As a result, the current study concentrates on the plant extracts ability to prevent diabetes while employing ethnopharmacological expertise as a guide (Bhattacharjee et al, 2016).

6.2. Material and Methods

6.2.1. Drugs and chemicals

Trichloroacetic acid (TCA) from Merck Ltd., Mumbai, India; Potassium dichromate, glacial acetic acid from Hi-media, Mumbai; Thiobarbituric acid (TBA), streptozotocin (STZ), 5,5´-dithio bis-2-nitro benzoic acid (DTNB), phenazonium methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), and reduced glutathione (GSH) from SISCO Research Laboratory, Mumbai, India; and metformin from Sigma-Aldrich, India. The remaining reagents were all of analytical reagent grade, purchased from a commercial source.

6.2.2. Experimental animals and housing conditions

In this investigation, the experimental animals were procured from registered breeder M/S Chakraborty Enterprise, Kolkata (Reg. no. 1443/PO/Bt/s/11/CPCSEA), adult male Wistar albino rats (160-180 g), were taken for the experiment. The experimental animals were housed for a 07-day acclimatization period in a typical setting with unlimited access to food and water. For the study period the rats were fed a typical high-fat diet and allowed full access to water while being maintained in a 25°C environment with a 12-hour light/dark cycle. The experimentation was approved (Approval Number JU/IAEC-22/15) by the Animal Ethical Committee of Jadavpur University, Kolkata, West Bengal, India.

6.2.3. Test sample preparation

The ethyl acetate fractions of the fruits of *S. heterophylla* and *C. pedata* were reconstituted in distilled water before administration to the experimental rats orally (Jana et al., 2024).

6.2.4. Acute toxicity study

Ethyl acetate fraction (EASH and EACP) is utilized for acute oral toxicity study from OECD (Organisation for Economic Co-operation and Development) recommendations 425 (OECD 2008). **(Anonymous 2008)**

6.2.5. Induction of diabetes

Rats were starved for 18 hours with water access before the induction of diabetes. After that, Streptozotocin (35 mg/kg body weight, i.p.) injections were administered and dissolved in 0.1 M citrate buffer (pH 4.5). The presence of diabetes was determined by estimating the fasting

blood glucose level. For the experiment, animals with fasting blood sugar levels under 250 mg/dl were used (Jana et al., 2024).

6.2.6. Experimental design

The experimental treatment schedule was designed after high-fat diet treatment for 28 days. With 7 groups of six rats each (n = 6), the rats were divided and the experiment was scheduled for 28 days (Jana et al., 2024).

Group-I: Normal control group: For 28 days, the rats were given 0.5 ml/kg of normal saline p.o.

Group-II: Diabetes control group (STZ+HFD): The diabetes group included rats given STZ (35 mg/kg, b.w., i.p.) and a high-fat diet.

Group-III: EASH Fraction (EASH 200 mg/kg) treated group: EASH (200 mg/kg b.w.) was given orally to the diabetic rats for 28 days.

Group-IV: EASH Fraction (EASH 400 mg/kg) treated group: The diabetic rats were orally treated with EASH (400mg/kg b.w.) for 28 days.

Group-V: EACP Fraction (EACP 200 mg/kg) treated group: EACP (200 mg/kg b.w.) was given orally to the diabetic rats for 28 days.

Group-VI: EACP Fraction (EACP 400 mg/kg) treated group: The diabetic rats were orally treated with EACP (400mg/kg b.w.) for 28 days.

Group-VII: Metformin-treated group: For 28 days, metformin (150 mg/kg, p.o.) was administered to the diabetic rats.

6.2.7. Oral glucose tolerance test

Overnight fasting rats with normal glycemia (85-90 mg/dl) underwent the OGTT. There were six groups of six rats each. EASH and EACP were administered to groups III, IV, V and VI at 200 and 400 mg/kg b.w., respectively, while group I acted as the standard control group and received distilled water (5 mL/kg b.w. p.o.). Following these procedures, oral glucose (2 g/kg b.w.) was given to all groups. Before and 30, 60, 90 and 120 minutes after oral glucose

delivery, blood was collected from the tail vein (Jana et al., 2024). A single-touch glucometer was utilized to evaluate blood sugar levels (Accu-Chek, India).

6.2.8. Glycosylated haemoglobin level (HbA1c)

A complete blood sample was used to using test kit available for purchase measure the amount of glycosylated haemoglobin (Coral clinical system, Tulip diagnostics Pvt. Ltd. India). This assay methodology is based on the use of an ion exchange resin (Jana et al., 2024).

6.2.9. Serum biochemical parameter and serum lipid profile

All rats received 28-day course of medication before being starved for 12 hours and given 0.3% isoflurane inhalation anaesthesia. Then, under anaesthesia blood is obtained through heart puncture. As soon as the blood was collected the animals were sacrificed using the cervical decapitation technique. Centrifugation was used to separate the serum for 10 minutes at 3000 rpm. Using a commercially available assay kit, collected serum was examined for number of biochemical factors including total protein, HDL cholesterol, triglyceride levels, serum alkaline phosphatase (SALP), serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) (Jana et al., 2024).

6.2.10. Tissue antioxidant parameters

Livers and kidneys collected from the sacrificed animals were homogenized separately in 10 ml of phosphate buffer (20mM, pH-7.4) and centrifuged at 12000 rpm for 30 min at 4° C. The supernatants were collected and Lipid peroxidation (LPO), reduced glutathione (GSH) and Super oxide dismutase (SOD) were estimated by Chakraborty et al, 2015.

6.2.11. Histopathological studies

Pancreatic tissue from sacrificed rats was collected and used in histopathological investigations. The tissues were prepared for microscopic photo analysis through being instantly fixed in 10% formalin for at least 24 hours after being rinsed in normal saline and immediately sliced into 4 to 5-m thick pieces, fixed in paraffin, and stained with haematoxylin and eosin dye after being dried with alcohol (Jana et al., 2024).

6.2.12. Statistical Analysis

The mean and SEM are used to express all results. Graph Pad Prism 8.02 software was utilized to perform a one-way analysis of variance (ANOVA) and post hoc Dunnett test to evaluate whether the results were statistically significant (Graph Pad Software, USA). Statistics were considered important at p <0.05.

6.4. Results

6.4.1. Acute toxicity study

The following administration of EASH and EACP up to 2000mg/kg b.w., no toxicity was observed. Two dosages of EASH and EACP 200 mg/kg and 400 mg/kg body weight were selected for investigation.

6.4.2. Oral glucose tolerance test

Rats with normal blood sugar levels (85–90 mg/dl) underwent the oral blood glucose tolerance test. Following glucose injection into healthy rats, blood glucose levels expanded during the first 30 minutes before steadily declining over the following 60, 90, and 120 minutes as shown in **(Figure 6.1.).**

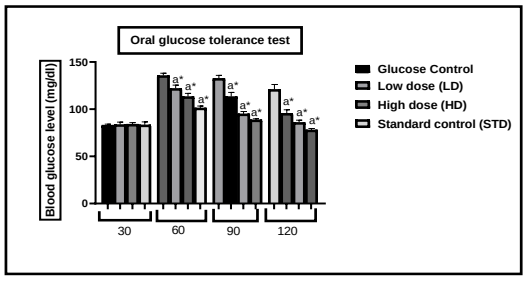


Fig 6.1.A. Effect of EASH on oral glucose tolerance test. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

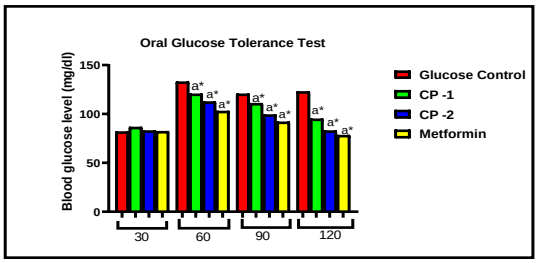


Fig 6.1.B Effect of EACP on oral glucose tolerance test. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

Fig 6.1. Oral glucose tolerance test

6.4.3. Body weight and fasting blood glucose

Low-dose STZ high fat instigated diabetic rats had significantly ($p< 0.05$) higher FBG levels than the standard control group. When EASH and EACP were fed to diabetic rats for 28 days at doses of 200 and 400 mg/kg, there was a substantial ($p <0.05$) decline in FBG level toward normal differentiated to the diabetic control group. The effect of EASH and EACP on Fasting Blood Glucose (mg/dl) is shown in **(Figure 6.2., 6.3.).** Differentiated to the normal control group, the end body weights in the diabetes control group were considerably ($p <0.05$) lower. In comparison to the diabetic control group, the body weight was broadly ($p <0.05$) improved after receiving EASH and EACP at doses of 200 and 400 mg/kg.

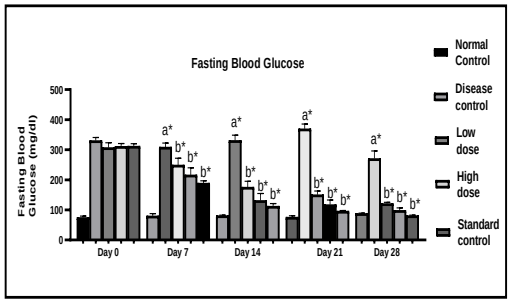


Fig 6.2.A. Effect of EASH on fasting blood glucose (mg/dl). Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

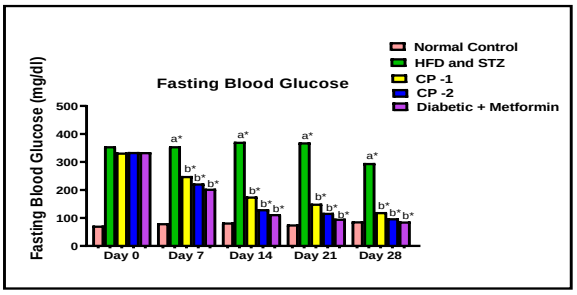


Fig 6.2.B. Effect of EACP on fasting blood glucose (mg/dl). Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

Fig 6.2. Fasting Blood Glucose Level

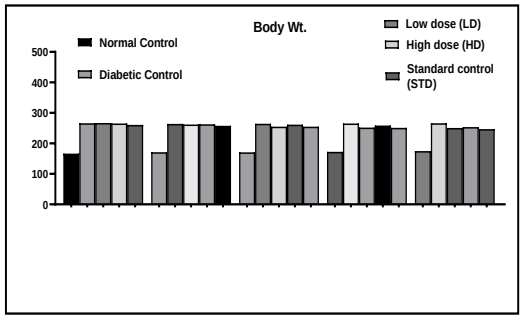


Fig 6.3.A. Effect of EASH on Body weight. Each value is expressed as mean ±SEM, where n=6.

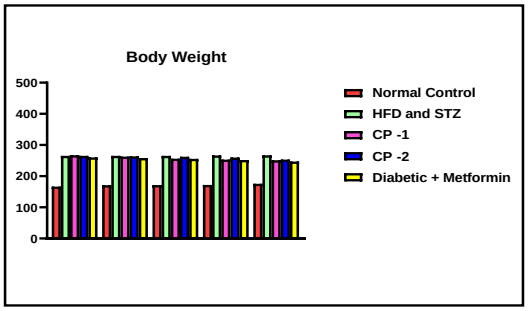


Fig 6.3.B. Effect of EACP on Body weight. Each value is expressed as mean ±SEM, where n=6.

Fig 6.3. Effect of extract on body weight

6.4.4. Glycosylated hemoglobin

When compared to diabetic rats, EASH and EACP treated rats had significantly reduced levels of glycosylated haemoglobin (p <0.05) effect of EASH on HbA1c **(Figure 6.4.).**

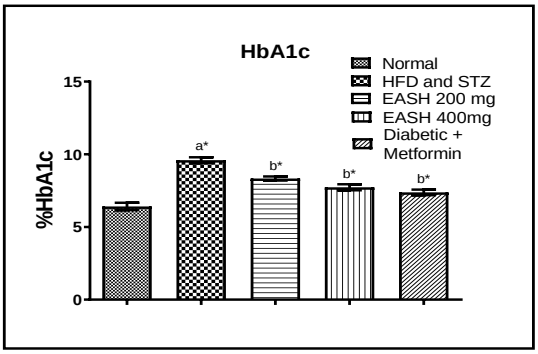


Fig 6.4.A. Glycosylated haemoglobin level of EASH. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

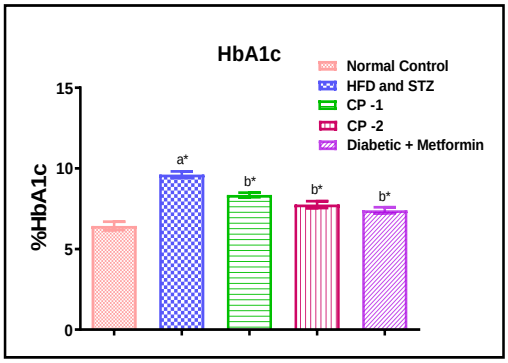
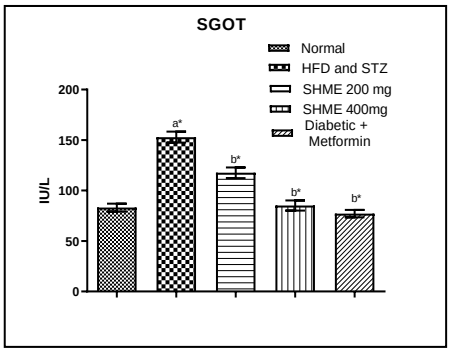


Fig 6.4.B. Glycosylated haemoglobin level of EACP. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

Fig 6.4. Glycosylated haemoglobin level

6.4.5. Serum biochemical parameter

Compared to the low dosage STZ high-fat control group, EASH and EACP treated groups demonstrated a substantial (p <0.05) decrease in blood biochemical parameters such as ALP, SGOT, and SGPT. Comparing the treated group to the low-dosage STZ high-fat diet control, the total protein level expanded in the treated group **(Figure 6.5.).**



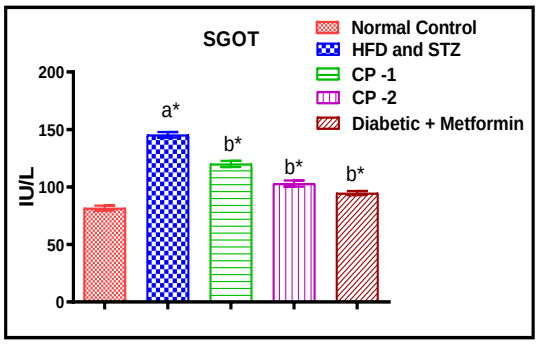
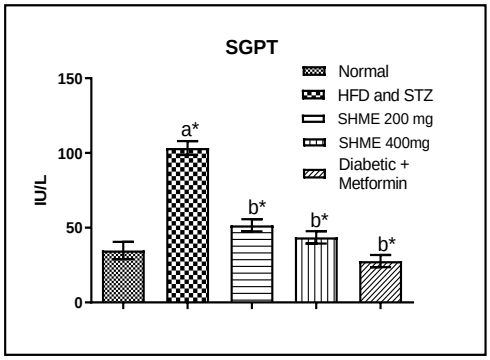


Fig 6.5.A. Effect of EASH on SGOT. Each value is expressed as mean ±SEM, where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

Fig 6.5.B. Effect of EACP on SGOT. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.



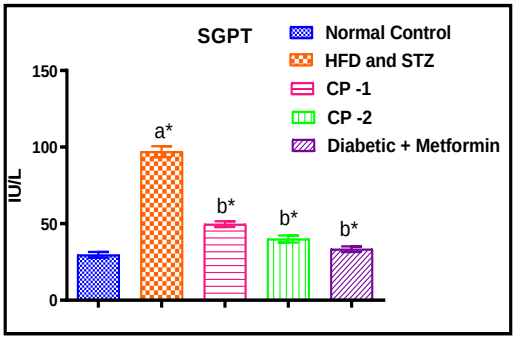
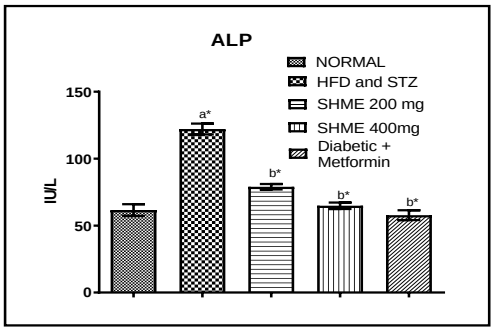


Fig 6.5.C. Effect of EASH on SGPT. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

Fig 6.5.D. Effect of EACP on SGPT. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.



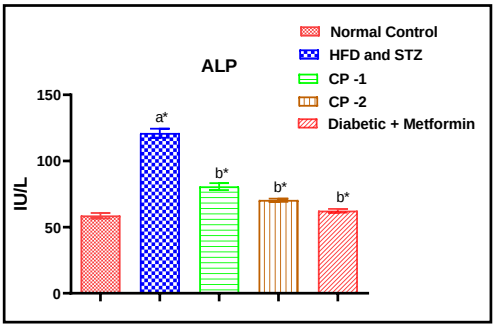


Fig 6.5.E. Effect of EASH on ALP. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

Fig 6.5.F. Effect of EACP on ALP. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

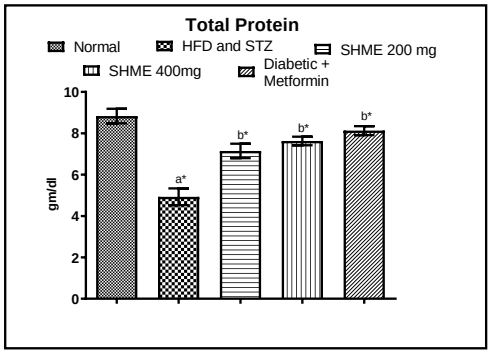


Fig 6.5.G. Effect of EASH on Total Protein. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

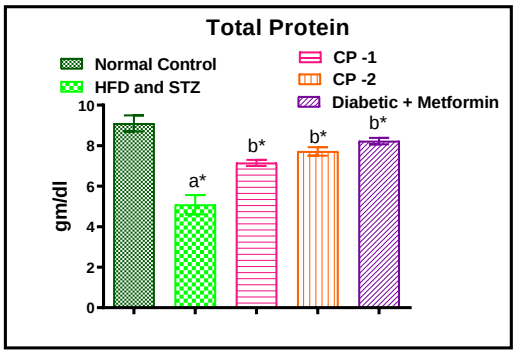


Fig 6.5.H. Effect of EACP on Total Protein. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

Fig 6.5. Serum biochemical parameter

6.4.6. Serum lipid profile

As opposed to the diabetic group, diabetic rats had serum lipid profiles that were considerably ($p < 0.05$) higher in triglycerides and total cholesterol and significantly ($p < 0.05$) lower in HDL. In comparison to the diabetes groups, the triglyceride and total cholesterol levels were especially ($p < 0.05$) decreased after EASH and EACP extracts feed at doses of 200 mg/kg and 400 mg/kg, while HDL levels were significantly (p <0.05) elevated **(Figure 6.6.).**

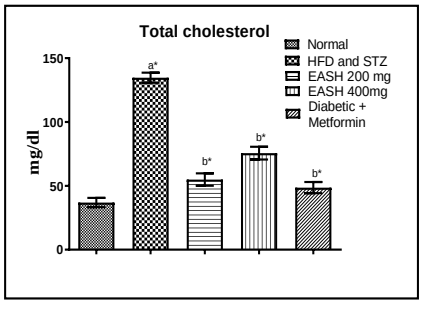


Fig 6.6.A. Effect of EASH on Total Cholesterol. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

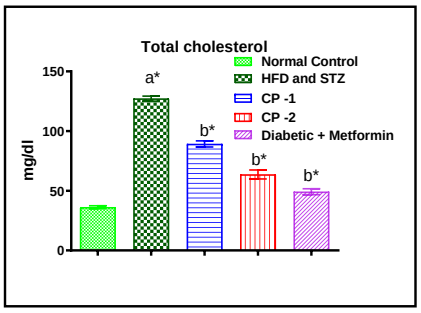


Fig 6.6.B. Effect of EACP on Total Cholesterol. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

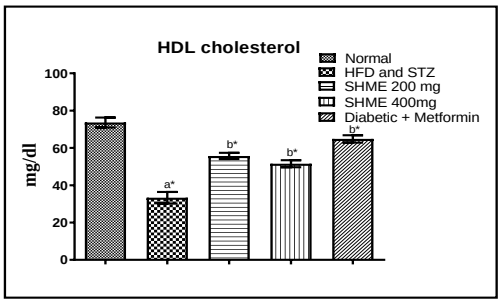


Fig 6.6.C. Effect of EASH on HDL Cholesterol. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

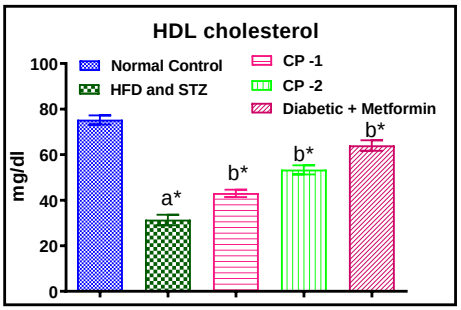


Fig 6.6.D. Effect of EACP on HDL Cholesterol. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

Fig 6.6. Serum lipid profile

6.4.7. Tissue antioxidant parameters:

In EASH and EACP treated rats compared to diabetic rats, the malonaldehyde level was significantly (p <0.05) decreased, SOD, and GSH level was significantly (p <0.05) enhanced **(Figure 6.7.).**

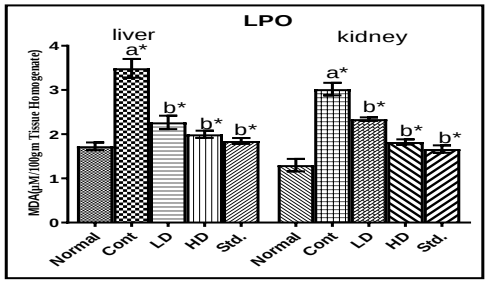


Fig 6.7.A. Effect of EASH on LPO. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

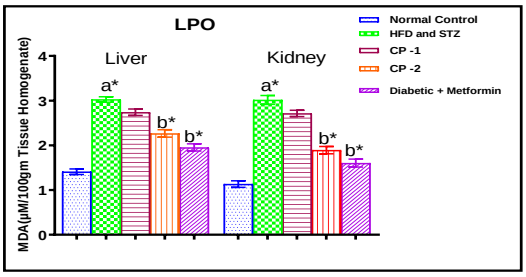


Fig 6.7.B. Effect of EACP on LPO. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to disease control.

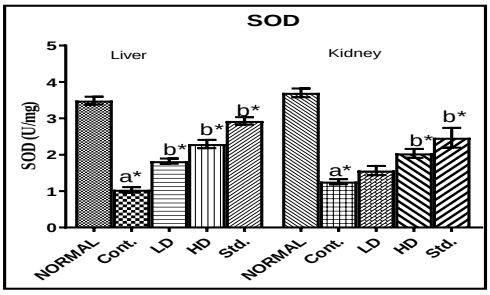


Fig 6.7.C. Effect of EASH on SOD. Each value is expressed as Mean ± SEM where n=6. a* $p < 0.05$ when compared to normal control and b* $p < 0.05$ when compared to disease control.

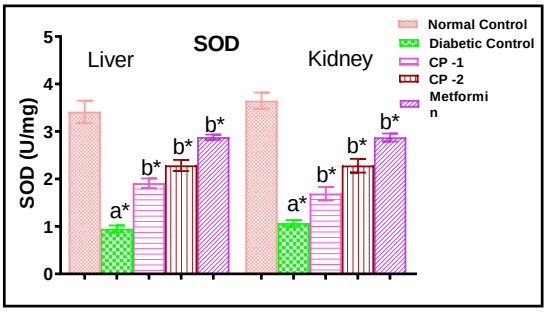


Fig 6.7.D. Effect of EACP on SOD. Each value is expressed as Mean ± SEM where n=6. a* $p < 0.05$ when compared to normal control and b* $p < 0.05$ when compared to disease control.

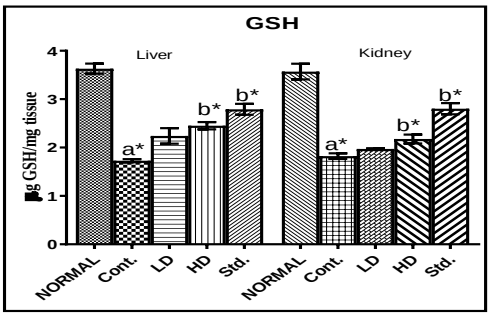


Fig 6.7.E. Effect of EASH on GSH. Each value is expressed as Mean ± SEM where n=6. a* $p < 0.05$ when compared to normal control and b* $p < 0.05$ when compared to disease control.

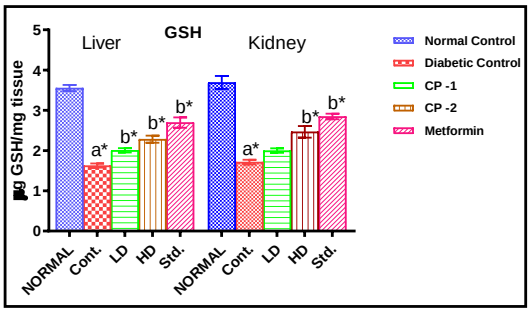
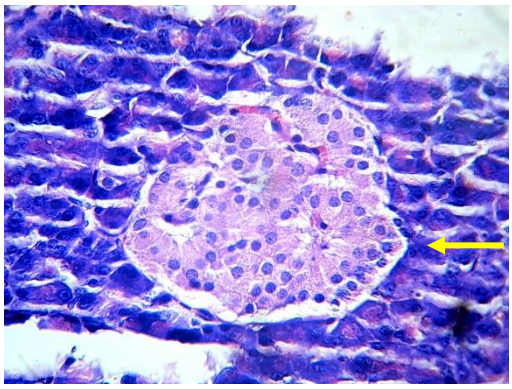
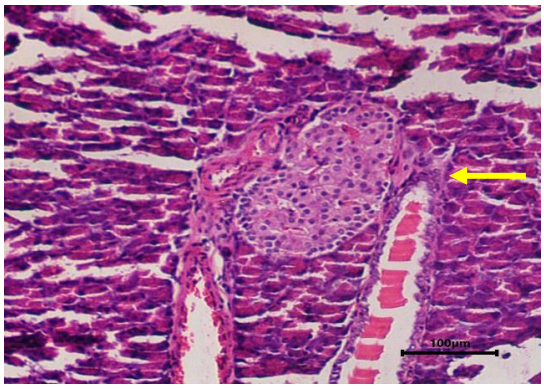
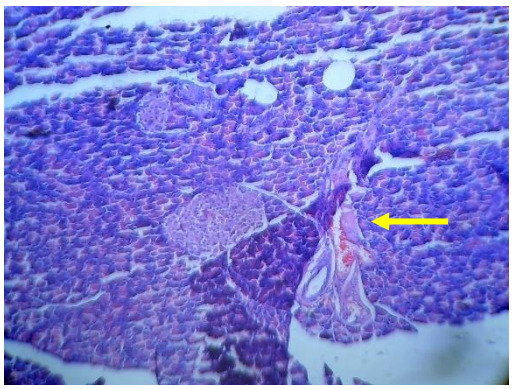
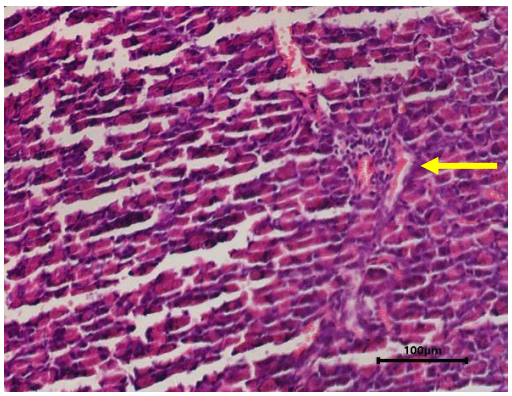
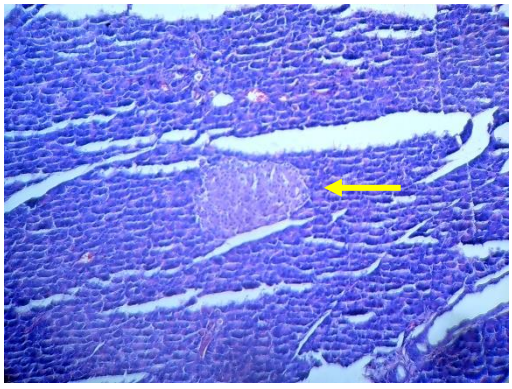
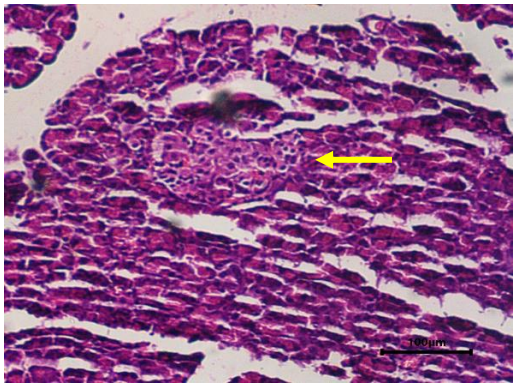


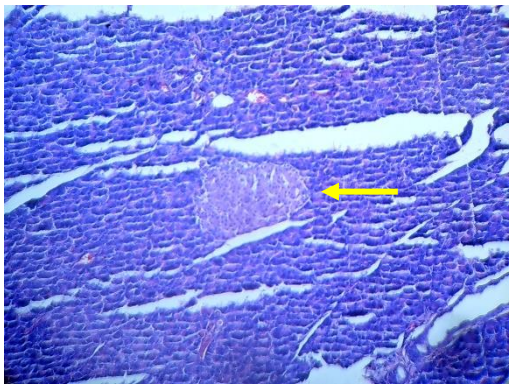
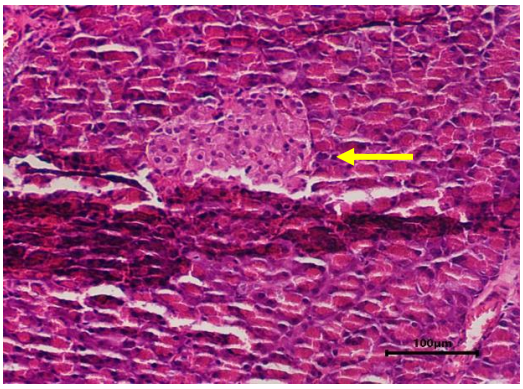
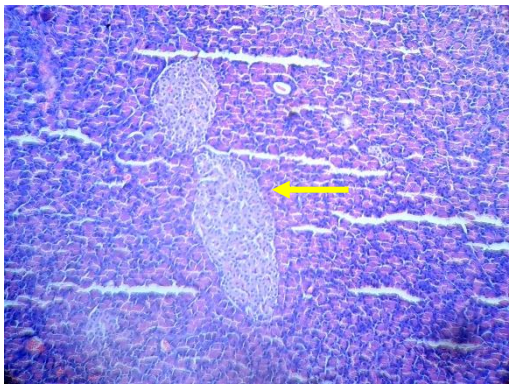
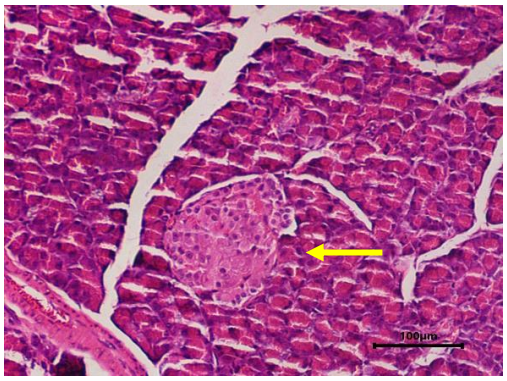
Fig 6.7.F. Effect of EACP on GSH. Each value is expressed as Mean ± SEM where n=6. a* $p < 0.05$ when compared to normal control and b* $p < 0.05$ when compared to disease control.

Fig 6.7. Tissue antioxidant parameters

Histopathology of pancreas

Studies on pancreatic histopathology revealed that groups treated with EASH and EACP had pancreatic beta cell protective action. Compared to diabetic rats, there was a dose-dependent gradual improvement in pancreatic beta cell density in groups treated with EASH and EACP. **(Figure 6.8, 6.9).**

	
6.8.A- NORMAL CONTROL PANCREAS	**6.9.A-NORMAL CONTROL PANCREAS**
	
6.8.B- DISEASE CONTROL PANCREAS	**6.9.B- DISEASE CONTROL PANCREAS**
	
6.8.C- 200 mg/kg of EASH PANCREAS	**6.9.C- 200 mg/kg of EACP PANCREAS**

	
6.8.D- 400 mg/kg of EASH PANCREAS	**6.9.D-400 mg/kg of EACP PANCREAS**
	
6.8.E- Standard drug (Metformin) treated PANCREAS	**6.9.E- Standard drug (Metformin) treated PANCREAS**
Fig 6.8. Histopathology of pancreas - EASH	**Fig 6.9. Histopathology of pancreas- EACP**

6.5. Discussion

The current investigation aimed to examine the anti-hyperglycaemic as well as antidiabetic effects of ethyl acetate fraction of *Solena heterophylla* and *Cyclanthera pedata* in diabetic induced wistar albino rats, had been given high-fat diet and low dose of STZ (35 mg/kg). The current study findings showed that EACP at of 200 and 400 mg/kg considerably lowering the elevated blood glucose levels back to normal range and effectively altered serum, liver, and kidney biochemical markers levels significantly compared with EASH at 200 and 400 mg/Kg. The results of the OGTT demonstrate that administration of the EACP possesses potent antihyperglycemic activity at both doses and inhibited the rise in serum glucose level as effectively as the reference medication Metformin ($P<0.05$) and EASH. This observation is similar to that the intestinal glucose transporter has decreased (Panigrahi et al., 2016).

Following seven days of low-dose STZ induction, hyperglycaemia was observed in experimental animals. After 7, 14, and 21 days, treatment of a HFD and low-dose STZ (35 mg/kg)-induced diabetic rats began to reduce fasting blood glucose levels in a dose-dependent manner with the treatment of both the plant extracts. After 28 days, the rats became significantly normoglycemic with the treatment of EACP. And also shown to be equivalent to those produced at 150 mg/kg by the reference medication metformin. When, STZ is administered IP route, it damages β cells by producing free radicals, gradually reducing the amount of insulin production and raising blood sugar levels. Due to the impairment of lipid metabolism caused by increased glucose levels, atherosclerosis and coronary heart disease and other diabetic related adverse effect are considered to be grown (Wahab et al., 2022). Treatment with EACP remarkably reversed all the parameters to normal.

It has long been known that dyslipidaemia plays a part in the emergence of diabetes macro vascular problems. Following treatment with EACP and EASH fraction, lipid profiles were diabetic induced rats improved at 200 and 400 mg/kg doses. When diabetic rats were treated with EACP at quantities of 200 and 400 mg/kg, hepatic lipid build up was significantly reduced. The lipolytic enzyme lipase hydrolyses triglycerides and phospholipids in circulating plasma lipoproteins (Kumar et al., 2013).

Increased plasma SGOT, SGPT, and ALP level, as well as a drop in total protein level, suggested that diabetes may cause hepatic dysfunction and causes liver necrosis. Therefore, it is possible that the release of these enzymes from the liver cytosol into the circulation is principally responsible for the elevation in SGOT, SGPT, and ALP plasma activity (El-Demerdash et al., 2005). EACP (200 and 400 mg/kg) treated diabetic rats, caused a reduction in the activity of these enzymes in plasma as compared to the mean values of the diabetic group. These findings support the notion that the plant has an excellent liver-protective effect in relative to EASH.

Type 1 and Type 2 diabetes mellitus have been linked to lipid peroxide-mediated tissue injury. Diabetes impairs insulin secretion, which may cause lipid peroxidation in biological systems. Increased levels of TBARS found in diabetic rats' livers and kidneys point to increased free radical production and activation of the lipid peroxidative system. The current study demonstrates that administering EACP doses along with metformin inhibits MDA generation to counteract lipid peroxidation. The two main scavenging enzymes SOD and GSH are crucial in defending the cell against the potentially harmful effects of ROS by eliminating dangerous free radicals. The build-up of superoxide radicals (O2-) and hydrogen

peroxide may cause various negative consequences when SOD and GSH activity is reduced. When metformin as well as EACP and EASH are administered to the in-vivo groups, SOD and GSH are activated and possess tissue antioxidant activity. EACP considerably contains more free radical scavenging activity, which could perform a protective action against pathological modifications brought on by the presence of O2- and OH-, according to the results of the SOD and GSH activity (Gupta et al., 2007). In Non-enzymatic endogenous antioxidant system, glutathione play a crucial role. Glutathione detoxifies hydrogen peroxide by acting as a reducing agent and have antioxidant properties. In HFD and STZ-induced hyperglycaemic rats, EACP precisely reduced the GSH may result a decrease in oxidative stress-induced breakdown of GSH. However, the GSH level in the kidney and liver tissues increased after treatment with EACP compared with EASH. In comparison to diabetic control rats, histopathological analysis of the pancreas of EACP-treated diabetic rats revealed a dose-dependent increase in β cell density, indicating that EACP treatment promotes cellular development. EACP revealed significant antidiabetic effect in albino rats in a dose-dependent manner in the current investigation. Whereas, EASH treated groups are comparatively generates less amount of β cell in pancreas.

6.6. Conclusion

In this present study shows that the EACP exhibits potent hypoglycemic effect (FBG) and also improves the pancreatic β cell, antioxidant defense system as Lipid peroxidation, SOD and GSH significantly in comparison with standard metformin and EASH. These findings point to a hopeful improvement in the histology of the pancreas as well as intestinal glucose transport (OGTT), glycosylated haemoglobin, and enzymatic liver biochemical markers. These outcomes can be helpful for the development of effective healthcare alternatives to manage diabetes and related disorders. The significant activity of EACP was further considered to assess its wound healing potential to validate the ethnopharmacological claim.

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Chapter 7

In-vivo antidiabetic wound healing activity

7.1. Introduction

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7.2.2. Experimental animals

7.2.3. Ointment preparation

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7.2.5. Induction of diabetes and wound

7.2.6. Excision wound model

7.2.7. Experimental design

7.2.8. Connective tissue parameters

7.2.9. Tissue antioxidant parameters

7.2.10. Histopathological studies

7.2.11. Statistical analysis

7.3. Results

7.4. Discussion

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7.1. Introduction

Wound healing is thought to be important for the elimination of harmed tissues and invasive pathogens from the body, as well as to restore the progression and building of a cutaneous or visceral defect. Haemostasis, inflammation, proliferation and remodelling are the four precisely timed steps that make up the normal biological process of wound healing in the human body (Guo et al., 2010). The synchronization of various cells, cytokines, and growth factors is necessary for the process of wound healing. All four steps must take place in the right order and amount of time for a wound to heal properly (Rodrigues et al., 2019, Sharma et al., 2021). Diabetes mellitus (DM) commonly impairs wound healing, which can cause chronic skin ulcers, delayed healing, or non-healing wounds (Parmar et al., 2018). Hyperglycaemia impedes wound healing by blocking the passage of nutrients because of its link to atherosclerosis. Re-epithelialization depends on the effects of hyperglycaemia on keratinocyte and fibroblast migration, proliferation, and protein synthesis besides endothelial cells. The formation of free radicals because of the antioxidant enzymes glutathione peroxidase and superoxide dismutase decreased activity is another way that hyperglycaemia impedes the healing of wounds (Burgess et al., 2021, Deng et al., 2021). Healing impairment in diabetes patients continues to be significant clinical concern and new era in wound healing research is necessary requiring innovative therapeutic options to address this rising problem. The therapeutic efficacy of medicinal plants has evolved to treat broad spectrum of pathological and physiological illnesses. Approximately 70 to 90% of the population in developing nations in traditional medicine provides the primary form of therapy (Anand et al., 2019).

Herbal medicines are also used for wound treatment includes cleaning, debridement and creating an atmosphere that promotes the natural healing process and herbal medicine is less toxic and also has fewer side effects than conventional medication (Maver et al., 2015). *Cyclanthera pedata* is suggested that those with high blood pressure, arteriosclerosis, diabetes, circulatory issues and high blood cholesterol use fruit juice or infusion as a treatment. Boiling fruit and leaves in olive oil can be applied topically as a topical analgesic and anti-inflammatory. Intestinal parasites can be treated with seeds that have been dried and ground into powder. For the treatment of diabetes, leaves are prepared as a decoction because they are hypoglycaemic (Antih et al., 2016)**.** "Caigua" has recently drawn interest from the pharmaceutical industry (Frigerio et al. 2021). Various effects have been ascribed to this

plant, with the most intriguing being its ability to reduce hypertension (Ranilla et al. 2010) and have hypoglycaemia and hypercholesterolemia (Egg 1999; Gonzales et al. 1995; Ranilla et al. 2010) qualities in addition to analgesic and antioxidant properties (Vasquez 2003; Garcia et al. 2010).

7.2. Material and methods

7.2.1. Drugs and chemicals

T-bact-ointment (15gm) [Mupirocin ointment I.P.] was bought from a local medical store. Hexosamine, Hexuronic acid, Hydroxyproline, Trichloroacetic acid A.R. (TCA) from Merck (Mumbai, India); Thiobarbituric acid (TBA), Nitrobluetetrazolium chloride (NBT) was obtained from LobaChemie (Mumbai, India); Phenazinemethosulphate (PMS), Reduced nicotinamide adenine dinucleotide (NADH), 5,5′-dithio bis-2-nitro benzoic acid (DTNB) from Sigma (St. Louis, MO). The chemicals and reagents utilised in the test were of analytical grade came from, LobaChemie (Mumbai, India), sigma (St. Louis, MO), Merck (Mumbai, India), Sisco (Mumbai, India) and Arkray Healthcare Pvt. Ltd. (Japan).

7.2.2. Experimental animals

In this investigation, Fasting blood glucose (FBG) readings of 85-90 mg/dL from the registered breeder West Bengal Livestock Development Corporation Limited, Buddha Park, Kalyani, Dist.-Nadia – 741 235, West Bengal (Reg. no. 2109/GO/ReRcBiBt/S/20/CPCSEA), male wistar albino rat (160-180 gm), were utilized. The animals had a 07th day of acclimatization in a typical setting with unlimited access to food and water. The rats were fed a typical high-fat diet and allowed full access to water while being maintained in a 25°C environment with a 12-hour light/dark cycle. The experimentation was approved (Approval Number JU/IAEC-22/15) by the Animal Ethical Committee of Jadavpur University, Kolkata, West Bengal, India.

7.2.3. Ointment preparation

Ointment was prepared of following doses of 5% and 10% as mentioned in the table below (Demilew et al., 2018).

Table 7.1: Ointment Formulation with ethyl acetate fraction of *Cyclanthera pedata*

Formula	Ingredients	Amount (%w/w)
5% w/w ointment preparation	Ethyl acetate fraction of *Cyclanthera pedata*	5
	Soft paraffin	80
	Hard paraffin	5
	Bees wax	5
	Cetostearyl alcohol	5
10% w/w ointment preparation	Ethyl acetate fraction of *Cyclanthera pedata*	10
	Soft paraffin	75
	Hard paraffin	5
	Bees wax	5
	Cetostearyl alcohol	5

7.2.4. Acute skin irritation study

To test the anti-inflammatory properties of *Cyclanthera pedata* ethyl acetate fraction on laboratory animals, the tested mice should be segregated from other mice and their dorsal hair should be removed, then they were anaesthetized with ketamine to create a wound. The experimental animals were then divided into three groups, and each group exposed area received applications of 5%, 10% ointment and 0.8% formalin as a standard irritant respectively (Ankomah et al., 2022). The animals were watched for seven days to search for any signs of swelling or inflammation, such oedema and erythema, in the region being investigated as per OECD guidelines 404. (Anonymous 2015)

When applied to the skin, all ointment formulations did not cause skin irritation, such as erythema and edema, for around a week. However, as depicted in **Fig 7. 1**, the 0.8% formalin group exhibited extreme redness and edema.

7.2.5. Induction of diabetes and wound

After inducing high fat diet for 28 days, on the 29th day with access to water, rats were starved for 18 hours before the induction of diabetes. An intraperitoneal dose of streptozotocin (35 mg/kg body weight) was administered. (i.p.) and dissolved in 0.1 M citrate buffer (pH 4.5). The presence of diabetes was determined by estimating the fasting blood

glucose level. For the experiment, animals with fasting blood sugar levels under 250 mg/dl were used.

7.2.6. Excision Wound Model

First, the test rats were given ketamine to make them unconscious (Rhea and Dunnwald, 2020). After marking a 500 mm2 region on the dorsal side of the experimental rats with a ring, hair was trimmed to expose the bare skin. After excision, measurements of the excised area were taken on every 4th day for the next 16 days using Image J software to calculate the % decrease of the wound area or the wound contraction rate. A proper validation of the test drug's ability to treat wounds was provided by a comparison of the wound area between the diabetic group with the ointment base group, test drug group and the standard control group (Pastar et al., 2014). The wound contraction rate was calculated as follows:

% wound contraction = [Healed area /Total wound area] × 100,

(% healed area = original wound area − present wound area)

7.2.7. Experimental design

The experimental treatment schedule was designed after high-fat diet treatment for 28 days. With 5 groups of four rats each (n = 4), the rats were split and the experiment was scheduled for 14 days.

Group - I: Normal control rat feed with 0.5 ml/kg of normal saline intraperitoneally

Group - II: Diabetic control rat feed with high fat diets + topical applications of ointment base

Group - III: Diabetic control rat feed with high fat diets + topical applications of 5% EACP ointment

Group - IV: Diabetic control rat feed with high fat diets + topical applications of 10% EACP ointment

Group - V: Diabetic control rat feed with high fat diets + topical applications of Mupirocin ointment

7.2.8. Connective tissue parameters

These studies were done in 96 well plates following the procedure as described in with slight modification. For this study, 250 mg of wet, granulated tissues were dried at 50° C for roughly 24 hours before being weighed and put into glass test tubes with stoppers. Each test tube contained 1ml of 6 N HCl after dissolving 40 mg of dry tissue in it. The test tubes were then placed in a pot of boiling water for 24 hours, with 12 hours per day, to allow for hydrolysis. The acid was then neutralised using 10N NaOH after cooling the hydrolysate, and phenolphthalein was used as an indicator. The neutral hydrolysates were diluted to a concentration of 20 mg/ml with distilled water. The standard curve method and the appropriate substrate were then used to determine whether hydroxyproline, hexosamine, and hexuronic acid were present in these hydrolysates (Murthy et al., 2013).

7.2.8.1. Tissue hexosamine (HXA) content

Each of the tubes received 0.05 ml of hydrolysate that had been made into 0.5 ml by diluting it with distilled water. Then, 0.5 ml of acetyl acetone, which had been added to this diluted solution, was added to each tube. In boiling water, these mixtures were heated. These mixtures got 0.5 mL of Ehrlich's reagent addition and 1 mL of 95% alcohol. These mixtures had 20 minutes to react. At 530 nm, the colour fidelity of each tube was evaluated against a blank. D (+) glucosamine hydrochloride (HiMedia Laboratories Pvt. Ltd., Kolkata, India), in concentrations ranging from 5 to 100 g/mL with a 1000 g/mL working solution was used to measure the amount of hexosamine in each test tube (Murthy et al., 2013).

7.2.8.2. Tissue hexuronic acid (HUA) content

0.150 mL of hydrolysate was diluted to 0.5 mL with distilled water. Stoppered tubes containing 2.5 mL of 0.025 M borax in concentrated sulfuric acid were put on a test tube rack to cool to 4 0C. Following the addition of 0.5 mL of diluted hydrolysate and keeping the temperature at 4 0C, the glass tube was sealed with a stopper and, after a slow first shake, was shaken vigorously to maintain the temperature. After that, the tubes were heated for 15 minutes in a hot water bath to return the temperature to normal. After each tube had received 0.1 mL of 0.125% carbazole in absolute alcohol, been thoroughly shaken, and spent 15 minutes in a hot water bath, the tissue's colour intensity was evaluated against a blank. The standard curve created using D (+) Glucurono-6, 3-lactone from 5 to 100 g/ mL by HI Media

Laboratories Pvt. Ltd., Kolkata, India. The quantity of hexuronic acid in the samples was determined using a 1000 g/mL working solution (Murthy et al., 2013).

7.2.8.3. Tissue hydroxyproline (HPR) content

0.3 ml of hydrolysate, 0.01 M CuSO4, 2.5 N NaOH, and 6% H_2O_2 were added to each tube. The tubes were shaken vigorously and then put in a water bath at a temperature of 70^0 C. Tubes were removed from the water bath after 10 minutes and placed in ice water to cool for 5 minutes. The test tube was then filled with 0.7 ml of a 5% solution of para dimethyl amino-benzaldehyde and 1.4 ml of 3 N H2SO4. The test tubes were again immersed in a water bath at 75 °C for 15 minutes, and then allowed to cool for 10 minutes under a regular water stream. UV spectroscopy at 540 nm was used to compare the tissue content colour intensity of each tube to a blank. The hydroxyproline content of the tissue was determined using the standard curve technique, which consisted of standard 4-hydroxy L-proline from 100 to 1000 g/mL using 3 mg/mL of working solution (Murthy et al., 2013).

7.2.9. Tissue antioxidant parameters

Skin tissues are collected from the sacrificed animals were homogenized separately in 10 ml of phosphate buffer (20mM, pH-7.4) and centrifuged at 12000 rpm for 30 min at 4° C. The supernatants were collected and Lipid peroxidation (LPO), reduced glutathione (GSH) and Super oxide dismutase (SOD) were estimated by Chakraborty et al, 2015**.**

7.2.10. Histopathological studies

Skin tissue from sacrificed rats was collected and used in histopathological investigations. The tissues were prepared for microscopic photo analysis through being instantly fixed in 10% formalin for at least 24 hours after being rinsed in normal saline and immediately sliced into 4 to 5-m thick pieces, fixed in paraffin, and stained with haematoxylin and eosin dye after being dried with alcohol (Jana et al., 2024).

7.2.11. Statistical evaluation

The mean and SEM are used to express all results. Graph Pad Prism 8.02 software was utilized to perform a one-way analysis of variance (ANOVA) and post hoc Dunnett test to evaluate whether the results were statistically significant (Graph Pad Software, USA). Statistics were considered important at $p < 0.05$.

7.3. Results

7.3.1. Acute Skin Irritation Assay

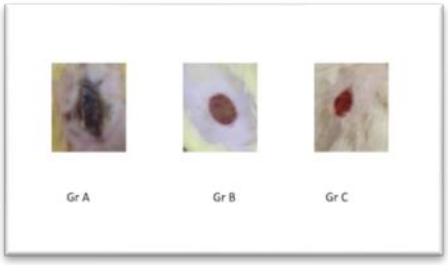


Fig 7.1: Photographic representation of effect of EACP ointment at the different concentrations over the skin after 7 days, where Gr A= Aqueous solution of 0.8% formalin , erythema and edema skin rash and inflammation was observed, Gr B= 100 gm of 5% w/w of ethyl acetate fraction of *Cyclanthera pedata* fruits ointment, no lesion occur. Gr C= 100 gm of 10% w/w ethyl acetate fraction of *Cyclanthera pedata* fruits ointment, no lesion occur.

7.3.2. Excision Wound Model

7.3.2.1. Percentage wound healing area

Following 16 days of monitoring, the percentage of wound contraction revealed that 90.22% was the normal control and topical applications of 5% and 10% EACP fruits fraction resulted in wound shrinkage percentages of 93.74 and 96.82% respectively, compared to 98.93% for conventional medication, mupirocin ointment (5g). The EACP ointment was used from the first day onward and observation revealed that this encouraged wound contraction more quickly than the diabetic control.

Table 7.2.: Effect of EACP on % wound healing area through excision wound model.

GROUPS	DAY 1	DAY 4	DAY 8	DAY 12	DAY 16
	MEAN±SEM	MEAN±SEM	MEAN±SEM	MEAN±SEM	MEAN±SEM
GR I	0±0	20.15004±5.92	42.26344±8.54	65.0399±12.1	90.22704±3.25
GR II	0±0	-77.271±16.32^{a*}	- 61.4866±14.82^{a*}	3.94±0.26^{a*}	66.43599±13.5^{a*}
GR III	0±0	43.82717± 4.34^{b*}	56.87944±10.26^{b*}	91.04839±9.1^{b*}	93.74882±2.51^{b*}
GR IV	0±0	14.17±3.06^{b*}	45.11±6.98^{b*}	93.60884±3.43^{b*}	96.8211±3.16^{b*}
GR V	0±0	30.81±1.04^{b*}	90.07314±3.17^{b*}	94.9539±7.26^{b*}	98.9382±1.58^{b*}

Each value is mean ± S.E.M (n=4). a= when compared with Normal Control group, b= When compare with Diabetic Control Group. (*) indicates statistically difference from respective group using ANOVA, followed by Dunnett's multiple comparison test ($p<0.005$).

7.3.2.2. Percentage wound healing area

Photographic representation of effect of EACP ointment, Group-I: Normal control rat feed with 0.5 ml/kg of normal saline intraperitoneally; Group-II: Diabetic control rat feed with high fat diets + topical applications of ointment base; Group-III: Diabetic control rat feed with high fat diets + topical applications of 5% EACP ointment; Group-IV: Diabetic control rat feed with high fat diets + topical applications of 10% EACP ointment; Group-V: Diabetic control rat feed with high fat diets + topical applications of Mupirocin ointment.

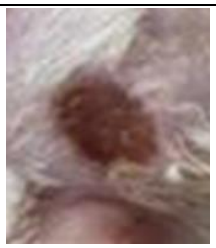
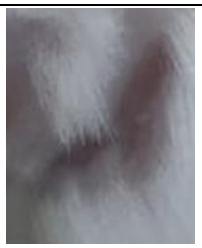
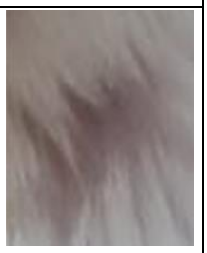
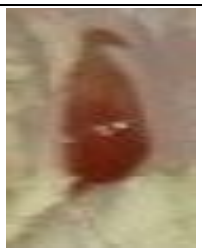
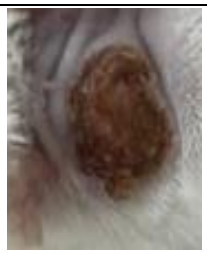
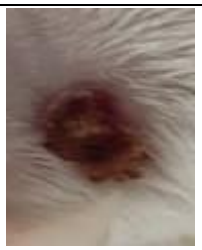
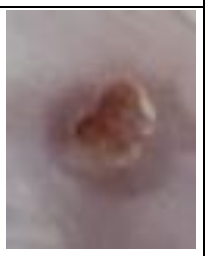
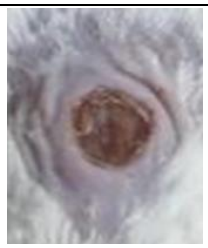
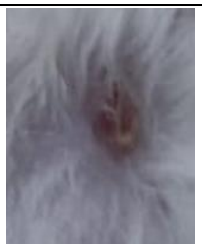
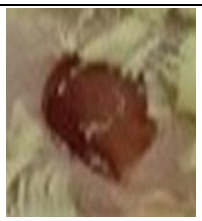
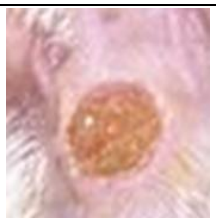
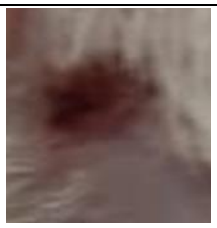
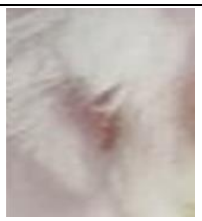
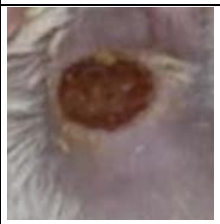
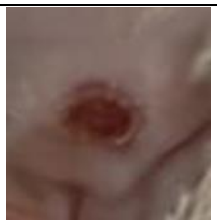
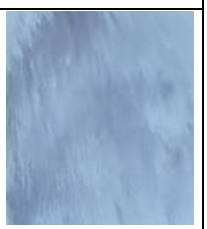
GROUPS	DAY 1	DAY 4	DAY 8	DAY 12	DAY 16
GR I					
GR II					
GR III					
GR IV					
GR V					

Fig 7.2.: Photographic representation of effect of EACP ointment on the wound contraction throughout the study after topical treatment.

7.3.2.3. Tissue antioxidants and free radicals

GSH, SOD, and CAT levels significantly increased whereas LPO and NO levels of free radicals reduced. Below **(Fig 7.3)**, the outcomes with EACP in contrast to diabetic control are shown.

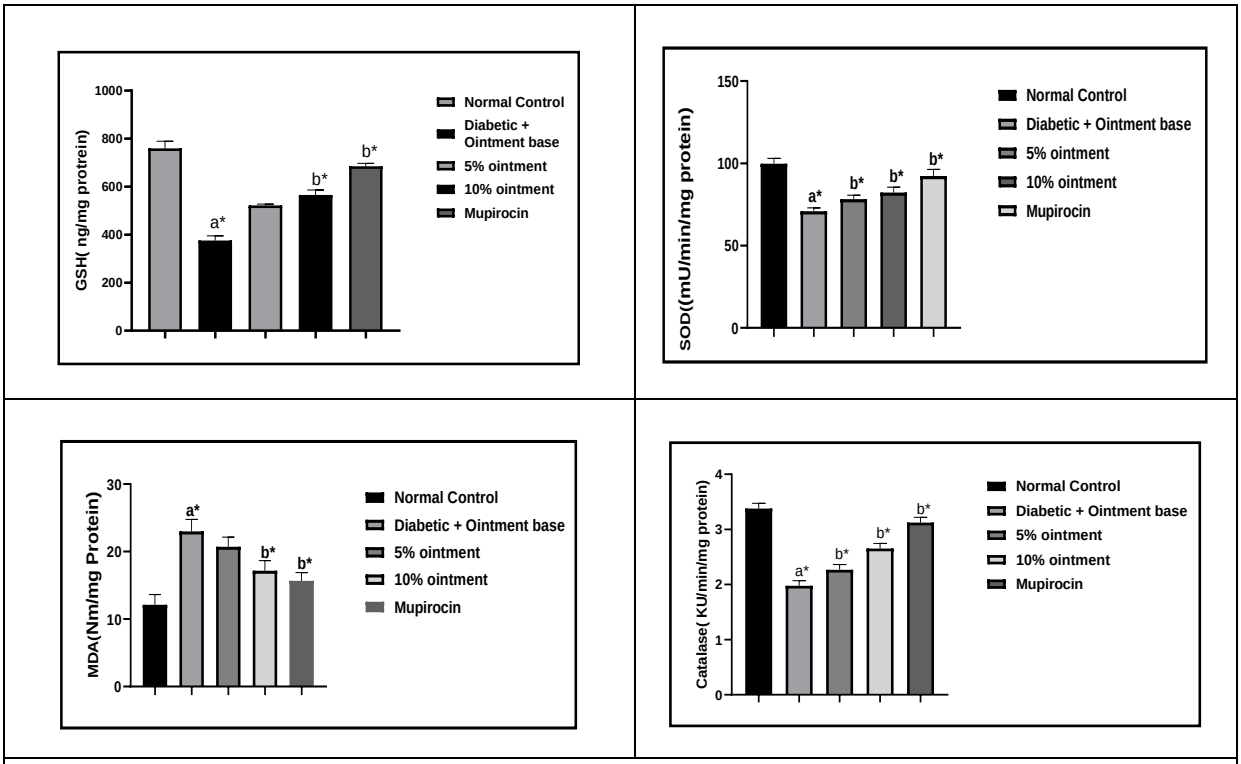


Fig 7.3: Effect of EACP on tissue antioxidants (Catalase, GSH, SOD) and free radicals (MDA). Each value is in Mean± S.E.M (n=4). a= when compared with Normal Control group, b= When compare with Diabetic Control Group. (*) indicates statistically difference from respective group using ANOVA, followed by Dunnett's multiple comparison test ($p<0.005$).

7.3.2.4. Connective tissue parameters

Hydroxyproline, Hexamine and Hexuronic acid levels significantly increased. Each value is in Mean± S.E.M (n=4).

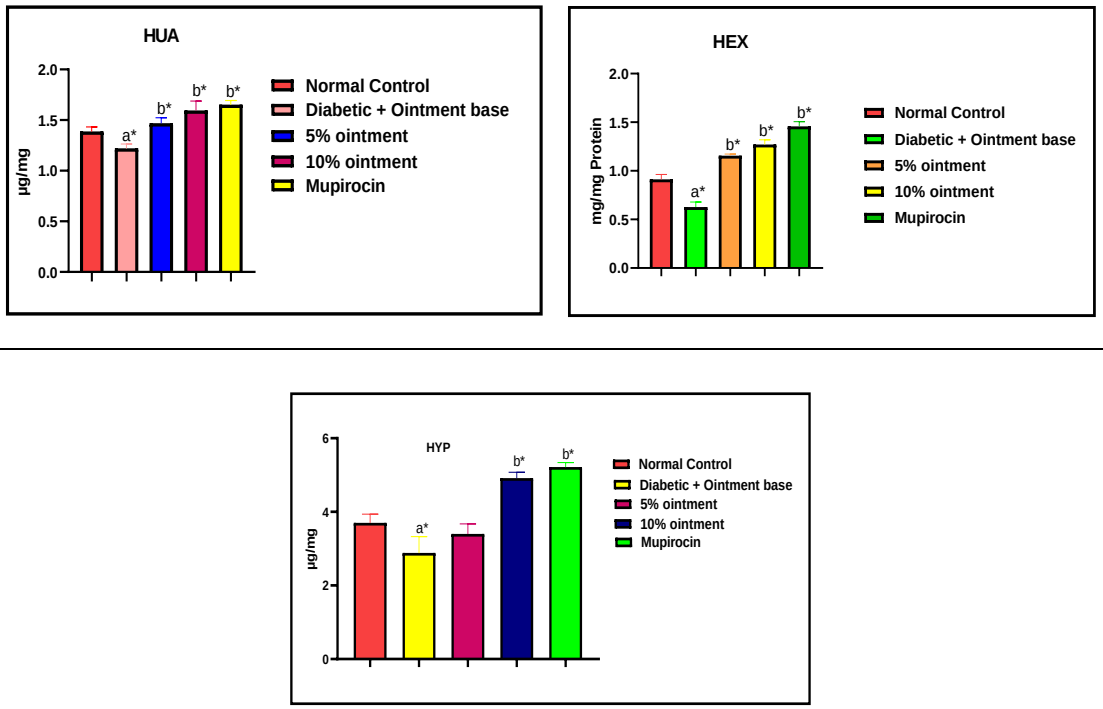


Fig 7.4.: Effect of EACP on tissue Hydroxyproline, Hexamine, Hexuronic acid. Each value is in Mean± S.E.M (n=3). a= when compared with Normal Control group, b= When compare with Diabetic Control Group. (*) indicates statistically no significant difference from respective group using ANOVA, followed by Dunnett's multiple comparison test ($p<0.05$).

7.3.3. Histopathological studies

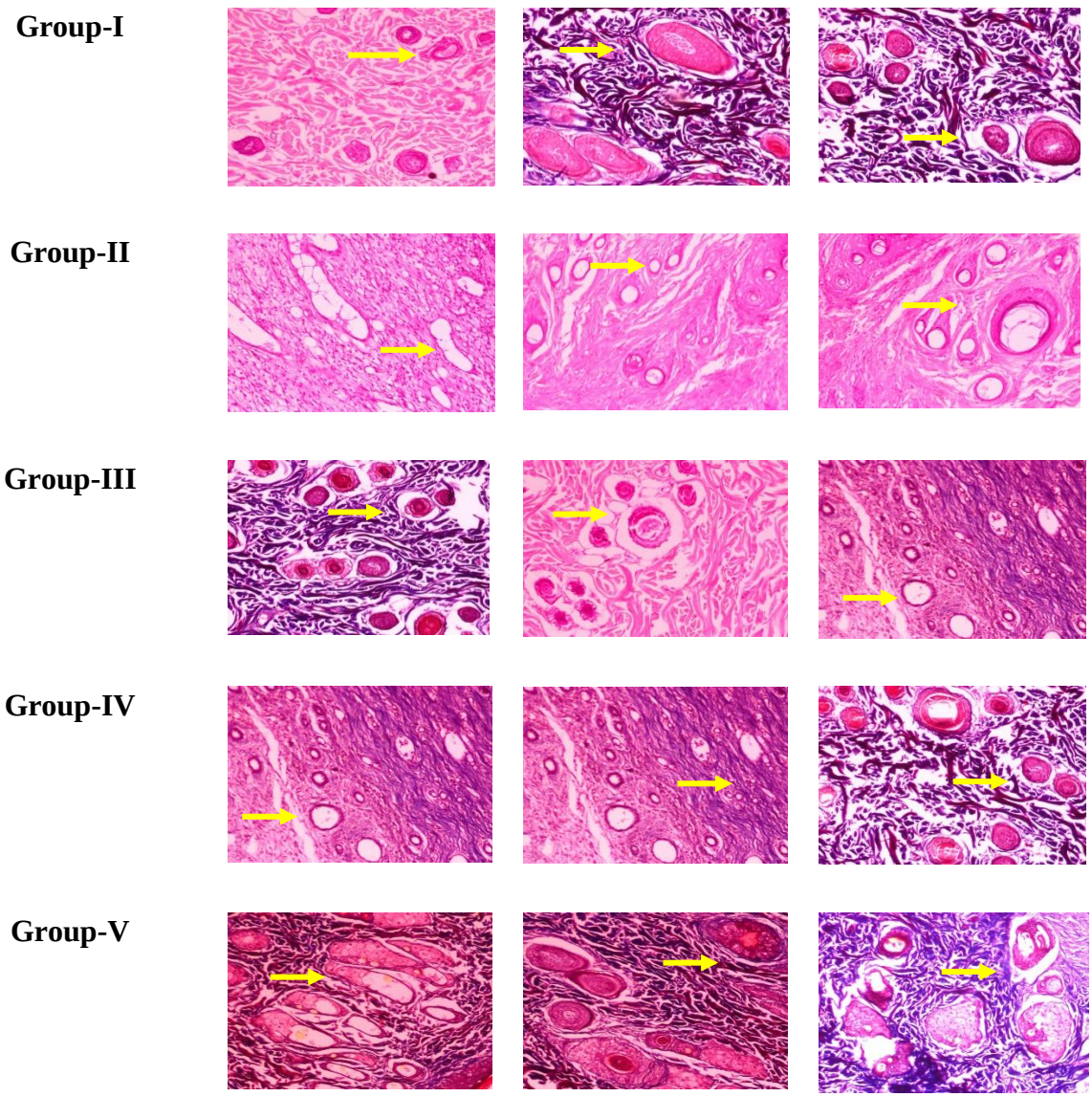


Fig 7.5: Skin histpathology phtographs of wound healing. Each value is in Mean ± S.E.M (n=4). a= when compared with Normal Control group, b= When compare with Diabetic Control Group. (*) indicates statistically difference from respective group using ANOVA, followed by Dunnett's multiple comparison test (*p<0.005).

7.4. Discussion

As a result, measuring this hydroxyproline has been utilised as a measure of collagen turnover. The higher concentration of hydroxyproline in the excision wound suggests quicker collagen turnover, which promotes quick healing and increases the breaking strength of the treated wounds. The matrix molecules hexosamine and hexuronic acids serve as the building blocks for the creation of new extracellular matrix. By strengthening electrostatic and ionic interactions with collagen fibers, glycosaminoglycans are known to stabilise them and may even regulate their final alignment and distinctive size.

They have been recognised as crucial determinants of cellular response in development, homeostasis, and illness due to their capacity to bind and modify protein-protein interactions. When compared to the diabetic control group in our study, the concentrations of hexuronic acid and hexosamine, which are parts of glycosaminoglycans, were significantly higher in the oral treatment group, indicating stabilisation of collagen fibre. Due to its extensive availability with low toxicity, lack of negative side effects, and potential for crude preparation, plant products have long been considered as promising wound healers. Under our current study, which used excision wound models under diabetes settings and included observation of many physical, histological, and biochemical data, the hydroalcoholic extract of EACP revealed wound healing activity. The research found that streptozotocin-induced diabetes in rats was accompanied by a considerable rise in blood glucose levels. Due to the destruction of the beta cells in the islets of Langerhans by streptozotocin, hyperglycemia was brought on. Without insulin, the adipose tissue and skeletal muscles are unable to absorb glucose from the blood, which prevents them from converting it to fat and glycogen. This causes the blood glucose level to rise. In the present investigation, diabetic rats' skin wound healing was markedly slowed down, which may be related to the high glucose levels seen in their blood plasma. As anticipated, the rats' diabetes circumstances favoured an aberrant physiological response that slowed the healing of their wounds. The diabetic animals' body weight gain was significantly reduced, which showed altered metabolic processes and may potentially be the result of irregular waste absorption and excretion. The animals' hyperglycemic condition caused them to become catabolic, breaking down protein and fat since there was insufficient glucose available for cell feeding, which did not help the healing process. After 16 days, it was seen that taking EACP orally dramatically lowered blood sugar levels. The current study shown that an EACP hydro-alcoholic extract accelerates wound

healing in diabetics. The findings suggested that EACP hydro-alcoholic extract treatment may be beneficial for many stages of wound healing, including the synthesis of fibrous tissues as well as the production and contraction of collagen, resulting in faster healing. The results also suggested that the extract may have hypoglycemic activity, as it has been demonstrated that diabetics' wound healing is enhanced by regulating blood glucose levels. Oral administration of EACP hydroalcoholic extract at doses of 200–400 mg/kg body weight per day to streptozotocin–induced diabetic rats for a period of 16 days resulted in a notable decrease in fasting blood glucose levels. On the other hand, fasting blood glucose levels changed after 16 days of topical administration of EACP at doses of 5% and 10% w/w ointment daily to streptozotocin-induced diabetic rats.

7.5. Conclusion

Chronic wounds exhibit oxidative stress induced by free radicals and neutrophil-derived oxidants, both of which significantly contribute to tissue damage during chronic wound inflammation, according to experimental and clinical studies. Reactive oxygen species (ROS) that are produced in excess cause oxidative stress, cytotoxicity, and sluggish wound healing. Therefore, eliminating ROS may be a crucial step in the healing of chronic wounds.

Because free radicals have been shown to slow down wound healing, assessment of free radicals like NO and MDA in skin tissue is important. Our research showed that the hydroalcoholic extract of EACP significantly reduced the stress on free radicals, helped to avoid inflammation, and reduced oxidative damage, all of which served to speed up the healing process of wounds. The histopathological changes in the skin from the wound area further backed up the results of the biochemical measures. The histological analysis revealed that oxidative stress had a role in the development of wound healing complications. The outcomes of this technique shed light on the possibility of an ointment comprising hydro-alcoholic extract of *Cyclanthera pedata* as a promising, secure, and efficient formulation to be employed in the management of wound healing, particularly in diabetes patients. The anti-inflammatory and anti-diabetic properties of the plant species also encourage their widespread use.These effects were mostly seen via enhancing antioxidant defences and altering connective tissue indicators. Overall, this research encourages the continued development of a novel pharmaceutical product or a novel raw material source for a topical ointment active ingredient.

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Chapter 8

Summary and Conclusion

8.1. Summary

8.2. Conclusion

8.3. Publications and Presentations

8.1. Summary

Diabetes is a disorder of carbohydrate, lipid, and protein metabolism ailment caused by decreased insulin production or increased insulin resistance. Herbal diabetes treatments have been used in patients with insulin-dependent and non-insulin-dependent diabetes, diabetic retinopathy, diabetic peripheral neuropathy, and other diabetes-related conditions. The usefulness of botanicals in lowering sugar levels has been scientifically validated using many Indian plant species.

This thesis is a pioneer of scientific investigations on hexane, chloroform, ethyl acetate and ethanol fractions of *Solena heterophylla* and *Cyclanthera pedata* in the research field. The introductory part of the thesis is designated by the concept of diabetes and pathological complications related with α-amylase and α-glucosidase enzymes. Pathophysiology of diabetic wound healing and a brief overview of Cucurbitaceae family were discussed. The fresh fruits of *Solena heterophylla* and *Cyclanthera pedata* were collected from North Eastern Regions of India. After collection fruits were cleaned, air dried and ground into a coarse powder. The powdered plant material was extracted by hydro alcoholic by cold maceration and further fractioned with hexane, chloroform, ethyl acetate and ethanol.

In-vitro antioxidant, α-amylase and α-glucosidase enzyme inhibitory activity were studied. The DPPH assay was quintessential in determining the free radical scavenging potential of all the fractions of both the plant under study. To terming the *in-vitro* antidiabetic activity α-amylase and α-glucosidase enzyme inhibition was performed and acarbose was used as standard for both the enzyme inhibition assay. In compare to all the fractions EASH and EACP showed significant antioxidant as well as *in-vitro* α-amylase and α-glucosidase inhibitory activity. The IC50 of free radical scavenging assay of EASH (0.102 ± 0.19 mg/ml) and for EACP (0.97 ± 0.26 mg/ml) which was comparable to the standard ascorbic acid (0.72 ± 0.15 mg/ml). In α-amylase enzyme inhibition assay EASH and EACP showed IC50 value of 0.578 ± 0.51 and 0.531 ± 0.32 mg/ml respectively. In α-glucosidase inhibition assay EASH showed inhibitory activity at 0.118 ± 0.34 mg/ml whereas EACP having the IC50 value of 0.116 ± 0.21 mg/ml. As per the results, EASH and EACP were further studied for metabolite profiling, HPTLC analysis and *in-vivo* assays.

In chapter 5, LC-Q-ToF-MS study was performed to compare the metabolite profiles of Ethyl acetate fractions of *Solena heterophylla* and *Cyclanthera pedata*. Compounds were

accurately identified with a precision of less than 5 ppm using negative ionisation, retention durations, molecular weights (m/z), and library searches. *Solena heterophylla* possesses a wide range of bioactive chemicals, such as isoquinoline alkaloids, diterpenoids, glycosides, and flavonoids. The main chemical compounds obtained from both the fractions were majorly triterpenoids, alkyl sulphates, benzenoids, phenolic acids, flavonoids, and glycosides. Polyphenols have the ability to hinder the activity of α-glucosidase and α-amylase enzymes, which leads to an enhancement in the absorption of glucose by muscles through the AMPK pathway. HPTLC analysis was performed for both the plants to analyse the quality. Quantification of cucurbitacin B, a major phytomarker of the Cucurbitaceae plant family was chosen at a concentration of 1 mg/ml. Both plants showed significant presence of Cucurbitacin B (2.29% in EASH and 0.014% in EACP). Previous studies mentioned the potential of Cucurbitacins, triterpenoids to decrease high blood sugar levels by stimulating intestinal AMPK and elevating plasma GLP-1 and insulin. The HPTLC study confirmed the reliability, correctness, and consistency of detecting Cucurbitacin B.
This study emphasises the potential of *Solena heterophylla* and *Cyclanthera pedata* in the treatment of diabetes and wound healing, specifically highlighting the therapeutic effects of their bioactive components.

The acute oral toxicity of EASH and EACP in male Swiss albino mice was studied as per 'up and down' method. The median lethal dose (LD50) was examined to be 2000 mg/kg body weight respectively, and indicating no acute oral toxicity in mice. The *in-vivo* antidiabetic activity of EASH and EACP was assessed by using high fat diet and low dose streptozotocin induced diabetic model on Wistar albino rats. Both the fractions were administered orally in a dose of 200mg/kg, 400mg/kg, respectively for consecutive 28 days. After 28 days of study rats were sacrificed and all biochemical parameters, tissue antioxidant parameters, immune histopathology were performed at the end of the study.

EACP was showing significant blood glucose lowering effect in fasting blood glucose and oral glucose tolerance test. EACP also exhibited its antidiabetic effect such as glycaemic control, serum SGOT, SGPT, ALP and total protein level, where increased dose convey more effect on these biochemical parameters. EACP also exhibited significant tissue antioxidant activity with increased dose and histopathology of pancreas reveals that EACP have β cell protective property during diabetic condition. For the outcome of the study, EACP was

further studied for diabetic wound healing as it was showed strong antidiabetic activity in compare with the EASH.

In chapter 7, *in-vivo* antidiabetic wound healing activity was examined to check the skin regeneration and wound healing potential of EACP. Hydroxypoline assay was done to analyse the collagen turnover by excision wound model. In this study, the concentration of hexuronic acid and hexosamine were found to be significantly higher in topical application (10%) as ointment. To asses the tissue antioxidant of the ointment lipid peroxidation, GSH and SOD assays with skin tissue was done. The removal of ROS by these methods was found to be vital in the recovery process of the wounds. The results suggest EACP at 10% a potential ingredient for ointment to treat diabetic topical wounds.

8.2. Conclusion

The present research thoroughly examines the antidiabetic activities of the ethyl acetate fractions of *Solena heterophylla* (EASH) and *Cyclanthera pedata* (EACP) and diabetes induced wound healing activity of EACP. The study shows that both EASH and EACP have considerable antioxidant and enzyme inhibitory activities in *in-vitro* manner. Metabolite profiling qualitative evaluation with LC-Q-ToF-MS and HPTLC suggested the presence of cucurbitacin B along with several other bioactive phytocomponents. Metabolite profiling revealed the presence of several bioactive chemicals, such as triterpenoids, flavonoids, and glycosides. Notably, significant amounts of Cucurbitacin B were detected, which is renowned for its ability to reduce glucose levels. EACP has better benefits in reducing blood glucose levels in diabetic rats. Empirical investigations have substantiated the minimal harmfulness and powerful therapeutic properties of EACP, which furthermore stimulated the safeguarding of beta-cells and heightened the antioxidant activity of tissues. In addition, the use of EACP resulted in a substantial enhancement of skin regeneration and wound healing in diabetic rats. This indicates that EACP has the potential to be used as a therapeutic agent for the treatment of diabetes and its related problems. It can be concluded that EACP has better activity in diabetic induced wound healing and promising agent for reducing diabetes. Further analysis to investigate the mechanism of action for wound healing study along with its antimicrobial potential can be studied to develop value added formulation for the benefit of human rights.

8.3. Publications and Presentations

8.3.1. List of Publications with first authorship:

1. **Sandipan Jana**, Srijon Gayen, Barun Das Gupta, Seha Singha, Jayashree Mondal, Amit Kar, Abhimanyu Nepal, Suparna Ghosh, Rajan Rajabalaya, Sheba R David, Ashok Kumar Balaraman, Asis Bala, Pulok Kumar Mukherjee, Pallab Kanti Haldar. Investigation on Anti-diabetic Efficacy of a Cucurbitaceae Food Plant from the North-East Region of India: Exploring the Molecular Mechanism through Modulation of Oxidative Stress and Glycosylated Hemoglobin (HbA1c). Endocrine, Metabolic & Immune Disorders - Drug Targets. 2024; 24(2): 220-234. doi: 10.2174/ 187153 0323666 230907 115818, PMID: 37691221.

2. **Sandipan Jana**, Nilanjan Sarkar, Mainak Chakraborty, Amit Kar, Subhadip Banerjee, Sanjib Bhattacharya, Pulok K. Mukherjee, Pallab K. Haldar. 2023. Combination synergy between β-carotene and lupeol against breast adenocarcinoma in vitro and in vivo using combination index, Phytomedicine Plus, Volume 3, Issue 1, 100392, ISSN 2667-0313, https://doi.org/10.1016/j.phyplu.2022.100392.

8.3.2. List of Presentations in National/International conferences:

1. Presented a **Poster** on "Determination of the potency of *in-vitro* α-glucosidase enzyme inhibition in some plants of *Momordica* species (Cucurbitaceae)". International conference on "Ethnopharmacology in development of Scientifically Validated Quality Products from Medicinal Plants & Regulatory Aspects" organized by SFEC, New Delhi, 15th - 17th February, 2020.

2. Presented an **Oral** on "Metabolite analysis and *in-vitro* α-glucosidase inhibitory potential *Luffa acutangula* (L.) Roxb (ridge gourd)". International conference on "Combating COVID-19 - Ethnopharmacology & Traditional Food and Medicine" organized by SFE, IBSD, Imphal, 17th - 19th December, 2020.

3. Presented a **Poster** on "Quality evaluation and in-vitro α-glucosidase enzyme inhibition potential of *Zehneria indica* – An important medicinal food plant of Cucurbitaceae family". International conference on "Ethnopharmacology & Medicinal

Plants – Approach towards product development" organized by SFEC, Pune, 27th - 29th August, 2021.

4. Presented an **Oral** on "Investigation of *in-vitro* and *in-vivo* anti-diabetic potential of *Solena heterophylla* Lour. fruits from Cucurbitaceae family". National conference on "Translational Research on Indian Medicinal Plants" organized by SFE, Jadavpur University, Kolkata, 23th - 24th September, 2022.

Chapter 9

Publications

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220 *Endocrine, Metabolic & Immune Disorders-Drug Targets,* **2024,** *24,* **220-234**

RESEARCH ARTICLE

Investigation on Anti-diabetic Efficacy of a Cucurbitaceae Food Plant from the North-East Region of India: Exploring the Molecular Mechanism through Modulation of Oxidative Stress and Glycosylated Hemoglobin (HbA1c)

Sandipan Jana[1], Srijon Gayen[2], Barun Das Gupta[2], Seha Singha[1], Jayashree Mondal[1], Amit Kar[3], Abhimanyu Nepal[1], Suparna Ghosh[2], Rajan Rajabalaya[4], Sheba R. David[5], Ashok Kumar Balaraman[6], Asis Bala[7], Pulok Kumar Mukherjee[3] and Pallab Kanti Haldar[1,2,*]

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ARTICLE HISTORY

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Abstract: ***Background*:** The medicinal plants of the Cucurbitaceae family, such as *Solena heterophylla* Lour. fruits, have significant ethnobotanical value and are readily accessible in North East India.

***Aims*:** We conducted a study on *Solena heterophylla* Lour. fruits to evaluate their anti-diabetic activity *in vivo*, standardize their HPTLC, and profile their metabolites using LC-QTOF-MS. We aimed to explore the molecular mechanism behind their effects on oxidative stress and glycosylated hemoglobin (HbA1c).

***Methods*:** Firstly, the ethyl acetate fraction of *Solena heterophylla* Lour. fruits was standardized using Cucurbitacin B as a standard marker by conducting HPTLC evaluation. Next, we delved into analyzing metabolite profiling. In addition, the standardized fraction was utilized in an experimental study to investigate the molecular mechanism of action in an *in vivo* high-fat diet and a low dose of streptozotocin-induced diabetic model.

***Results*:** We have reportedly identified 52 metabolites in the ethyl acetate fraction of *Solena heterophylla* (EASH). In the *in vitro* tests, it has been observed that this extract from plants possesses notable inhibitory properties against α-amylase and α-glucosidase. *Solena heterophylla* fruits with high levels of Cucurbitacin B (2.29% w/w) helped lower FBG levels in animals with EASH treatment. EASH treatment reduced HbA1c levels and normalized liver lipid peroxidation and antioxidant enzyme levels. SGOT, SGPT, and SALP serum enzyme levels also returned to normal.

***Conclusion*:** Based on the current evaluation, it was found that EASH exhibited encouraging hypoglycemic effects in diabetic rats induced by a low dose of STZ and high-fat diet, which warrants further investigation.

Keywords: Cucurbitaceae food plant, *Solena heterophylla*, anti-diabetic activity, glycosylated hemoglobin, modulation of oxidative stress, EASH treatment.

1. INTRODUCTION

Many people consume plants from the Cucurbitaceae family as part of their diet. *Solena heterophylla* Lour. fruits, also known as Golkakri which are easily found in North East India. The leaves of these fruits are commonly used to soothe irritated skin. In contrast, root juice has been historically used to treat dysuria, spermatorrhoea, the common cold, infant pneumonia, throat discomfort, and fever. Tribal health practitioners in Sikkim, Assam, and other states of North East India have been using this herb as a traditional remedy for diabetes [1]. Most commonly used traditional anti-diabetic plants include: Apiaceae (*Daucus carota*), Apocynaceae (*Catharanthus roseus*), Anacardiaceae (*Anacardium occidentale*), Asteraceae (*Taraxacum officinale*), Bignoniaceae (*Tecoma stalbus*), Liliaceae (*Aloe vera*) (*Allium cepa*) (*Allium sativum*), Myrtaceae (*Syzygium cumini*) (*Eucalyptus globulus*), Meliaceae (*Azadirachta indica*), Rosaceae (*Poterium ancistroides*), Urticaceae (*Urtica dioica*) [2]. Research has proved that the plants such as *Zingiber officinale*, *Cinnamomum cassia*, *Mangifera indica*, and *Panax ginseng* can

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enhance insulin sensitivity and help manage diabetes [3]. Since the Indian Cucurbitaceae family includes some of the most well-known vegetables in the world, including watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), pumpkin, and squash (*Cucurbita pepo*), melon (*Cucumis melon*) and bitter gourd (*Momordica charantia*), it was necessary to update the two checklists of this family. Cucumis (11 species), Momordica (8 species), Trichosanthes (22 species), and Zehneria (5 species) are the genera with the most species [4].

Diabetes is becoming more common in day-to-day life due to urbanization and poor lifestyle management. Diabetes often worsens with age in Western nations but primarily affects young to middle-aged persons in Asian countries. This situation is severely impacting the economics and health of the developing world. The International Diabetes Federation (IDF) calculates that there were around 50.8 million diabetics in India in 2010, which would progressively rise to roughly 87 million by 2030. Recent data gathered from several regions of the country revealed the invasiveness of diabetes in cities. Furthermore, due to recent socio-economic expansion, diabetes prevalence is rising concurrently in rural areas [5].

Several oral hypoglycemic medications are available on the market, each with side effects. For example, some of these medications can cause congestive heart failure if taken with glitazones. When taken with glucosidase inhibitors, sulfonylureas, and meglitinides, they may result in gastrointestinal problems. Sulfonylureas are known to cause weight gain and also can lead to cardiac issues [6]. As a solution to the harmful effects of current therapeutics, there is a need to develop multi-target agents that can effectively treat Type 2 Diabetes Mellitus (T2DM) and its associated pathogenesis, considering the various pathophysiological factors involved. Plant extracts, containing several beneficial chemicals, have shown their medicinal efficacy in various ways. Based on ethnopharmacological knowledge, this study focuses on exploring the potential of plant extracts in treating diabetes [7-9].

Cucurbitacin B is a natural substance that can lower blood sugar levels. It is found in plants from the Cucurbitaceae group and has been used as a marker in the HPTLC technique during studies. Daily exposure to cucurbitacin B has been shown to reduce hemolymph glucose levels in Drosophila melanogaster on a high-carbohydrate diet, similar to high blood glucose levels in humans. Its effectiveness is comparable to that of metformin, a popular medication used to lower blood sugar levels. Additionally, cucurbitacin B has been found to reduce levels of triacylglycerol, glycogen, and tissue sugar. In diabetic rats, a dose of 0.1 mg/kg of cucurbitacin B significantly lowered blood glucose levels. This effect was mainly achieved by controlling intestinal AMPK levels, which triggered the release of plasma insulin and glucagon-like peptide-1. Subsequently, this impacted the rat's appetite and eating habits. Plasma glucose levels of rabbits without normal or high blood sugar were examined using *Citrullus colocynthis* (also known as Bitter apple) extracts. Results showed an insulinotropic effect at a dose of 300 mg/kg [10]. After being taken orally, *Coccinia indica* showed hypoglycemic effects at a dose of 200 mg/100 g per day. This resulted in a significant decrease in blood sugar and an increase in liver glycogen levels. After being administered to STZ-induced diabetic rats at a dose of 200 mg/kg for 45 days, the ethanolic leaf extract of *Coccinia indica* effectively hindered the build-up and cross-linking of collagen. This resulted in a reduction of aortic collagen levels and displayed hypolipidemic properties [11-14]. *Momordica cymbalaria* extract effectively lowers blood glucose in diabetic rats by increasing hepatic glycogen. *Momordica charantia* also has hypoglycemic properties.

People with pre-diabetic HbA1c levels ranging from 5.7 to 6.4% have higher blood sugar levels compared to normal levels of HbA1c > 6.5%, indicating impaired glucose tolerance. Insulin dosages are required to lower HbA1c levels, but they should be used in conjunction with other medications, not as a standalone treatment.

The preparation of a hydro-alcoholic fruit extract from *Solena heterophylla* Lour. was carried out through cold maceration in this particular study. To determine the relationship between phenolic and flavonoid content, antioxidant potential, and α-amylase and α-glucosidase inhibitory potential, the "Pearson" correlation was used. The ultra-performance liquid chromatography and quadrupole time-of-flight tandem mass spectrometry analysis of the ethyl acetate fraction revealed 52 phytoconstituents. High-performance thin-layer chromatography was used to quantitatively determine Cucurbitacin B as a standard marker in the potent bioactive ethyl acetate fraction. The effective fraction was also utilized to investigate the molecular mechanism of regulating oxidative stress and glycosylated hemoglobin (HbA1C) in an experimental in-vivo anti-diabetic animal model established by a high-fat diet and low-dosage streptozotocin. This investigation aims to develop innovative alternative therapies for improving human health.

2. MATERIALS & METHODS

2.1. Chemicals

Streptozotocin (≥ 99% (HPLC), powder] from Sigma (St. Louis, MO); Trichloroacetic acid, A.R. (TCA) from Merck (Mumbai, India Thiobarbituric acid (TBA), Nitrobluetetrazolium chloride (NBT) from LobaChemie (Mumbai, India); Phenazinemethosulphate (PMS); Cucurbitacin B [≥95% (HPLC), powder) from Sigma (St. Louis, MO); Reduced nicotinamide adenine dinucleotide (NADH), 5,5′-dithiol bis-2-nitro benzoic acid (DTNB) and standard Cucurbitacin B (≥95% pure) from Sigma (St. Louis, MO). The chemicals and reagents utilized in the test were of analytical grade and came from, LobaChemie (Mumbai, India), Sigma (St. Louis, MO), Merck (Mumbai, India), Sisco (Mumbai, India), and Arkray Healthcare Pvt. Ltd. (Japan).

2.2. Plant Ingredients Collection, Authentication, and Extraction

In July 2020, *Solena heterophylla* Lour. plant fruits were gathered from Bermiok, South Sikkim, India. The Botanical Survey of India verified and validated the plant, which is a part of the Ministry of Environment, Forest, and Climate Change in the Government of India. This organization is located in Gangtok, Sikkim, India. The voucher specimen (SHRC-5/40/2021-22/Tech) was preserved at the laboratory for future reference. *Solena heterophylla* fresh fruits were

washed, shade dried, mechanically crushed, and stored in an airtight container. The maceration extraction method was used with 70:30 aqueous ethanol for 3-5 days at room temperature to obtain crushed dried fruit extract. Next, liquid-liquid fractionation was done with 800 ml of ethanol, n-hexane, chloroform, ethyl acetate, and their polar counterparts. To evaporate the fractions and determine the percentage yield of each one (hexane, chloroform, ethyl acetate, and ethanol), we used a rotary evaporator from Eyela in Japan. The yields for each fraction were 2.8% (w/w) for hexane (HFSH), 10.3% for chloroform (CFSH), 12.6% for ethyl acetate (EASH), and 13.29% for ethanol (EFSH). We stored the samples in borosilicate glass vials at 4°C before analysis.

2.3. Assessment of the Total Phenolic and Flavonoid Content

We measured the total phenolic and flavonoid content of the aqueous ethanol extract (AESH) partitioned fractions of *S. heterophylla*, based on a previous approach published [15]. We created the samples by using gallic acid as the reference standard and a concentration of 1 mg/mL ethanol. The reaction mixture comprised 18 L of sample, 90 L of 10% Folin-Ciocalteu reagent, and 90 L of 7.5% Na_2CO_3. The samples were analyzed using a spectrophotometer (SpectraMax Plus, Molecular Devices LLC, USA) and incubated in a BOD incubator at 45°C for 45 minutes. The absorbance was measured at 725 nm.

To measure the total phenolic content (TPC), a spectrophotometric assay was utilized and quercetin was utilized as the reference substance [16]. Originally, 170 µL of ethanol was introduced into the reaction mixture after the inclusion of 10 µL of 3% $NaNO_2$, 50 µL of 1 M NaOH, and 10 µL of 15% $AlCl_3$. At 415 nm, the mixture's absorbance was measured. Milligrams equivalents of quercetin (QE)/g of the sample were used to measure the total flavonoid content (TFC). Means with standard deviations (SD) were used to illustrate both outcomes (n = 3). To assess the correlation between the total phenol and flavonoid levels in *S. heterophylla* fractions (SHF) and the inhibitory potential (IC_{50} value) of α-amylase and α-glucosidase, the Pearson correlation coefficient was utilized.

2.4. Evaluation of the *In-Vitro* Antioxidant Activity

We evaluated the antioxidant capacities of *S. heterophylla* fruit using the DPPH free radical scavenging assay. We combined the sample and DPPH solution in a microplate, measured the reduced absorbance, and used ascorbic acid as a reference marker. The IC_{50} value represented the free radical scavenging potential. We also conducted Pearson correlation analysis to understand the relationship between antioxidant and α-amylase/α-glucosidase inhibitory activities.

2.5. *In-vitro* α-amylase, α-glucosidase Enzyme Inhibitory Activity

The test was conducted according to the protocol [17] with minor adjustments. Different concentrations of AESH partitioned fractions and standard marker acarbose (50, 100, 300, and 500 g/ml) were prepared in pre-labeled test tubes. 20 µl of α-amylase was added to each test tube, followed by incubation at 37°C for 10 minutes. After the initial incubation, 200 µl of 1% starch solution was added to each test tube, followed by another 1 hour of incubation at 37°C. Then, each test tube was filled with 10 ml of distilled water and 200 µl of 1% iodine solution. The absorbance of the mixture was measured at 565 nm. The samples, substrate, and α-amylase blank were treated under the same conditions. The experiment was repeated three times, and the IC_{50} value was calculated using regression analysis. The test sample was combined with 50 µL of α-glucosidase (0.5 U/ml), 50 L of 0.2 M K_3PO_4 buffer (pH 6.8), and 50µL of the buffer. After a 15-minute pre-incubation period at 37°C, 3 mM PNPG (100 µl) was added. After being allowed to continue for 10 minutes at 37°C, the enzymatic process was stopped by adding 750 µL of 0.1 M Na_2CO_3. A spectrophotometer was used to detect the 4-nitrophenol absorption at 405 nm. To ensure accuracy, a solution without the sample and a blank solution without the substrate were used as controls. Additionally, the anti-diabetic medication acarbose was measured as a standard reference [17].

$$\text{Inhibitory activity (\%)} = (1 - A_S/A_C) \times 100$$

Where,

A_S= absorbance of the sample

A_C = absorbance of control

2.6. UPLC-QTOF-MS Analysis

The analysis was carried out using the UPLC-QTOF-MS method that has been previously reported [18]. The Agilent 1260 Infinity II LC System and Agilent 6530 LC/Q-TOF equipment were used to identify the phyto molecules in the EASH. ACN (A) and H_2O (B), consisting of 0.1% formic acid, comprised the mobile phases. The flow rate used was 0.5 ml/min. The gradient profile was adjusted from 0 to 1 minute to 5% B, 35% B at 3 minutes, 50% B at 5 minutes, 80% B at 7 minutes, 90% B for 8 minutes, and 10% B for 9 minutes. A sample of 5 µl, 200 µg/ml was injected. The mass of compounds was measured using negative ionization mode, and a score between 0-100 was assigned based on the connection between the measured mass (m/z) and its theoretical formula. The analysis was conducted using Agilent Mass Hunter Acquisition B.06.01 software. Individual peaks were identified using Agilent Mass Hunter Qualitative Analysis v. 10.0. Identification of compounds were made possible by a custom library uploaded into the algorithm of Mass Hunter Qual software.

2.7. HPTLC Analysis

2.7.1. Reference and Sample Solutions Preparation

For the HPTLC analysis of *Solena heterophylla* fruit, the pure Sigma Cucurbitacin B (0.1 mg) was weighed in an Eppendorf microcentrifuge tube and then diluted in 1 mL of Ethanol. We made a 10 mg/mL EASH solution by separately vortexing and filtering standard and EASH using a syringe filter with a pore size of 0.45 mm from Millipore in Burlington, Massachusetts, USA. To assess the linearity of the response-made references, we created a calibration curve using 5 different volumes of the reference (Cucurbitacin B, 1 mg/mL) in the 2-10 L range. The HPTLC analysis solutions were then

properly vortexed, filtered, and sonicated as per standard procedure [19].

2.7.2. Chromatographic Conditions

A 1 mg/mL solution of Cucurbitacin B was injected using a Hamilton syringe into a 100 L syringe. Then, using a CAMAG Linomat V sample applicator in five tracks, 5 different volumes (2, 4, 6, 8, and 10 L) were applied band-wise to 5 distinct 1 cm x 10 cm additions on 60 F254 silica gel plates. Next, 10 mg/mL of EASH solutions were used in three different ways and in three different amounts (10, 12, and 14 L). Pet Ether, Ethyl Acetate, Formic Acid, and Acetic Acid (in a ratio of 5:4:0.5:0.5 V/V) were used as the solvent system to produce the plates in a CAMAG twin-trough chamber after they had been dried in warm air (40-45°C). Finally, at 254 nm, compounds were identified and scanned [20].

2.7.3. Method Validation

During the validation of the HPTLC method, the International Conference on Harmonisation (ICH) recommendations for robustness, precision, linearity, and limits of quantification and detection were adhered to as outlined in 2005.

2.7.3.1. Specificity

In order to prevent errors caused by sample contamination, the accuracy of the standardization results was assessed using the ICH criteria for specificity. Both the standard and the test samples were analyzed to determine the method's specificity. A multivariate analysis was conducted by comparing the peak areas and retention periods of the standard compound with those of the extract and fractions. This was done to confirm the purity of the peaks.

2.7.3.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The formulae LOD = 3:1 /S and LOQ = 10:1 /S were used to calculate the LOD and LOQ using a technique based on standard deviation (σ) and the slope (S) of the calibration plot.

2.7.3.3. Accuracy

To measure the accuracy of the approach, the % recovery of markers in the plant extract and fractions was calculated. The standard addition approach was used to investigate the procedure and the results were reported in percentages of analogous standard deviations (%RSD) from the hypothesized concentrations' mean recovery. Prior to injection, the tests were spiked with three separate reference drugs at established amounts. Analyses were performed to determine the overall average recovery in an ambient environment. The achieved mean levels of the markers were used as absolute values to calculate the spike recuperations.

2.7.3.4. Precision

To ensure the accuracy of the procedure, six duplicates of the standard material, fractions, and extract were injected at three different concentrations. The percent relative standard deviation (% RSD) values for both intra-day and inter-day runs were calculated, along with the mean amount. Intra-day precision was determined by testing three concentrations in one day, while intra-day accuracy was assessed by studying the same concentrations over three consecutive days. The process's repeatability was measured by conducting six injections.

2.7.3.5. Robustness

We tested the durability of the proposed method by analyzing samples in different experimental situations. To determine how they affect retention time, we studied the test solutions using various flow rates, mobile phase compositions, detection wavelengths, column temperatures, and columns with the same design but different materials.

2.8. *In vivo* Anti-Diabetic Activity

2.8.1. Acute Toxicity Study

In order to conduct an acute oral toxicity study, the Ethyl Acetate Fraction (EASH) was used in accordance with the guidelines outlined by the Organisation for Economic Co-operation and Development (OECD) recommendations 425 (OECD 2008) [21].

2.8.2. Animals

We used adult male Wistar albino rats (160-180 g) with FBG readings of 85-90 mg/dL from M/S Chakraborty Enterprise, Kolkata (Reg. no. 1443/PO/Bt/s/11/CPCSEA). During their acclimatization period (07 days), they were provided with free access to food and water for seven days. They were fed a high-fat diet and kept in a 25°C environment with a 12-hour light/dark cycle. The Animal Ethical Committee of Jadavpur University approved the experimenta-tion (Approval Number JU/IAEC-22/15).

2.8.3. Induction of Diabetes

To induce diabetes, rats were deprived of water for 18 hours and then given an intraperitoneal dose of streptozotocin dissolved in 0.1 M citrate buffer (pH 4.5). The injection was given at a dose of 35 mg/kg body weight. The rats were considered to have diabetes if their fasting blood sugar level was above 250 mg/dl. Rats with a fasting blood sugar level above 250 mg/dl were only used for the experiment [22].

2.8.4. Experimental Design

The schedule for the experimental treatment was created based on a high-fat diet treatment that lasted 28 days. With 5 groups of six rats each (n = 6), the rats were split and the experiment was scheduled for 14 days [23].

Group –I: Normal control group: For 14 days, the rats were given 0.5 ml/kg of normal saline intravenously.

Group-II: Diabetes control group (STZ+HFD): The diabetes group included rats given STZ (35 mg/kg, b.w., i.p.) and a high-fat diet.

Group-III: EASH Fraction (EASH 200 mg/kg) treated group: EASH (200 mg/kg b.w.) was given orally to the diabetic rats for 14 days.

Group-IV: EASH Fraction (EASH 400 mg/kg) treated group: The diabetic rats were orally treated with EASH (400mg/kg b.w.) for 14 days.

Group-V: Metformin-treated group: For 14 days, metformin (150 mg/kg, p.o.) was administered to the diabetic rats.

2.8.5. Oral glucose Tolerance Test (OGTT)

Overnight fasting rats with normal glycemia (85-90 mg/dl) underwent the OGTT. There were three groups of six rats each. EASH was administered to groups II and III at 200 and 400 mg/kg b.w., respectively, while group I acted as the standard control group and received distilled water (5 mL/kg b.w. p.o.). After these procedures, all groups were given oral glucose (2 g/kg b.w). Blood was collected from the tail vein in 30, 60, 120, and 240 minutes after oral glucose delivery [24]. A single-touch glucometer was utilized to evaluate blood sugar levels (Accu-Chek, India).

2.8.6. Estimation of Glycosylated Hemoglobin Level (HbA1c)

A Coral clinical system test kit, manufactured by Tulip Diagnostics Pvt. Ltd. in India, was used to conduct a complete blood sample analysis to measure glycosylated hemoglobin levels. The test employs ion exchange resin methodology [25].

2.8.7. Estimation of Serum Biochemical Parameters

All rats received a 28-day course of medication before being starved for 12 hours and given 0.3% isoflurane inhalation anesthesia. Then, under anesthesia, bloodwas obtained through a heart puncture. The animals were sacrificed using the cervical decapitation technique as soon as the blood was collected. Centrifugation was used to separate the serum for 10 minutes at 3000 rpm. Using a commercially available assay kit, serum collected was examined for a number of biochemical factors, including total protein, HDL cholesterol, triglyceride levels, serum alkaline phosphatase (SALP), serum glutamic pyruvic transaminase (SGPT), and serum glutamic oxaloacetic transaminase (SGOT) [26].

2.8.8. Histopathological Studies

We collected pancreatic tissue from rats that were sacrificed for the purpose of conducting histopathological investigations. The tissue was rinsed in normal saline and then instantly fixed in a 10% formalin solution for at least 24 hours. Afterward, the tissue was sliced into 4 to 5-m thick pieces, fixed in paraffin, and stained with hematoxylin and eosin dye. Prior to staining, the tissue was dried using alcohol [27].

2.8.9. Statistical Analysis

The mean and SEM are used to express all results. Graph Pad Prism 8.02 software was utilized to perform a one-way analysis of variance (ANOVA) and post hoc Dunnett test to evaluate whether the results were statistically significant (Graph Pad Software, USA). Statistics were considered significant at $p < 0.05$.

2.9. Results

2.9.1. α-amylase, α-glucosidase Enzyme Inhibitory Activity

The *in-vitro* inhibitory activity of various *S. heterophylla* fruit fractions against α-amylase and α-glucosidase was measured as an inhibitory half-maximal concentration (IC_{50}). The inhibitory activity (IC_{50} value) of EASH was highest among the four fractions for α -amylase and α -glucosidase at 0.578 ± 0.51 mg/ml and 0.118 ± 0.34 mg/ml, respectively; on the other hand, acarbose had IC_{50} values of 0.37 ± 0.73 mg/ml and 0.055 ± 0.52 mg/ml, respectively. Other subfractions of α -amylase and α-glucosidase had IC_{50} values of 1.130 ± 0.25, 0.161 ± 0.41 mg/ml (HFSH), 0.948 ± 0.63, 0.150 ± 0.27 mg/ml (CFSH), and 0.778 ± 0.16, 0.122 ± 0.75 mg/ml (EFSH) (Table **1**). There was no discernible difference between EASH and the common inhibitor acarbose ($P < 0.05$). Inhibitor concentrations between 25-500 µg/ml were shown to have a dose-dependent effect on the inhibition (Figs. **1A** and **B**).

2.9.2. Correlation between α-Amylase, α-glucosidase Enzyme Inhibitory Potential, and Total Phenolic and Flavonoid Content

The highest TPC and TFC were showed in EASH, with values of 3.70 ± 0.31 mg GAE/g and 2.50 ± 0.54 mg QE/g of material, respectively. Other TPC and TFC fraction values fell within the EFMC > CFMC > HFSH range. The Pearson correlation coefficient (R-value) was used to examine the communication between the inhibitory potential of the enzymes α-amylase and α-glucosidase and the TPC and TFC of all the fractions. The TPC of the ethyl acetate fraction of α-amylase and α-glucosidase enzyme inhibition potential (IC_{50} value) were shown to be statistically significantly correlated (Table **1**). The phenolic and flavonoid content in other fractions had a weaker connection.

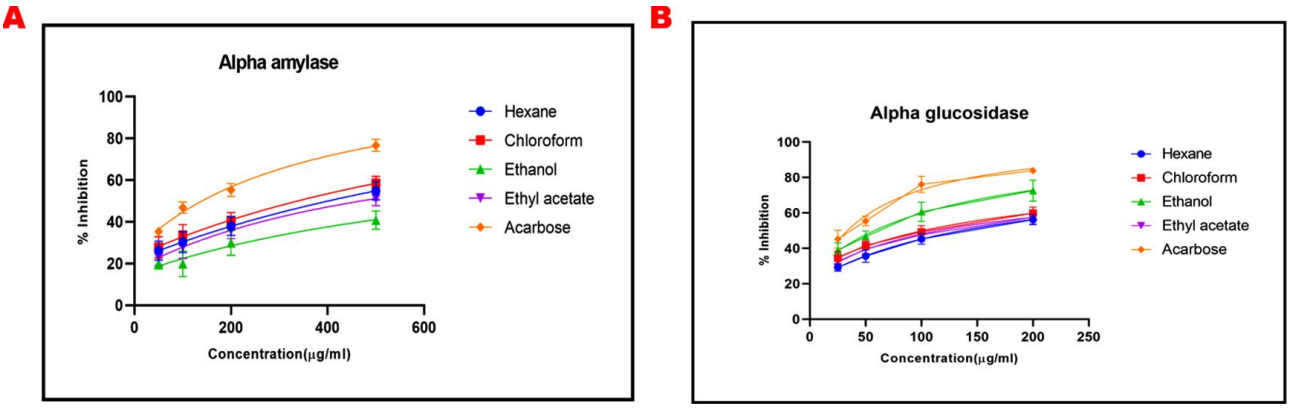


Fig. (1). The % inhibition of α-amylase was shown at different concentrations of various fractions against standard marker acarbose **1A.** % inhibition of α-glucosidase was shown at different concentrations of various fractions against standard marker acarbose **1B**. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 1. Correlation analysis between the TPC/TFC and DPPH values of the fractions of *Solena heterophylla* fruits and α-amylase, α-glucosidase and antioxidant potential (IC_{50} value).

Parameters	Pearson Correlation (R) Coefficient (IC_{50} Value of α-Amylase and α-glucosidase Inhibition)								α-amylase and α-glucosidase ; TPC, TFC and DPPH Free Radical Scavenging Potential of Fraction of *Solena heterophylla* Fruit			
	n-Hexane Fraction		Chloroform Fraction		Ethyl Acetate Fraction		Ethanol Fraction		n-hexane Fraction	Chloroform Fraction	Ethyl Acetate Fraction	Ethanol Fraction
	αM	αG	αM	αG	αM	αG	αM	αG				
Total phenol content (mg GAE/g)	- 0.933ns	- 0.877ns	0.975ns	0.924ns	0.997*	0.999*	0.996ns	0.944ns	1.96 ± 0.26	1.74 ± 0.39	3.70 ± 0.31	3.40 ± 0.19
Total flavonoid content (mg RE/g)	0.960ns	0.989ns	- 0.955ns	- 0.952ns	0.997*	0.999*	0.962ns	0.989ns	1.26 ± 0.27	0.48 ± 0.41	2.50 ± 0.24	2.20 ± 0.67
DPPH free radical scavenging potential (IC50 mg/ml)	0.998*	0.997*	- 0.905ns	- 0.985ns	0.998*	1.000**	0.983ns	0.823ns	0.208 ± 0.62	0.139 ± 0.71	0.102 ± 0.19	0.105 ± 0.28

Note: #Values are represented as mean ± SEM (n=3). # **Abbreviations:** αM = α-amylase, αG = α-glucosidase, GAE = Gallic acid equivalent, RE = Rutin equivalent.

2.9.3. Effect of Antioxidant Potential on α-amylase, α-glucosidase Inhibition

Based on the DPPH free radical scavenging potential of the samples, which is represented as the IC_{50} value of the samples, the antioxidant potential of the extract and fractions of *S. heterophylla* fruits was ascertained. When analyzed with ascorbic acid, the EASH had the highest antioxidant property (0.102 ± 0.19 mg/ml), which meant it was more effective at scavenging free radicals. In the case of EASH, the strongest positive linear correlation was found between the DPPH free radical scavenging activity, α-amylase, and α-glucosidase inhibitory activity (R = 0.9986, 1.000) (Table **1**).

2.9.4. Utilizing UPLC-QTOF-MS to Identify Bioactive Compounds

Based on their respective retention times, m/z values, error in parts per million (ppm), categorization of the suggested compounds, score times, molecular formulas, and molecular weights, evaluation of UPLC-QTOF-MS was used for identifying 52 key phytoconstituents (Table **2**) found in EASH. The total ion chromatogram of EASH is shown in Fig. (**2**). The analysis was done within the mass error window of -346737.478 to 696598.52 ppm to establish their elemental composition.

2.9.5. HPTLC Analysis

The fresh fruit extract of *Solena heterophylla* L. contained a high level of Cucurbitacin B (2.29% w/w), according to a densitometric analysis by HPTLC. Fig. (**3B**) displays the HPTLC of the standard cucurbitacin B in the extracts. In Fig. (**3D**), the plate below 254 nm is seen. The calibration plot linearity was determined to be 200–1000 ng/spot. Figs. (**3A** and **C,** and **3E**) show different aspects of the HPTLC analysis of Cucurbitacin B. These include the HPTLC chromatogram of a calibration curve, the chromatogram of EASH, and photographs of the standard cucurbitacin B and the sample under 366 nm respectively. The calibration curve revealed a correlation value of > 0.99. This information demonstrates how closely the data follow the best-fit line. The precision and accuracy of the cucurbitacin B assay were evaluated on the basis of LQC (low-quality control), MQC (medium-quality control), and HQC (high-quality control), Six times each experiment was run, and the percent recovery values ranged from 99.68 - 99.93%. The incredible accuracy of the approach was evidenced by the low %RSD values, which ranged from 0.076 - 2.63%. Table **3** displays the findings of the recovery study. Standard cucurbitacin B was utilized at two different concentrations 200 low %RSD µg and 400 µg, to gauge the method accuracy. For intra-day and inter-day precision, the average area and RF values for the same were calculated. Table **2** displays the results of the inquiry on intra-day and interlay precision. The 6 repetitions for each type of determination produced %RSD values of 0.180 - 0.28% (intra-day) and 0.150.26% (inter-day), which are incredibly low and show the method's accuracy. The LOD ranged between 623.20 ng and 622.12 ng per spot and the LOQ between 1950.12 ng and 1951.32 ng per spot. The approach also qualified for the robustness test.

2.9.6. In vivo Anti-diabetic Activity

2.9.6.1. Acute Toxicity Study

No signs of toxicity were observed following the administration of EASH up to a dosage of 2000 mg/kg body weight. For further investigation, two dosages of EASH were chosen: 200 mg/kg and 400 mg/kg body weight.

2.9.6.2. Oral glucose Tolerance test

Healthy rats with normal blood sugar levels (85-90 mg/dl) were subjected to the oral blood glucose tolerance test. After the injection of glucose, blood glucose levels increased during the first 30 minutes and then gradually decreased over the next 60, 120, and 240 minutes, as illustrated in Fig. **4A**.

Table 2. Compounds identified in ethyl acetate fraction of *Solena heterophylla* fruit by UPLC-QTOF–MS.

S. No.	Retention Time	Molecular Formula	Theoretical Mass	Expected Mass	Error (ppm)	Score	Compounds	Class
1	8.827	C_{19} H_{17} N O_3	307.3	308.128	2694.435405	98.03	N-Acetylanonaine	Isoquinoline alkaloid
2	8.925	C_{28} H_{40} O_{12}	568.5	586.2847	31283.55	85.92	Musabalbisiane C	Diterpenoids
3	0.53	C_{14} H_{15} N	197.28	220.1092	116172.413	85.43	3-Phenyl-4-propylpyridine	Alkaloids
4	0.627	C_{18} H_{26} O_{10}	402.4	425.1419	56515.65	83.51	Benzyl O-(arabinofuranosyl-(1->6)-glucoside)	Glycoside
5	9.316	C_{24} H_{4}2 O_{11}	506.6	507.2771	1336.557	82.8	4-Megastigmene-6a,9R-diol 9-(apiosyl-(1->6)-glucoside)	Fatty acyl glycosides of mono and disaccharides
6	0.627	C_{15} H_{21} N O	355.4	232.1695	-346737.478	82.09	Rotundine A	Alkaloids
7	0.822	C_{20} H_{32} O_4	336.5	337.2382	2193.759	81.28	9alpha-(3-Methylbuta-noyloxy)-4S-hydroxy-10(14)-oplopen-3-one	Sesquiterpenoid
8	0.627	C_{41} H_{64} O_{17}	828.937	851.4062	27106.040	80.31	Digitoxigenin 3-(glucosyl-(1->6)-glucosyl-(1->4)-2,6-dideoxyribohexoside)	Cardenolide glycoside
9	0.432	C_{24} H_{32} O_{12} S	544.57	545.1723	1106.0102	77.51	Estriol 3-sulfate 16-glucu-ronide	Steroidal glycosides
10	0.53	C_{16} H_{21} N O_7	339.34	357.1656	52530.205	77.26	5-Hydroxytryptophol glucuronide	Glycoside
11	8.437	C_{27} H_{36} O_3	408.6	409.2744	1650.513	76.84	Apo-10'-violaxanthal	Sesterterpenoid
12	0.53	C_{18} H_{22} N O_2	284.373	302.1999	62688.440	76.58	6,7-Dihydro-4-(hydroxyme-thyl)-2-(p-hydroxy-phenethyl)-7-methyl-5H-2-pyrindinium	Alkaloids
13	6.68	C_{16} H_{32} O_2	256.42	274.2758	69634.973	74.97	Trimethyltridecanoic acid	Sesquiterpenoids
14	6.485	C_{32} H_{37} N_5 O_5	571.7	594.27	39478.747	73.78	Endomorphin-2	isoflavonoids
15	9.608	C_{29} H_{48} O	386.65	413.378	39478.7	72.9	Clerosterol	Tetracyclic triterpene
16	8.437	C_{24} H_{42} O_{11}	436.5	507.2782	162149.369	71.38	4-Megastigmene-6a,9R-diol 9(apiosyl-(1->6)-glucoside)	Fatty acyl glycosides of mono and disaccharides
17	0.627	C_{26} H_{28} O_6	506.6	437.1983	-136995.065	69.75	Kanzonol K	6-prenylated isoflavanoids
18	0.53	C_{15} H_8 O_5	268.22	286.073	66561.031	69.67	Coumestrol	Isoflavonoids
19	0.53	C_{11} H_{20} O_5	232.2735	250.167	77036.338	69.42	1,2-dibutyrin	Glycerolipids
20	0.627	C_{20} H_{16} O_5	342.3	354.135	34574.934	68.75	Sojagol	Isoflavonoids
21	0.627	C_{16} H_{10} N_2 O_3	278.2622	296.105	64122.255	68.22	Dehydroxymethylflazine	Harmala alkaloids
22	0.627	C_{25} H_{31} N O_3	393.5	416.219	57735.705	66.14	Acidissiminol	Aromatic monoterpenoids
23	0.53	C_{15} H_{10} N_2 O_2	250.25	268.11	71368.631	65.85	2-Methoxycanthin-6-one	Alkaloid

(Table 2) contd….

S. No.	Retention Time	Molecular Formula	Theoretical Mass	Expected Mass	Error (ppm)	Score	Compounds	Class
24	0.725	$C_{16}H_{10}O_5$	282.24	283.059	2901.785	65.6	Pseudobaptigenin	Isoflavonoid
25	0.237	$C_{32}H_{42}O_9$	570.7	593.269	39546.1713	64.23	Ganoderic acid F	Triterpenoid
26	2.677	$C_7H_8O_2$	124.1372	142.088	144604.518	62.78	4-Methyl-1,2-benzenediol	Catechol
27	0.627	$C_{11}H_{12}O_2$	176.21	194.118	101628.738	62.44	Ethyl cinnamate	Cinammic acid and derivatives
28	4.63	$C_{15}H_{24}O_3$	252.349	275.165	90414.465	61.98	Bisacurone C	Sesquiterpenoids
29	0.627	$C_{19}H_{21}NO_7S$	407.44	425.142	43446.887	61.71	Erysothiopine	Erythrina alkaloids
30	0.627	$C_{27}H_{36}O_6$	460.6	457.262	-7247.069	61.61	Lucidenic acid F	triterpenoids
31	6.875	$C_{15}H_{16}O_3$	244.28	245.119	3434.583	60.66	3-(1,1-Dimethylallyl) herniarin	Coumarins and derivatives
31	0.627	$C_{21}H_36O_9$	432.5	433.245	1722.543	60.62	Glucosyl (2E,6E,10x)-10,11-dihydroxy-2,6-farnesadienoate	Terpene glycosides
33	9.316	$C_{37}H_{64}O_4$	260.24	573.492	54426.50	60.53	Coronin	acetogenins
34	0.432	$C_{10}H_{13}N_3$	175.23	176.116	5056.2	60.38	Debrisoquine	Tetrahydroisoquinoline alkaloids
35	0.53	$C_{30}H_{39}ClO_7$	547.1	547.251	276.000731	59.93	Physagulin B	Withanolide glycosides
36	8.535	$C_{15}H_{22}O_2$	234.33	235.171	3588.9557	59.76	Fukinanolide	Terpene lactone
37	0.53	$C_{16}H_{13}NO_6$	315.28	316.085	2553.285	59.65	Avenanthramide K	Phenolic alkaloids
38	9.706	$C_{27}H_{48}O_3$	420.668	443.353	53926.136	59.24	(3alpha,5alpha,22R,23R)-Cholestane-3,22,23-triol	Vitamin e compound
39	0.53	$C_{14}H_{25}NO_{10}$	367.35	368.155	2191.370	58.86	2-Acetamido-2-deoxy-6-O-alpha-L-fucopyranosyl-D-glucose	Fatty acyl glycoside
40	8.925	$C_{36}H_{62}O_{11}$	670.9	688.459	26172.305	58.76	Ginsenoside Rh6	Triterpene saponin
41	7.461	$C_{30}H_{27}O_{14}$	611.5	634.131	37008.994	58.59	Cyanidin 3-(caffeoylglucoside)	Anthocyanidin glycoside
42	9.706	$C_{30}H_{46}O_4$	324.4	493.332	520752.157	58.59	Glabranin A	Flavonoid
43	0.53	$C_{15}H_{10}O_3$	238.24	261.053	95756.380	58.47	2-Hydroxymethylanthraquinone	Anthraquinones glycoside
44	0.53	$C_{16}H_{30}O_9$	366.40	389.1824	62179.039	58.32	(2R,6x)-7-Methyl-3-methylene-1,2,6,7-octanetetrol 2-glucoside	Fatty acyl glycosides
45	0.53	$C_{21}H_{24}O_9$	258.27	438.1805	696598.520	57.91	2',4',6'-Trihydroxydihydrochalcone 4'-glucoside	Flavonoids
46	9.901	$C_{29}H_{48}O_2$	414.7	429.3702	35375.452	57.44	4-hydroxymethyl-4-methyl-5-cholesta-8,24-dien-3-ol	Triterpenoid
47	0.53	$C_{32}H_{46}O_8$	558.7	581.3048	40459.638	55.98	Cucurbitacin B	Tetracyclic triterpenoids
48	0.53	$C_{26}H_{32}O_{12}$	536.5	554.2225	33033.550	55.13	8-Hydroxypinoresinol 4-glucoside	Lignan glycosides
49	0.92	$C_{11}H_{13}NO_4$	223.22	224.0926	3909.147	53.92	Salsolinol-1-carboxylate	Alkaloids

(Table 2) contd….

S. No.	Retention Time	Molecular Formula	Theoretical Mass	Expected Mass	Error (ppm)	Score	Compounds	Class
50	0.53	C_{23} H_{28} O3	352.5	375.1913	64372.482	51.66	Methyl (9Z)-8'-oxo-6,8'-di-apo-6-carotenoate	Diterpenoid
51	0.53	C_{36} H_{58} O_9	634.8	635.4178	973.219	51.11	Momordicin II	Steroidal gycoside
52	8.437	C_{26} H_{32} O_3	392.530	393.2445	1820.243	50.95	Methyl (9Z)-6'-oxo-6,5'-di-apo-6-carotenoate	Diterpenoid

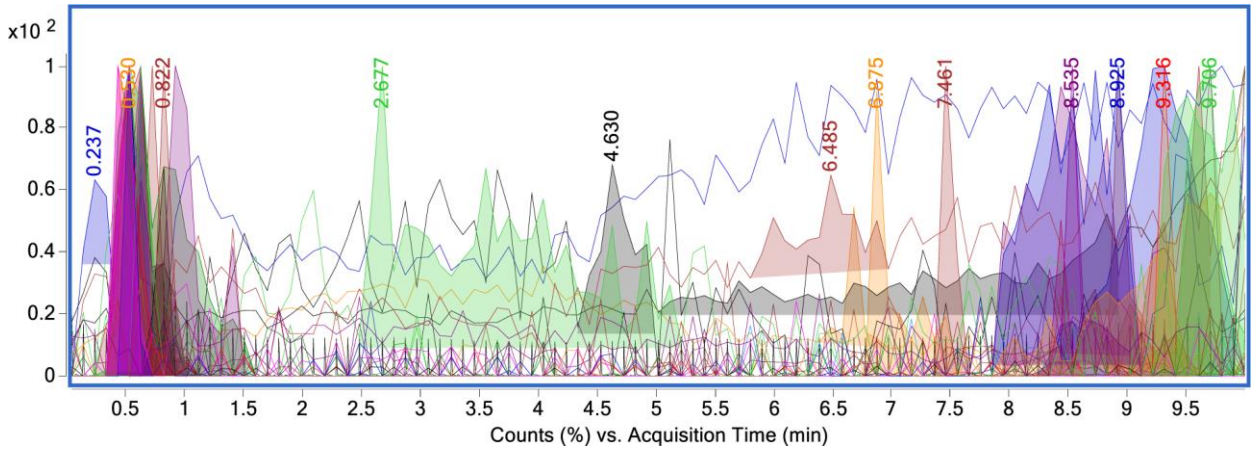


Fig. (2). UPLC-QTOF–MS total ion chromatogram of EASH in the negative ionization mode. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

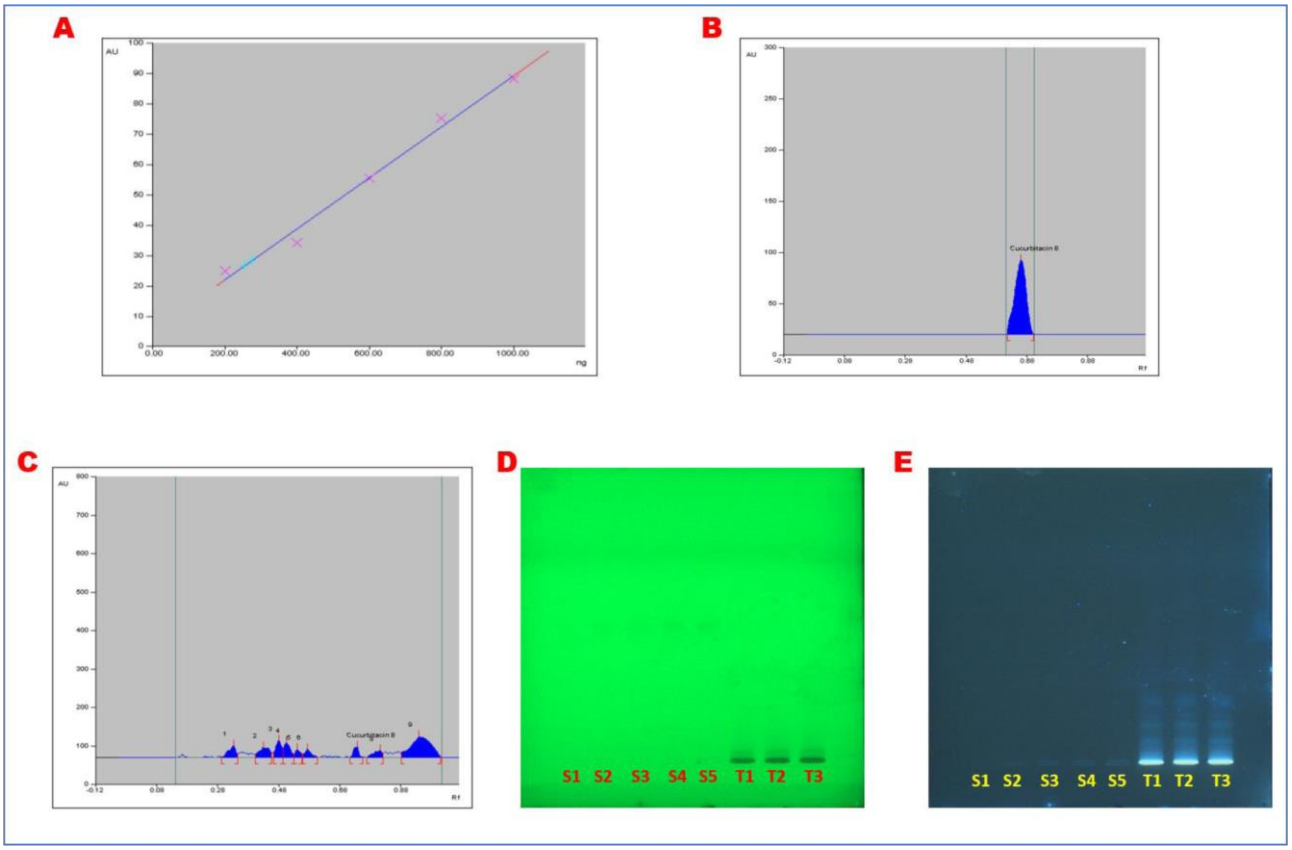
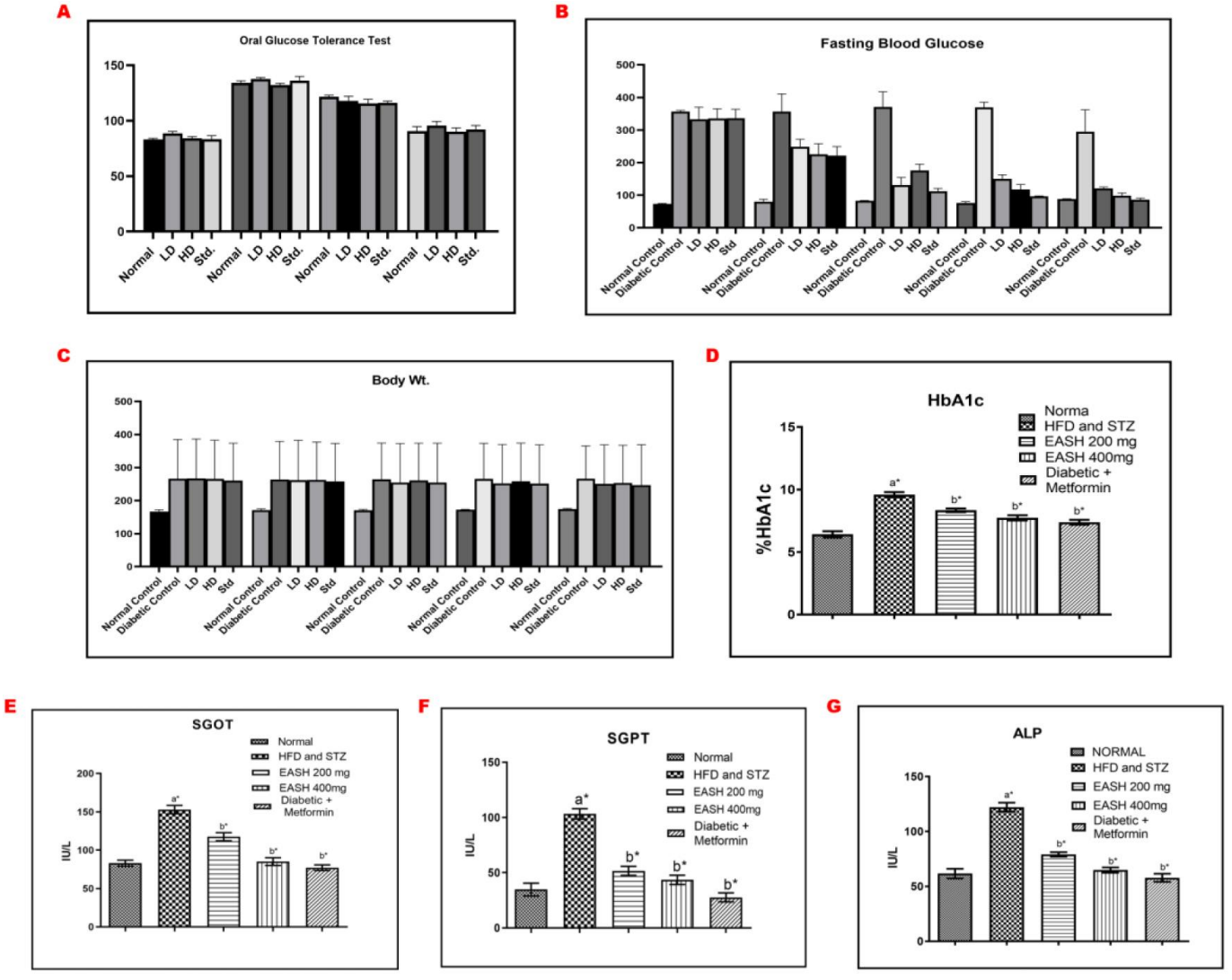


Fig. (3). HPTLC chromatogram of standard cucurbitacin B and EASH. **3.A** HPTLC chromatogram of calibration curve **3.B** HPTLC chromatogram of Cucurbitacin B **3.C** HPTLC chromatogram of EASH **3.D** HPTLC plate photographs of standard cucurbitacin B with sample extract under 254 nm. **3. E** HPTLC plate photographs of standard cucurbitacin B with sample under 366 nm. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 3. Recovery studies of the selected plants & intra-day and inter-day precision studies of the selected plants with Cucurbitacin B.

The Amount Added (ng)	Theoretically Expected Amount (ng/spot)	Experimentally Obtained Amount (ng/spot)	*%RSD*	Percentage Recovery
Recovery Studies				
0	1562.00	1560.83	0.08	99.93
200	1762.00	1758.62	0.14	99.81
400	1962.00	1959.67	0.12	99.88
600	2162.00	2155.06	0.23	99.68

-	Intra-day Precision Study Response (Area)		Inter-day Precision Study Response (Area)	
	Mean	***%RSD***	**Mean**	***%RSD***
200	9494.69	0.28	9499.13	0.26
400	18981.19	0.18	19321.25	0.15



(Fig. 4) Contd

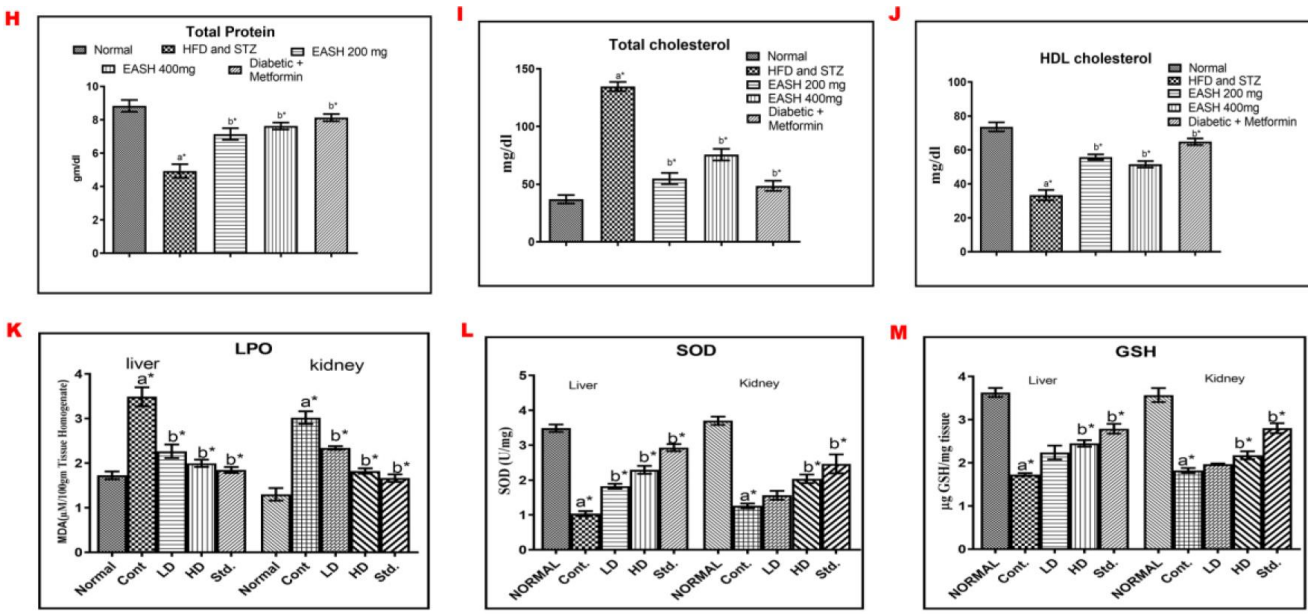


Fig. (4). Effect of EASH on oral glucose tolerance test **4A**; Effect of EASH on fasting blood glucose (mg/dl) **4B**; Effect of EASH on Body weight **4C**; Glycosylated hemoglobin level **4D**; Serum biochemical parameter (**4E**, **4F**, **4G**, and **4H**); Serum lipid profile (**4I** - **4J**); Tissue (Liver and kidney) antioxidant parameter (**4K**, **4L**, and **4M**). Each value is expressed as mean ± SEM, where n = 6, $p < 0.05$ when compared to normal control. **4D** - **4M**: Each bar represents mean ± standard error of the mean, n = 6; a* indicates diabetic control group *vs*. normal control group, b* indicates all treated groups *vs*. diabetic control group, where all bars are statistically significant from each other $p < 0.05$ (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

2.9.6.3. Body Weight and Fasting Blood Glucose

Low-dose STZ high-fat-instigated diabetic rats had significantly ($p< 0.05$) higher FBG levels than the standard control group. When EASH was fed to diabetic rats for 28 days at 200 and 400 mg/kg doses, there was a substantial ($p <0.05$) decline in FBG level toward normal differentiation to the diabetic control group. The effect of EASH on Fasting Blood Glucose (mg/dl) is shown in (Figs. **4B** and **C**). When compared to the normal control group, the diabetes control group had significantly lower-end body weights ($p <0.05$). However, after receiving doses of 200 and 400 mg/kg of EASH, the body weight of the diabetic control group improved significantly ($p <0.05$).

2.9.6.4. Glycosylated Hemoglobin

In comparison to diabetic rats, rats treated with EASH showed a significant decrease in levels of glycosylated hemoglobin ($p <0.05$). This is evidenced by the effect of EASH on HbA1c, as shown in Fig. (**4D**).

2.9.6.5. Serum Biochemical Parameter

The rats that received EASH treatment showed a notable decrease ($p <0.05$) in blood biochemical parameters such as SALP, SGOT, and SGPT, in comparison to the control group that was given a low dose of STZ and a high-fat diet. Furthermore, the treated group had a greater total protein level than the control group that received the same low dose of STZ and high-fat diet, as depicted in Figs. (**4E-H**).

2.9.6.6. Serum Lipid Profile

Compared to the group of rats without diabetes, the diabetic rats had significantly higher levels of triglycerides and total cholesterol in their serum, while their HDL levels were significantly lower ($p < 0.05$). However, after being fed with EASH extract at doses of 200 mg/kg and 400 mg/kg, the levels of triglycerides and total cholesterol were significantly reduced ($p < 0.05$), while the levels of HDL were significantly increased ($p < 0.05$) when compared to the diabetic groups (Figs. **4I** and **4J**).

2.9.6.7. Tissue Antioxidant Parameters:

Compared to diabetic rats, rats that received EASH treatment showed a significant decrease ($p <0.05$) in malondialdehyde levels and a significant increase ($p <0.05$) in SOD and GSH levels, as shown in Fig. (**4K-4M**).

2.10. Histopathology of Pancreas

Research on pancreatic histopathology showed that the groups treated with EASH exhibited protection for pancreatic beta cells. In comparison to diabetic rats, the groups treated with EASH showed a gradual improvement in pancreatic beta cell density that was dependent on the dosage (Figs. **5A–5E**).

3. RESULTS AND DISCUSSION

The high-fat diet and low doses of STZ-induced diabetic rat is used as an animal model for non-insulin-dependent diabetes mellitus (NIDDM) or type 2 diabetes mellitus in humans [28]. Studies based on this experimental T2DM model have shown that diet plays a significant role in developing diabetes, hypertension, hyperlipidemia, and nephropathy over time [28, 29]. This study aimed to investigate the potential of the ethyl acetate fraction of *Solena heterophylla* to reduce blood glucose levels in rats with high-fat diets and low doses of STZ-induced diabetes. Taking high doses of STZ can significantly affect insulin secretion, resembling type 1 diabetes. However,

when combined with a high-fat diet, low doses of STZ can lead to mild impairment of insulin secretion, comparable to the later stage of type 2 diabetes [30]. Our experiment on animal models showed that the control diabetic group had a notable increase in blood sugar levels. However, administering EASH at 200 and 400 mg/kg were able to significantly reduce the blood glucose levels.

The research aimed to explore the molecular mechanism of action of EASH. Various plant secondary metabolites have been identified and isolated for their effectiveness in treating DM and other metabolic diseases [31]. During the study, a significant and positive correlation was found between the levels of α-amylase, α-glucosidase, overall phenolic and flavonoid content, and the antioxidant capabilities of EASH. The chemical components of EASH were identified using LC-QTOF-MS analysis. Through this analysis, different classes of compounds have been identified as potential plant secondary metabolites that could be responsible for antidiabetic activity.

Glycohemoglobin, or glycosylated hemoglobin or hemoglobin A1C (HbA1c), forms when glucose combines with hemoglobin in cells without enzymes [26, 31, 32]. HbA1c measures average blood glucose levels over the past three months [32]. This study, HbA1C levels were examined as a crucial marker for T2DM [26, 31, 32]. Diabetic animals were found to have higher levels of HbA1c when compared to non-diabetic animals. Monitoring HbA1c levels is important for diabetes management but doesn't give a complete picture. Lowering HbA1c levels may not always lead to a significant decrease in microvascular events in vital organs, which are complications of diabetes often associated with oxidative stress [33]. Long-term high blood sugar levels are linked to the development of microvascular and macrovascular complications associated with diabetes [34]. This happens because of the increased production of reactive oxygen species (ROS), the formation of advanced glycation end products from glucose, a heightened polyol pathway, and the activation of protein kinase C (PKC) isoforms by glucose [34]. Diabetes is characterized by high blood sugar and oxidative stress, and this ongoing metabolic condition can result in tissue damage and degenerative issues in different organs, including the kidneys and liver [35]. The findings revealed that administering EASH at doses of 200 mg/kg and 400 mg/kg significantly decreased oxidative stress in liver tissue. When STZ is administered intravenously, it can harm beta cells and cause a gradual reduction in insulin production. This can result in high blood sugar levels due to the generation of free radicals. Furthermore, the histopathology of the pancreas revealed that the groups treated with EASH had safeguarded pancreatic beta cells. This confirms that Cucurbitaceae food plants have protective properties in reducing oxidative stress. Earlier reports have shown that the antioxidant and antiradical capacities of 18 cultivars of Cucurbitaceae food plants are related in the same way [36].

It has been established that dyslipidemia plays a role in the development of macrovascular complications related to diabetes [35, 36]. Treatment with EASH fraction resulted in improved blood lipid profiles in diabetic rats at doses of 200 and 400 mg/kg. The bitter principles of the Cucurbitaceae family are steroidal natural products, that are derived from the triterpene cucurbitane, are mainly the bitter principles of the family Cucurbitaceae responsible for. These compounds are known for their anti-inflammatory, hepatoprotective, antihyperlipidemic, and anti-cancer activities [37]. A densitometric analysis by HPTLC revealed that *Solena heterophylla* L.'s fresh fruit extract has a high level of Cucurbitacin B (2.29% w/w).

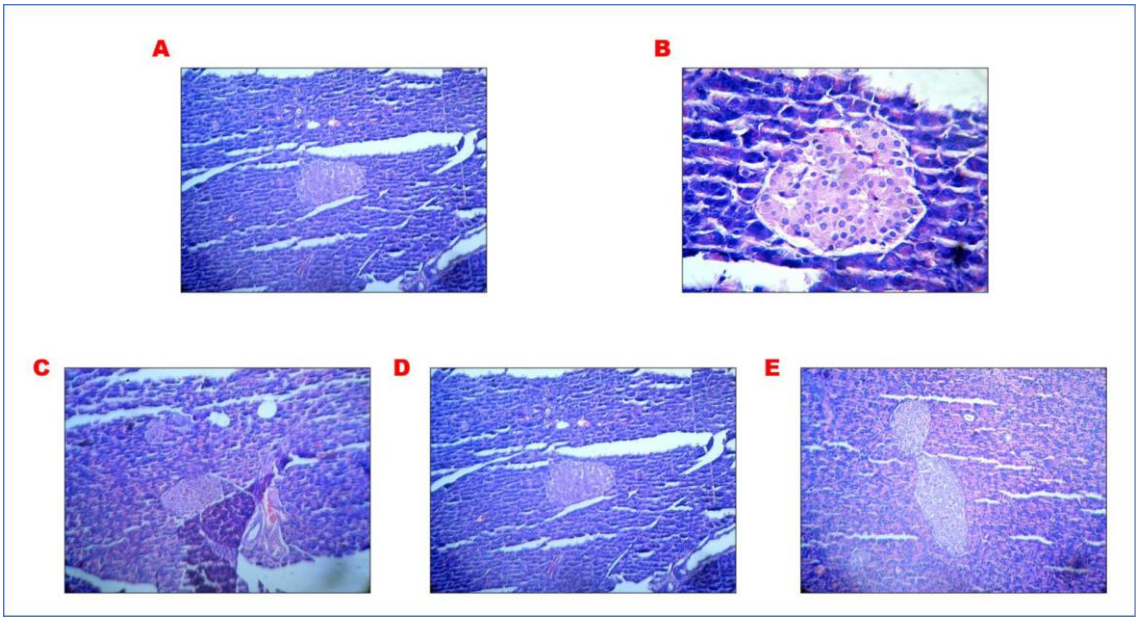


Fig. (5). Representative profile of histopathological analysis of pancreas tissues. **(5.A)** Normal control Rat pancreas; **(5.B)** Diabetic control rat after induction of STZ; **(5.C)** STZ-induced rat pancreas after low dose treatment; **(5.D)** STZ-induced rat pancreas after high dose treatment; **(5.E)** STZ induced rat pancreas after Standard drug (Metformin) treatment. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

It has been noticed that individuals with diabetes may encounter liver dysfunction. Studies have shown that there is an increase in plasma AST, ALT, and ALP activity and a decrease in total protein levels [38, 39]. It is believed that liver cytosol enzymes may leak into the bloodstream, resulting in an increase of AST, ALT, and ALP levels [39-41]. This could indicate that the livers of diabetic patients have undergone necrosis. To evaluate liver damage in diabetic rats caused by streptozotocin, the markers AST and ALT were utilized. After administering EASH at doses of 200 and 400 mg/kg to diabetic rats, we observed a reduction in enzyme activity in the plasma when compared to the average values of the diabetic group.

According to a study, *Solena heterophylla's* ethyl acetate fraction exhibited powerful antidiabetic properties in albino rats in a dose-dependent fashion. The plant was discovered to have multiple secondary metabolites, including terpenoids, flavonoids, and alkaloids, as revealed through preliminary phytochemical, HPTLC, and mass spectrophotometric analyses. The presence of flavonoids may have contributed to the antioxidative effects observed *in vivo*. In experimental animals, EASH was found to significantly reduce the HbA1C levels while also managing tissue oxidative stress in the liver caused by diabetes. These results suggest that EASH has a significant potential in treating diabetes by reducing the oxidative stress and serum lipid levels, which supports its traditional use in Indian traditional medicine in North-East India.

CONCLUSION

According to a recent study, an extract derived from *Solena heterophylla* fruit could potentially help reduce hypoglycemia, as well as improve the pancreatic β cell and antioxidant defense system. The extract has been found to positively impact the pancreas histology, intestinal glucose transport, glycosylated hemoglobin, and enzymatic liver biochemical markers. These findings could be beneficial in developing effective healthcare options for managing diabetes and related disorders. However, further research is needed to understand the mechanisms and synergistic interactions of the identified secondary metabolites in the active fraction responsible for these effects.

LIST OF ABBREVATIONS

CAN	= Acetonitrile
AESH	= Aqueous Ethanol Extract *Solena heterophylla*
ALP	= Alkaline Phosphatase
ALT	= Alanine Transaminase
ANOVA	= Analysis of Variance
AST	= Aspartate Transaminase
CAT	= Catalase
CFSH	= Chloroform Fraction *Solena heterophylla*
CPCSEA	= Committee for the Purpose of Control and Supervision of Experiments on Animals
CuB	= Cucurbitacin B
DTNB	= 5, 5′-dithio bis-2-nitro Benzoic Acid
EASH	= Ethyl Acetate Fraction *Solena heterophylla*
EFSH	= Ethanol Fraction *Solena heterophylla*
FBG	= Fasting Blood Glucose
GSH	= Glutathione
HFD	= High Fat Diet
HFSH	= Hexane Fraction *Solena heterophylla*
HPLC	= High Performance Liquid Chromatography
HPTLC	= High-Performance Thin Layer Chromatography
IDDM	= Insulin Dependent Diabetes Mellitus
IDF	= International Diabetes Federation
LC-QTOF-MS	= Liquid Chromatography-Quadrupole Time-of-Flight Tandem Mass Spectrometry
MDA	= Malondialdehyde
NADH	= Reduced Nicotinamide Adenine Dinucleotide
NBT	= Nitrobluetetrazolium Chloride
NIDDM	= Non-Insulin Dependent Diabetes Mellitus
OGTT	= Oral Glucose Tolerance Test
PKC	= Protein Kinase C
PMS	= Phenazinemethosulphate
SALP	= Serum Alkaline Phosphatase
SEM	= Standard Error of Mean
SGOT	= Serum Glutamic Oxaloacetic Transaminase
SGPT	= Serum Glutamic Pyruvic Transaminase
SHF	= *Solena heterophylla* Fraction
SOD	= Superoxide Dismutase
STZ	= Streptozotocin
T1DM	= Type 2 Diabetes Mellitus
T2DM	= Type 2 Diabetes Mellitus
TBARS Assay	= Thiobarbituric Acid Reactive Substances
TBA	= Thiobarbituric Acid
TCA	= Trichloroacetic Acid

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Institutional Animal Ethical Committee (IAEC) reviewed and approved the animal study at Jadavpur University, Kolkata, West Bengal, India, Protocol Approval Number JU/IAEC-22/15.

HUMAN AND ANIMAL RIGHTS

All the animal experimentation were performed according to the Guide for the Care and Use of Laboratory Animals.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

All the data and supporting information is provided within the article.

FUNDING

None.

CONFLICT OF INTEREST

Dr. Asis Bala is the Associate Editorial Board Member of the journal Endocrine, Metabolic & Immune Disorders-Drug Targets.

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Combination synergy between β-carotene and lupeol against breast adenocarcinoma *in vitro* and *in vivo* using combination index

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ABSTRACT

Background: Breast adenocarcinoma is one of the leading causes of death among women regardless of development in the field of diagnosis, treatment and prevention. Synergistic approaches are emerging as a strategy to overcome monotherapy resistance in cancer treatment.

Purpose: Evaluation of the combinatory activity of β-carotene (BC) and lupeol (LUP) for anti-proliferative activity by median effect equation and combination index. Validation was performed by fixed dose combination against murine mammary adenocarcinoma namely Ehrlich ascites carcinoma (EAC).

Methods: Cytotoxicity assay was performed using the MTT and Trypan blue methods. Isobologram and combination index analysis were used to analyze the type of interaction between β-carotene (BC) and lupeol (LUP). Based on the synergistic dose combination determination of in-vivo antitumor activity was performed using EAC cells inoculated in mice treated with BC, LUP and their combination (BC+LUP). Tumor volume, cell count, weight along with hematological, biochemical and antioxidant parameters were evaluated.

Results: The combination (BC+LUP) exhibited the highest inhibition (IC_{50}=54.8 ± 1.84) compare to the standard inhibitor, wher eas BC and LUP showed moderate inhibition with an IC_{50} of 101.5 ± 2.66, and 84.08±3.56 respectively. The hematological, biochemical, and tissue antioxidant parameters were reverted to normal levels in the treated animal groups. BC and Lupeol reduced the volume, weight, and viable cell count of the tumor; increased the life span of EAC tumor-bearing mice.

Conclusion: β-Carotene and lupeol were found to be more effective in combination for the anti-proliferative activity. Thus combination techniques must be employed to find out the synergistic activity of the molecules.

List of abbreviations

% ILS	percentage increase in life span
5-FU	5-fluorouracil
ANOVA	analysis of variance
BC	β-carotene
BC + LUP	β-carotene + lupeol (combination)
CI	combination index
Dc	dead EAC cells
DMSO	dimethyl sulfoxide
DMSO	dimethyl sulfoxide
DTNB	5,5′-dithio bis-2-nitro benzoic acid
EAC	ehrlich ascites carcinoma
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechin-3-gallate
GSH	reduced glutathione
Hb	haemoglobin
IC_{50}	half-maximal Inhibitory concentration
LPO	Lipid peroxidation
LUP	lupeol
MDA	malondialdehyde assay
MST	mean survival time
MTT	3-(4, 5-dimethylthiazol-2-yl)−2, 5-diphenyl tetrazolium bromide

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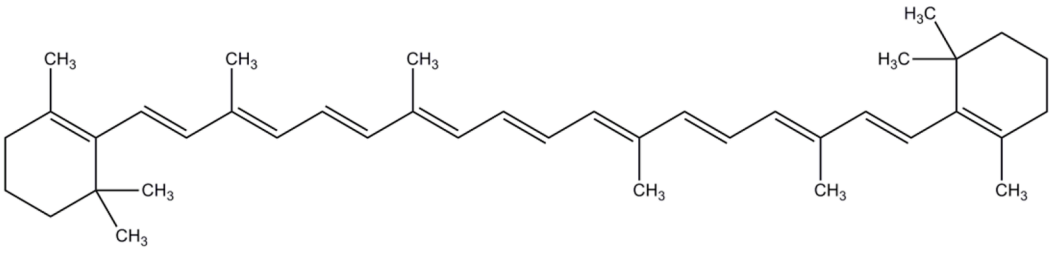
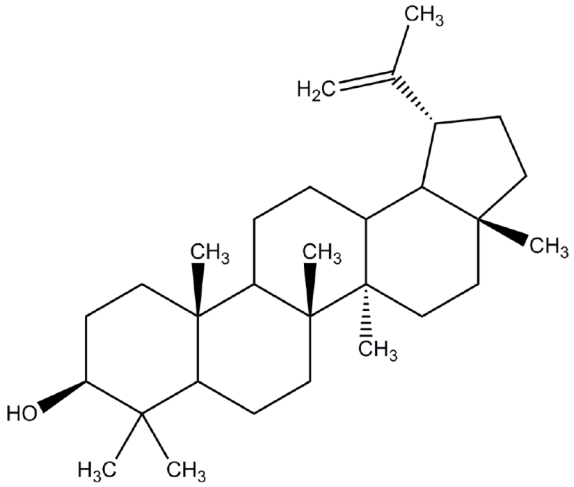
NADH	reduced nicotinamide adenine dinucleotide hydrogen
NBT	nitroblue tetrazolium chloride
OECD	organisation for economic co-operation and development
pH	potential of hydrogen
PMS	phenazine methosulphate
RBC	red blood corpuscle
RPMI	Roswell Park memorial institute medium
SALP	serum alkaline phosphatase
SEM	standard error mean
SGOT	serum glutamate oxaloacetate transaminase
SGPT	serum glutamic pyruvic transaminase
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid by reactive substances
Tc	total EAC cells
TCA	trichloroacetic acid
WBC	white blood corpuscle

Introduction

Cancer is a group of ailments that cause death before the age of 70 globally and is a significant impediment to extending life expectancy (Anonymous, 2019). By the year 2030, each year approximately 17 million deaths and 26 million new cases due to cancer will be reported (Thun et al., 2010). Despite significant efforts, cancer continues to be a deadly executioner on a global scale hence a continuing quest for new anticancer alternatives that are both effective and economical (Feitelson et al., 2015). Cancer cells are usually proliferated abnormally and have unusual morphology, membranes, and also cytoskeletal proteins. Medicines used in cancer treatment are mainly cytotoxic and exhibit a higher level of anti-proliferative effect (Baskar et al., 2014). Chemotherapy is used to treat, cure and improve the health condition of the cancer patient and also treated various tumor cells, but it also affects non-tumor cells, especially the skin, hair, and gastrointestinal epithelium (Coseri, 2009). Normal growth control of tumor cells is disrupted not just during carcinogenesis, but also during cancer cell metastasis. Combination therapy is an efficient treatment modality of cancer therapy. This approach potentially reduces drug resistance, while simultaneously providing therapeutic anti-cancer benefits (Bayat Mokhtari et al., 2017). Phytomolecules originating from medicinal plants have been utilized to treat human ailments since the birth of the humankind (Kar et al., 2021). Natural product-based chemotherapeutics have been popularized for its safety-related aspect (Mukherjee, 2019). Natural antioxidants mainly from plant sources have been proposed as anti-cancer adjuvant therapy by inhibiting the induction of cancer (Singh et al., 2012). A vast range of phytomolecules has been the source



(a)



(b)

Fig. 1. Chemical structures of Phytoconstituents (a) β-Carotene (b) Lupeol.

of around 60% of medications currently used in cancer treatment such as vinca alkaloids, taxus diterpenes, camptotheca alkaloids, podophyllum lignans, etc. (Cragg and Pezzuto, 2016). Combination of EGCG and curcumin has inhibited the growth of carcinoma cells through dose reduction index (DRI). In this study, dose depletion of EGCG (4.4–8.5-fold) and curcumin (and 2.2–2.8-fold) at ED_{50}s was found to be effective (Khafif et al., 1998). Synergistic effects of andrographolide and melatonin using the Chou-Talalay combination index method validated their efficacy in metastatic CRC cells, colospheroids, and drug resistant CRC cells reducing the effective dose of anti-CRC drug treatment and the mechanism is ER stress induction and angiogenic inhibition (Banerjee et al., 2021).

β-Carotene (Fig. 1a) is a carotenoid, the precursor of Vitamin A mainly responsible for the pigment of the fruit or flowers β-Carotene possess an effective role to treat cancer for their potent antioxidant activity (Das et al., 2015). β-carotene also inhibited the growth of human colon adenocarcinoma cell lines, by initiating cell-cycle arrest, apoptosis, and cell proliferation by reducing an important regulator of cell-cycle progression cyclin A (Palozza et al., 2002). Lupeol (Fig. 1b) is a pentacyclic triterpenoid also known as fagarsterol having potential anti-inflammatory, anti-arthritic, antiprotozoal, antitumor, cardioprotective, antioxidant as well as hepatoprotective activities (Gallo et al., 2009; Saleem, 2009).

The study was designed to explore the phytomolecules and their fixed dose combination with anti-proliferative activity against murine mammary adenocarcinoma *viz.* Ehrlich ascites carcinoma (EAC) cells. Isobologram and combination index analysis were used to analyze the type of interaction between β-carotene (BC) and lupeol (LUP). Based on the synergistic dose combination, *in vivo* antitumor activity was carried out using EAC cells. Thus the goal of this study was to assess the anti-proliferative activity of β-carotene (BC) and lupeol (LUP) and its combination by *in vitro* and *in vivo* models.

Materials and methods

Chemicals

5-Fluorouracil [≥ 99% (HPLC), powder] from Sigma (St. Louis, MO); β-Carotene [≥ 97% pure] (BC) from HIMEDIA (Mumbai, India); Trichloroacetic acid, A.R. (TCA) from Merck (Mumbai, India); Lupeol [≥94% pure] (LUP) from Sigma (St. Louis, MO); Thiobarbituric acid (TBA), Nitroblue tetrazolium chloride (NBT) from Loba Chemie (Mumbai, India); Phenazine methosulphate (PMS), Reduced nicotinamide adenine dinucleotide (NADH), 5,5′-dithio bis-2-nitro benzoic acid (DTNB) from Sigma (St. Louis, MO). All other chemicals or reagents used for the experiment were of analytical grade obtained from Sigma (St. Louis, MO), Loba Chemie (Mumbai, India), Sisco (Mumbai, India), Merck (Mumbai, India).

Animals

Healthy male Swiss albino mice aged 4–6 weeks and weighed between 22 and 25 g were procured from M/s Chakraborty Enterprise, Kolkata, India. The mice were kept in poly acrylic cages and perpetuated under standard laboratory conditions (temperature 25 ± 2 °C and dark/light cycle 12/12 h). The animals were subjected to a standard dry pellet diet and water *ad libitum*. Before the final experiment acclimatization was done to laboratory conditions for 7 days. The described procedures were conducted in an ethical manner and approved by the Institutional Animal Ethical Committee, NSHM Knowledge Campus, Group of Institutions, Kolkata-700,053 (Ref. No: NCPT/IAEC-04/18).

Dose selection

The dose of BC and LUP was selected as per the thorough literature search; the dose of BC (10 mg/kg p.o.) and LUP (200 mg/kg p.o.) was found to be effective against EAC-induced tumor was 10 mg/kg and 200 mg/kg p.o. respectively (Das et al., 2015; Saleem et al., 2009). Both the doses did not exhibit any kind of adverse reactions or behavioral changes in experimental animals and no signs of mortality were also not observed. The ratio of 1:2 was used as the dose combinations for the drug interaction analysis using the Chou-Taley median effect equation for which six different dose concentrations were taken for further analysis (Chou and Martin, 2005).

Implantation of tumor

EAC cells used for this study were collected from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The procured EAC cells were transplanted in Swiss albino mice through the intraperitoneal (i.p.) route. Administration of 0.2 ml of tumor cell suspension containing 2×10^6 tumor cells (counted by Trypan blue assay method) per mouse for a period of 10 days. Ascites were drawn out from the EAC tumor located at peritoneal cavity of mice at the log phase [day 7–8 of tumor bearing] (Dolai et al., 2012).

Determination of in vitro cytotoxicity

In vitro cytotoxicity was performed by utilizing both MTT {3-[4, 5-*dimethylthiazol*-2-*yl*]−2,5-diphenyl-2H-tetrazolium bromide} and Trypan blue assay method. In Trypan blue exclusion assay, 2×10^6 EAC cells were suspended in 0.2 ml of 0.2 M phosphate buffer saline (pH 7.4) and mixed with 100 μL of various concentrations of both β-carotene (BC) and lupeol (LUP) (5–200 μg/ml). The Phosphate buffer (1 ml) was used to maintain the final volume and the cells were incubated for 3 h at 37 °C. The cell viability was estimated using trypan blue reagent which caused the non-viable cells to turn blue. The percentage of cytotoxicity was assessed by calculating% inhibition (IC_{50}) as per the validated method (Karmakar et al., 2013; Manojkumar et al., 2009).

The% of cell inhibition was determined using the following formula:

$$\text{Cell count} = \text{Number of cells} \times \text{dilution factor/Area} \times \text{thickness of liquid film}$$

$$\%\ \text{Inhibition} = (\text{Dc} / \text{Tc}) \times 100\ldots\ldots \quad (1)$$

Where, Tc = Total EAC cells and Dc = Dead EAC cells

In MTT assay, 0.2 ml of cell suspension (2×10^6 cells/well) was seeded into 96-well microtiter plates and supplemented with RPMI 1640 medium (200 μL) along with the penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cells were then treated with different concentrations of BC and LUP (5–200 μg/ml) and incubated for 24 h at 37 °C (5% CO_2, 98% relative humidity). 20 μL of MTT (5 mg/mL in phosphate buffer saline) solution was poured into each well of the plate followed by incubation at 37 °C for 4 h. The colored formazan crystals, produced from MTT were dissolved in 150 μL of dimethyl sulfoxide (DMSO) and the absorbance was measured by Spectra Max iD3 Multi-Mode Microplate Reader at the wavelength of 570 nm and the% of cytotoxicity was assessed by the following equation (Kar et al., 2021).

The% of cell inhibition was determined using the following formula:

$$(\%)\ \text{Growth inhibition} = 100 - (\text{A/B}) \times 100\ldots. \quad (2)$$

Where A is the mean absorbance of the individual test group and B is the mean absorbance of the control group

Determination of acute toxicity

Acute oral toxicity of BC, LUP, and BC+LUP was performed by up and down procedure as per OECD guidelines no. 425. Before administration of the dose of test samples the mice were kept fasting for 12 h. After administration of the dose, the animals were observed individually for 14 days (Anonymous, 2008).

In vivo experimental design

Swiss albino mice were divided equally into six groups ($n = 12$). Group I served as normal saline control (5 ml/kg, p.o.). All groups except group I received EAC cells through the intra-peritoneal route (2×10^6 cells/mouse). Group II was denoted as the EAC control group. After 24 h, group III-V were being injected BC (10 mg/kg b.w., p.o.); LUP (200 mg/kg b.w., p.o.), and combination [(10+200) mg/kg b.w., p.o.] for nine consecutive days. BC, LUP and the combination dose were dissolved in corn oil for the oral administration to the experimental animals. 5-FU was given to group VI as a positive control at a dose of 20 mg/kg p.o. On the 10th day, six mice from each group were retained for fasting (18 h) and blood was collected by cardiac puncture for estimation of anti-tumor, hematological, and biochemical parameters. Rest of the animals were kept under observation to determine the% increase in life span (Haldar et al., 2010).

Treatment schedule

Group I: Normal saline control (5 ml/kg, p.o.)
Group II: EAC control (2×10^6 cells /mouse, i.p.)
Group III: EAC + BC (10 mg/kg body weight, p.o.)
Group IV: EAC + LUP (200 mg/kg body weight, p.o.)
Group V: EAC + Combination (BC+LUP) [(10 + 200) mg/kg body weight, p.o.]
Group VI: EAC + 5-FU (20 mg/kg body weight, p.o.)

Determination of tumor volume, packed cell volume, tumor weight, and (viable / non-viable) tumor cell count

The mice were sacrificed and then dissected on the 10th day; the ascites were collected from the peritoneal cavity of six mice and the other six mice were used for the estimation of tumor volume, packed cell volume, and tumor weight was measured and the viable and non-viable cells were counted in the Neubauer chamber as per the validated methods in Phytotherapy and Pharmacology Research Laboratory from the Department of Pharmaceutical Technology, Jadavpur University, Kolkata (Karmakar et al., 2013).

Determination of percentage increase life span (% ILS)

The% increase in life span was estimated based on the mortality rates of the experimental mice as reported earlier by (Karmakar et al., 2013) with slight modifications. Percentage increase in life span (% ILS) was calculated by the following formula (Karmakar et al., 2013).

Mean survival times (MST) in days = (Day of the first death + Day of the last death)/2

ILS (%) = [(MST of the treated group / MST of the control group) −1] × 100

Determination of hematological parameters

Hemoglobin (Hb) content, red blood cell count (RBC), and white blood cell (WBC) were estimated from the collected blood according to the standard methods used in Phytotherapy and Pharmacology Research Laboratory from the Department of Pharmaceutical Technology, Jadavpur University, Kolkata (Kumar et al., 2013).

Determination of serum parameters

Commercially available kits (Arkray Healthcare Pvt. Ltd, Mumbai, India) were used for the quantification of total proteins (TP), alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in the treated animals. The absorbance was measured by Spectra Max iD3 Multi-Mode Microplate Reader (Molecular Device, USA).

Antioxidant potential

Antioxidant assay was executed by using 200 mg liver tissues and homogenized in 10 ml and 5 ml Tris buffer (20 mM, pH: 7) respectively. Further, the tissue was subjected to centrifugation at 12,000 rpm for 30 min at a controlled temperature at 4 °C. The evaluation was carried out by collecting the supernatant for estimation in lipid peroxidation assay, superoxide dismutase and non-enzymatic antioxidant systems such as reduced glutathione (Kakkar et al., 1984; Bhattacharya et al., 2011).

Thiobarbituric acid reactive substances (TBARS) in the isolated liver tissue were used to measure lipid peroxidation (LPO). The tissue was suspended in saline and 10% w/v trichloroacetic acid and kept idle for 30 mins. The mixture was again centrifuged at 3000 rpm for 10 mins. 1 ml supernatant was taken and mixed in thiobarbituric acid (1% w/v). The mixture was heated in water bath until color changes; the absorbance was measured at 532 nm (Dolai et al., 2012).

Reduced glutathione (GSH) level was measured by mixing 100 μL of tissue homogenate and EDTA (0.02 M) on an ice bath. 1 ml of distilled water and 50% TCA were added to the homogenate. After centrifugation at 3000 rpm for 15 min 1 ml of supernatant was mixed with 0.4 M tris buffer and DTNB. The absorbance was measured at 412 nm.

1 ml of nitroblue tetrazolium solution, 1 ml of NADH and 0.1 ml of tissue homogenate were mixed with 100 μg of PMS solution, and incubated at 25 °C for 5 mins. Superoxide dismutase (SOD) level was measured at an absorbance of 560 nm (Dolai et al., 2012).

Interaction analysis by isobologram method

Interaction potential of BC and LUP combination was evaluated by using isobologram and median effect method Chou and Martin, 2005). Different dose combinations of BC+LUP were plotted against ED_{50}, ED_{75} and ED_{90} effect in the form of% proliferative effect which was mentioned in Eqs. (1) and ((2). ED_{50}, ED_{75}, and ED_{90} stand for the therapeutic effect of 50%, 75% and 90% respectively (Dimmitt et al., 2017). The doses are connected via a line of additivity and the combination is taken as antagonistic, additive or synergistic when the combination dose falls above, on the line or below the line of additivity respectively. Synergism, antagonism, and additivity for BC and LUP were also analyzed by effect analysis of Chou Talalay using COMPUSYN software 2.0. Less than 0.3 of the combination index exhibit strong synergism whereas value of 0.3–0.69 possess synergism, 0.70–0.84, 0.85–0.89 shows moderate synergism and mild synergism respectively, 0.9–1.09 exhibit additive effect, 1.10–1.19 indicates slight antagonism, 1.20–1.44 possess antagonism and >1.45 indicates moderate strong antagonism (Motiwala and Rangari, 2015).

Statistical analysis

Graph Pad Prism software (version 8.02, Graph Pad Software Inc., San Diego, CA) was used for the Statistical analysis. All the experimental data were represented in mean ± standard error of the mean (SEM). One-way ANOVA analysis followed by Dunnett's *post hoc* test was performed to determine statistical significance, where the 'p-value' of < 0.05 was significant.

Results

Determination of in vitro cytotoxicity

In vitro cytotoxicity assay by Trypan blue exclusion method on β-carotene (BC), lupeol (LUP), combination (BC+LUP), and standard (5-FU) showed dose-dependent cytotoxic effect on the EAC cell with IC_{50} value of 101.5 ± 2.66, 84.08±3.56, 54.8 ± 1.84 and 50.5 ± 0.52 μg/ml

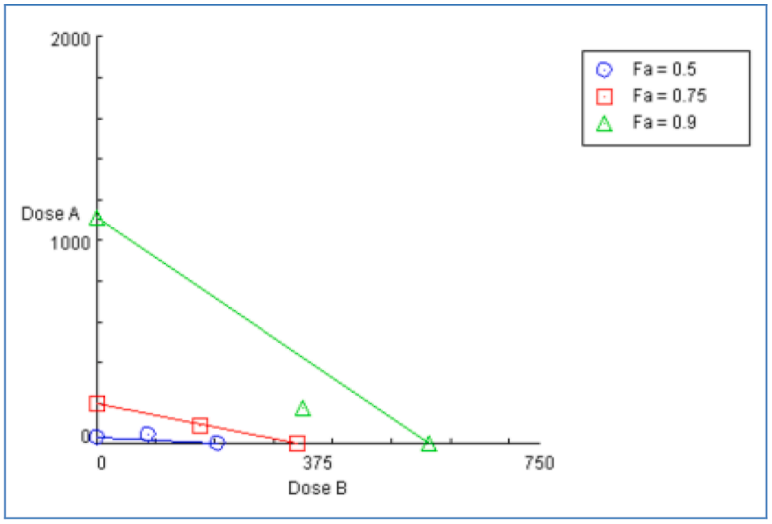
respectively and β-carotene (BC) and lupeol (LUP) by *in vitro* cytotoxicity (using MTT assay) after 48 h by color analysis respectively (Figs. 3 and 4).

Interaction between BC and LUP

The studied dose of BC, LUP, and their combination exhibited potent anti-proliferative activity (Figs. 3, 4) compared to the EAC control group. The interaction between the doses (synergism, antagonism, or additivity) is presented graphically in Fig. 2. In Fig. 2 (A, B) it was observed that ED_{50}, ED_{75}, ED_{90}, and ED_{95} effect levels lie at the left side of the line of additively. The effective levels and dose combination showed anti-proliferative activity in terms 50%, 75%, 90% and 95%. The combination index (CI) was calculated at specified endpoint of effect to determine the degree of interaction between two molecules, quantitatively. From the median effect method analysis, the CI obtained and represented in Table 1. BC+LUP combination with a CI value of less than 0.3 showed strong dose-dependent synergism for Trypan blue exclusion assay and MTT assay. The combination of both agents represented synergistic interactions in growth inhibition and expanded sigmoidicity (steepness) of the dose-effect curves, a response that was dose and cell type dependent. Combination of BC and LUP at Fa 0.5 the dose of BC was reduced 4.9 fold whereas the dose of LUP reduced 3.4 fold at a 2:1 dose combination ratio as determined by the dose reduction index (DRI) at ED_{50}. Higher DRI values above ED_{50s} were also observed The findings of this study, represent that this model can be useful for the analysis of various chemopreventive agents. BC and LUP showed inhibition potential through intervention of different mechanistic approaches, which may be considered a key factor for their synergistic effect.

Acute toxicity study

Acute toxicity study of β-carotene (BC), lupeol (LUP), and a combination of both was performed as per the OECD guideline (OECD guideline no. 425, 2008). Doses were given to experimental animals up to 2000 mg/kg body weight. After observation of 14 days, the mice did not produce any significant alteration in the behavior, breathing,



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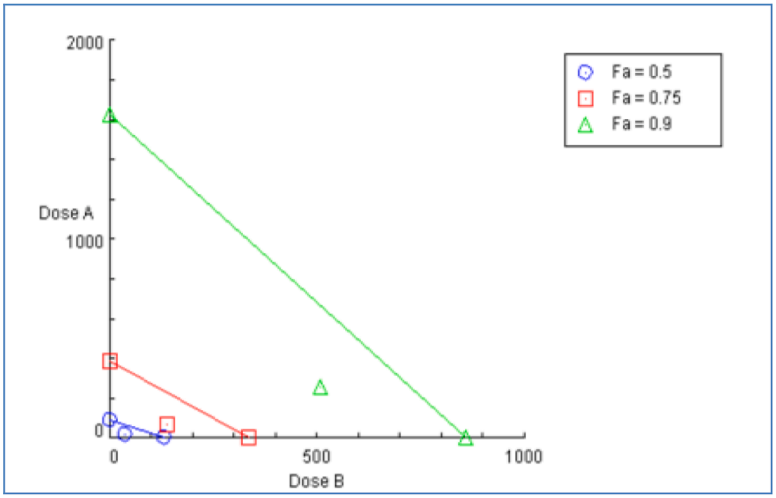

B

Fig. 2. Isobologram denoting the effect of combinations (BC+LUP) on Trypan blue assay [(A) MTT assay (B) 50% (Fa = 0.5), 75% (Fa = 0.75) and 90% (Fa = 0.9) effect levels. The line shows alignment of theoretical value of an synergistic interaction between β-carotene (BC) and lupeol (LUP). Values above the diagonal line of additive effects in the isobole suggest antagonism and below line suggest synergism.].

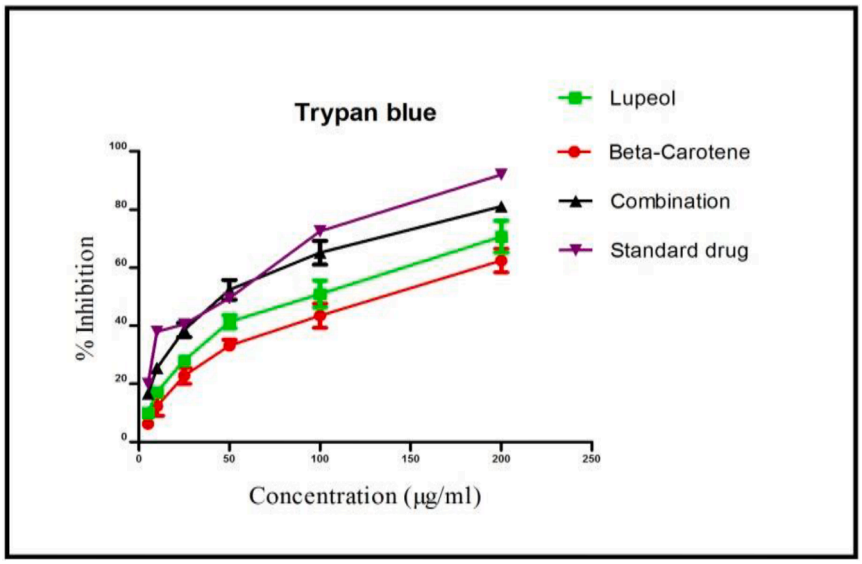

Fig. 3. Cytotoxic effect of BC, LUP, the combination (BC+LUP) and standard (5-FU) by the Trypan blue exclusion method. [Values are mean ± SEM.].

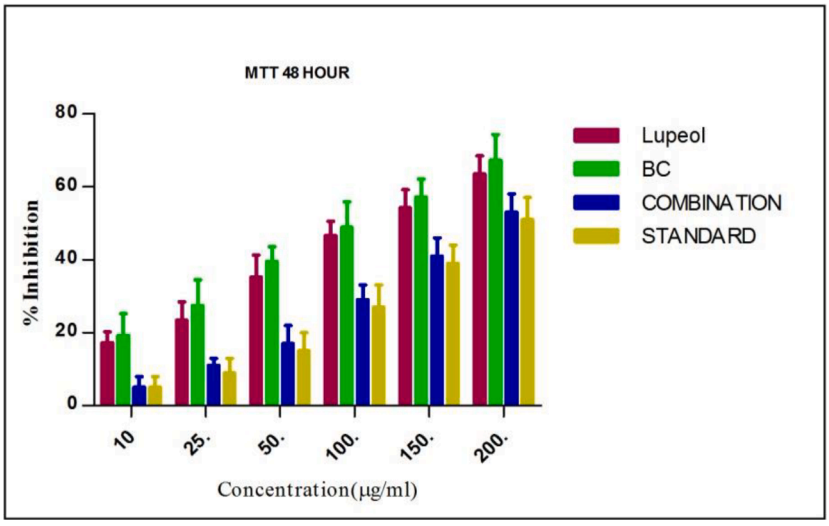

Fig. 4. Cytotoxic effect of BC, LUP, the combination (BC+LUP) and Standard (5-FU) by the MTT assay after 48 h.

Table 1
Combination index (CI) at the affected fractions of 50% (ED_{50}), 75% (ED_{70}), 90% (ED_{90}) and 95% (ED_{95}) of BC, combined with LUP.

CI Values ± SEM Cell viability Indicator	ED_{50}	ED_{75}	ED_{90}	ED_{95}
MTT assay	0.4965	0.5952	0.7502	0.8988
Trypan blue exclusion assay	1.6849	0.9614	0.7806	0.7858

cutaneous effects, sensory nervous response or gastrointestinal effects. During the toxicity study no deaths occurred inferring that BC, LUP, and the combination are safe up to the dose of 2000 mg/kg body weight.

Determination of direct tumor-related parameters

Anti-proliferative activity of β-carotene (BC) and lupeol (LUP) and combination (BC+LUP) at different dosages [10 mg/kg, 200 mg/kg, and (10+200 mg/kg)] against EAC tumor-bearing mice was evaluated in terms of tumor weight, tumor volume, viable and non-viable cell count, mean survival time together with the increase in life span. The result showed that the tumor volume, weight, and viable cell count were significantly increased, whereas the non-viable cell count was significantly decreased ($p < 0.05$) in EAC control in comparison with the normal control and treated group (Table 1).

Hematological parameters

Hematological parameters in mice treated with β-carotene (BC), lupeol (LUP), and different combination (BC+LUP) doses were found to be reduced in comparison with the control group (Table 2).

Tissue antioxidant parameters

EAC control mice showed a significant increase in lipid peroxide levels in the liver tissue as compared to normal control animals. After treatment with β-carotene (BC) and lupeol (LUP) and the combination (BC+LUP) (10 mg/kg and 200 mg/kg, and (10+200) mg/kg b.w.), MDA content of the liver and was significantly reduced as compared to EAC control mice (Fig. 5). SOD activity was reduced as compared to the normal controls in the Liver homogenates of EAC control group. The

Table 2
Effect of BC, LUP and the combination (BC+LUP) on tumor volume, packed cell volume,% of viable and non-viable cells, mean survival time and increase in life span.

Groups	Tumor Volume (ml)	Packed cell volume (ml)	Cell count ($\times 10^7$ /ml) Viable	Non-viable	%Viable	%Non- viable	MST (Day)	ILS %
Normal control	–	–	–	–	–	–	All animals alive	
EAC Control	2.18±0.04	1.84±0.07	7.75±0.25	0.45±0.01	95.91	4.08	21	00
EAC+ BC (10 mg/kg)	1.43±0.08*	0.74±0.03*	4.30±0.15*	3.26±0.21*	62.13	36.87	28	33.33
EAC+ LUP (200 mg/kg)	1.60 ± 0.11*	0.93±0.04*	5.89±0.09*	2.83±0.48*	57.84	41.16	34	61.90
EAC+[BC (10 mg/kg)+LUP (200 mg/kg)]	0.82±0.07*	0.55±0.05*	3.57±0.29*	4.16±0.33*	44.69	54.31	38	80.95
EAC+5-FU (20 mg/kg)	0.68±0.03*	0.36±0.05*	2.76±0.06*	5.29±0.24*	35.29	63.71	45	114.28

Values are represented as mean± SEM. *p<0.05 when EAC control compared with treated groups.

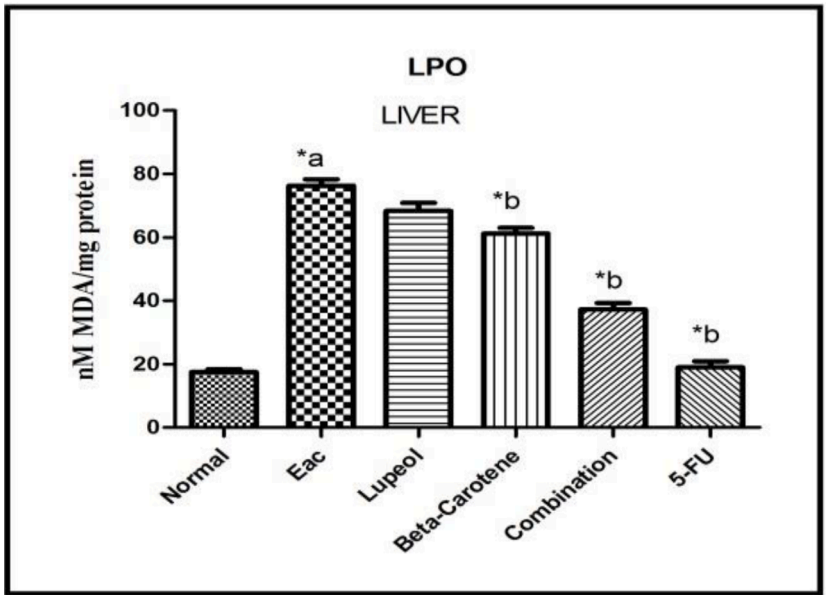

Fig. 5. Effects of different concentrations of BC, LUP and the combination (BC+LUP) on lipid peroxidation. [Values are represented as mean ± SEM. *[a]P < 0.05 EAC control compared to normal liver, *[b]P <0.05 when treated groups compared to EAC liver.].

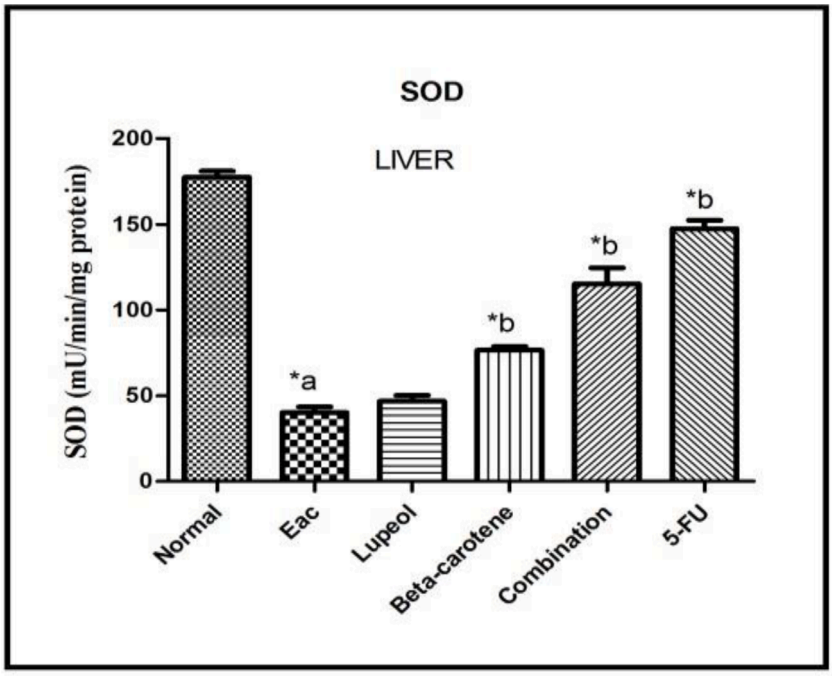

Fig. 6. Effects of different concentrations of BC, LUP and the combination (BC+LUP) on SOD. [Values are represented as mean ± SEM. *[a]P < 0.05 EAC control compared to normal liver, *[b]P <0.05 when treated groups compared to EAC liver.].

dose-dependent increase in SOD level was observed in BC, LUP and combination (BC+LUP) treatment group, as compared with the EAC control group (Fig. 6). The reduced GSH level in the liver was detected in the EAC control group. Treatment with BC and LUP and combination (BC+LUP) significantly elevated reduced GSH level in a dose-dependent manner (Fig. 7).

Discussion

The advantages of combinationdrugs are well recognized, and activity in the area has increased dramatically thanks to the opportunities provided by the enhanced understanding of the systems biology of disease (Foucquier and Guedj, 2015). The present investigation was carried out to evaluate the antitumor activity of the combination of β-carotene (BC) and lupeol (LUP) via *in vitro* assay and validating the combination in the EAC tumor bearing mice. The synergistic combination (BC+LUP) treated animals at (10+200) mg/kg significantly inhibited the tumor volume, packed cell volume, viable tumor cell count and brought back the hematological parameters to normal based on the combination index (CI) theorem of Chou-Talalay. The combination index (CI) depicts a quantitative definition of a drug combination for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) (Chou, 2010). Results of this study demonstrate that BC and LUP in combination show synergism with CI values of 0.49658 and 0.743 for the MTT assay and the Trypan blue assay, respectively. Result also showed a Fractional inhibition of Fa = 0.50–0.95, as reflected in the Fa-CI plot and isobologram. The synergism of the phytomolecules also protected the hepatic lipid peroxidation of free radical scavenging enzymes GSH as well as antioxidant enzymes in tumor-bearing mice (Haldar et al., 2011). In the EAC tumor bearing mice, a regular rapid increase in ascetic tumor volume was observed. The ascitic fluid serves as a direct nutritional source for tumor cells and rapid increase in ascetic fluid with tumor growth would be means to meet the nutritional requirement of tumor cells (Dai and Mumper, 2010). Treatment with BC, LUP, and combination (BC+LUP) decreased the tumor volume, the viable tumor cells count and increased the life span of tumor-bearing mice. The reliable criteria for judging the value of any anticancer drug is the prolongation of the life span of animals (Bhattacharya et al., 2011). It may be concluded that BC, LUP and combination (BC+LUP) increased the life span of EAC-bearing mice by decreasing the nutritional fluid volume and arresting the tumor growth.

In cancer chemotherapy, the reduction in RBC or hemoglobin percentage due to either iron deficiency or hemolytic or myelopathic conditions raises a major concern (Sundar and Hariharan, 2013). Treatment with BC, LUP, and the combination (BC+LUP) restored the hemoglobin content, RBC and WBC count. This indicated protective action of BC, LUP, and combination (BC+LUP) on the hemopoietic system (Das et al., 2012). TBARS levels were found to be higher in the cancerous tissues than those in normal tissues (Jemal et al., 2002; Sönmez et al., 2003). This emphasizes the reduction in free radical yield and MDA production resulting in a subsequent decrease in harm and damage to the cell membrane (Jemal et al., 2002) EAC control group showed elevated levels of serum parameters, *i.e.*, SGPT, SGOT, SALP, serum bilirubin and serum protein indicating impaired liver functions (Lala et al., 2022). Biochemical measurements of these parameters suggested hepatotoxicity after 9 days of inoculation with EAC. Treatment with BC, LUP, and the combination (BC+LUP) restored the elevated biochemical parameters of the order of normal range, indicating protection against tumor-induced hepatotoxicity (Fig. 8, Tables 3–5).

Conclusion

The present investigation suggests that β-carotene, lupeol, and thier combination have potent antiproliferative activity against Ehrlich ascites carcinoma *in vitro* and *in vivo*. The combination (BC+LUP) exhibited the highest inhibition in comparison to the standard inhibitor, whereas β-carotene (BC) and lupeol (LUP) showed moderate inhibitions respectively.

Combination of BC and LUP at Fa 0.5 the dose of BC was reduced 4.9 fold whereas the dose of LUP reduced 3.4 fold at a 2:1 dose combination ratio at ED50s as indicated by the dose reduction index (DRI).

The antioxidant studies elucidated that the phytochemicals having free radical scavenging activity which leads to the prevention of cancer could also be a probable reason for its anti-proliferative activity. This study suggests the probable synergistic activity of both the studied phytoconstituents. This study reveals that the effectiveness of phytomolecules in combination has been used as a promising alternative to

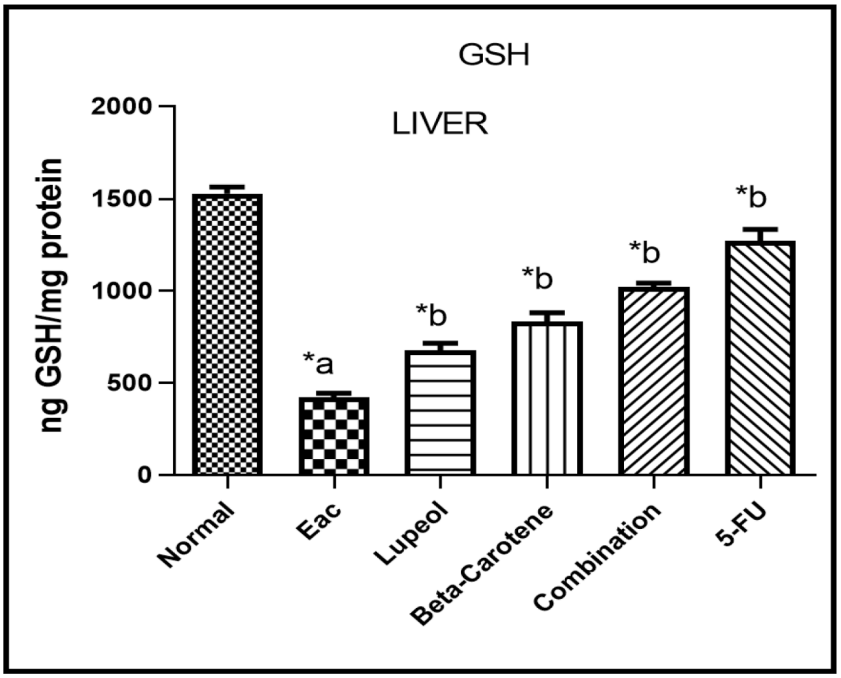

Fig. 7. Effects of different concentrations of BC, LUP and the combination (BC+LUP) on GSH. [Values are represented as mean ± SEM. *[a]P <0.05 EAC control compared to normal liver, *[b]P <0.05 when treated groups compared to EAC liver.].

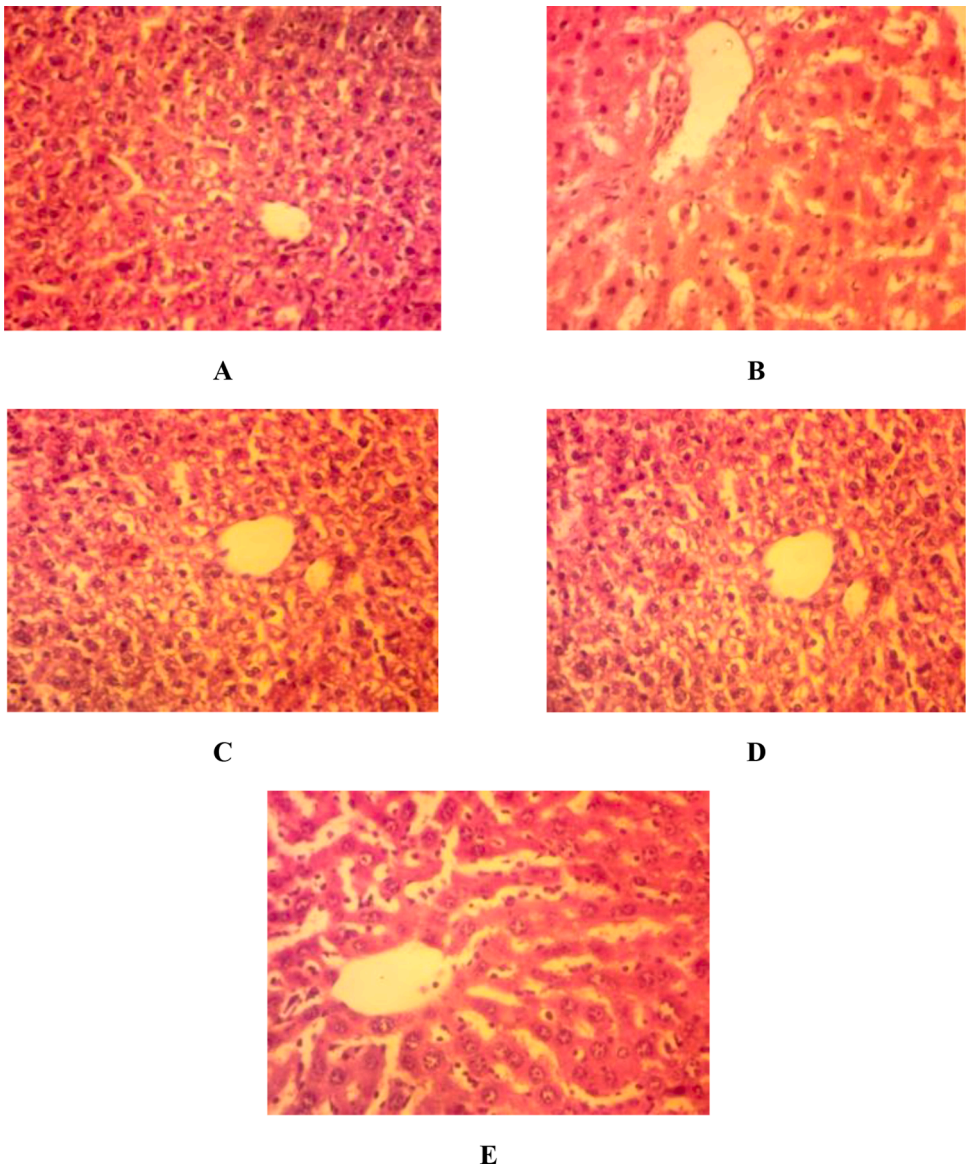

Fig. 8. Histopathological analysis of liver tissues, (A) EAC cell control mice liver. (B) EAC cell induced mice liver after treatment of standard (5-flurouracil). (C) EAC cell induced mice liver after β-carotene treatment. (D) Section of EAC cell induced mice liver after treatment of lupeol. (E) Section of EAC cell induced mice liver after treatment in fixed dose combination (BC + LUP).

Table 3
Effect of BC, LUP and the combination (BC+LUP) on hematological parameters.

Parameters	Normal control	EAC control	LUP (200 mg/kg)	BC (10 mg/kg)	[BC (10 mg/kg)+LUP (200 mg/kg)]	5-FU (20 mg/kg)
RBC (cell×10^6/mm^3)	4.78±0.20	2.94±0.11[a]	3.82±0.17	4.51±0.30[b]	5.49±0.28[b]	6.00±0.10[b]
WBC (cell×10^6/mm^3)	4.85±0.20	7.53±0.29[a]	6.55±0.27	5.81±0.23[b]	5.00±0.14[b]	4.73±0.47[b]
Hb (g/dl)	11.79±0.26	4.72±0.36[a]	6.91±0.14[b]	7.89±0.39[b]	8.84±0.37[b]	10.92±0.14[b]

Values are represented as mean ± SEM. [a]$p<0.05$ when compared to normal control.
[b] $p<0.05$ when compared with EAC control group.

treat and manage cancer. Different other experimental models including *ex vivo* studies together with the integration of molecular biological assays and techniques need to be performed for a better understanding of the probable mechanism of action.

CRediT author contribution statement

SJ, NS and MC collected the literature and conducted the experiments, AK interpreted the data and drafted the manuscript. SB and SB coordinated the work and revised the manuscript; PKM and PKH conceived and supervised the whole work. All authors read and approved the final manuscript. All data were generated in-house, and no

Table 4
Effect of BC, LUP and the combination (BC+LUP) on serum biochemical parameters.

Parameters	Normal control	EAC control	LUP (200 mg/kg)	BC (10 mg/kg)	[BC (10 mg/kg)+ LUP (200 mg/kg)]	5-FU (20 mg/kg)
TP (g/dl)	9.06 ±0.15	4.90 ±0.48*a	6.32 ±0.25*b	6.95 ±0.04*b	7.74 ±0.24*b	8.43 ±0.23*b
SGOT (IU/L)	87.00 ±2.64	144.7 ± 4.25*a	129.4 ± 4.24*b	123.0 ± 4.81*b	87.20 ±2.88*b	91.69 ±2.49*b
SGPT (IU/L)	39.00 ±3.46	99.00 ±7.63*a	74.67 ±4.97*b	62.67 ±4.80*b	48.67 ±4.05*b	36.33 ±3.75*b
SALP (KA/U)	69.33 ±3.48	132.3 ± 4.97*a	121.7 ± 2.60	89.67 ±5.60*b	78.33 ±3.75*b	68.00 ±2.88*b

Values are represented as mean± SEM. *a$p<0.05$ when compared to normal control.
*b $p<0.05$ when compared with EAC control group.

Table 5
Effect of BC, LUP and the combination (BC+LUP) on liver biochemical parameters.

Groups	Lipid peroxidation (nM MDA/mg protein)	Superoxide dismutase (mU/min/mg protein)	Reduced glutathione (ng/GSH/mg protein)
Normal control	17.48±0.90	177.4 ± 3.55	1528±34.75
EAC control	76.31±1.99*a	40.10±3.37*a	422.5 ± 22.03*a
LUP (200 mg/kg)	68.35±2.47	46.78±3.40	679.7 ± 38.27*b
BC (10 mg/kg)	61.15±1.87*b	76.53±2.12*b	832.2 ± 50.56*b
[BC (10 mg/kg)+ LUP (200 mg/kg)]	43.89±4.49*b	85.84±5.68*b	1022±19.34*b
5-FU (20 mg/kg)	18.96±1.95*b	147.4 ± 4.95*b	1273±61.93*b

Values are represented as mean± SEM. *a$p<0.05$ when compared to normal control,*b$p<0.05$ when compared with EAC control group.

paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Journal Name: Phytomedicine Plus

Article title: Combination Synergy between β -Carotene and Lupeol against Breast Adenocarcinoma *in vitro* and *in vivo* using Combination Index

Certified that all authors have seen and approved the final version of the manuscript being submitted and further warranting that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

Prof. Dr. Pallab K Haldar

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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