Analysis of the Efficacy of Novel Drug Based on Natural Plant Extract for the Treatment of Type II Diabetes

Zainab Riaz

Fall 2017-2019

NUST-2017-MS-BMS-00000206337

Supervised by

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DEPARTMENT OF BIOMEDICAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY,

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A thesis submitted in partial fulfillment of the requirements for the degree of

MS BIOMADICAL SCIENCES

Thesis Supervisor's Signature: _____

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MASTER THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: **Zainab Riaz (00000206337).**
Titled: **Analysis of the efficacy of novel drug based on natural plant extract for the treatment of type II diabetes** be accepted in partial fulfillment of the requirements for the award of degree of **MS in Biomedical Sciences.** (Grade : A)

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Abstract

Type 2 diabetes is the most common form of diabetes mellitus. The aim of this study was to develop and standardize the polyhedral formulation (granule) and checked its efficacy with regards to type 2 diabetes. The alcoholic extract of each plant *(H. antidysentrica, Prunus dulcis and Cicer arietinum*) and oleic acid was mixed and then formulated by wet granulation method. FTIR was done to investigate the presence of active compounds. Physicochemical properties of granules were evaluated, and antidiabetic potential was substantiated through inhibition of carbohydrate digestive enzyme such as α- amylase and α-glucosidase, glucose uptake activity in yeast cells and antioxidant activity. IR spectra indicated the presence of active compounds such as phenols and amines and showed the absence of interaction between drug and excipients. The dissolution profile of active pharmaceutical ingredient (API) from granules showed more 72–80% release in 2hrs. Granules exhibited better inhibition of α-amylase and α-glucosidase as on comparison with the standard drug and found to be dose dependent. The enhanced uptake of glucose was observed with decrease in drug concentration. Moreover, the DPPH scavenging activity was high (98%) at 1mg/ml. The stabilized formulation (granules) was formed and the presence of active compounds is responsible for the better antidiabetic activity by inhibiting carbohydrate digesting enzymes. Hence, it could lower the post prandial hyperglycemia and has the potential to use for the treatment of type II diabetes prior to determine the dose regime.

Key Words: *Type II diabetes, insulin, granules, herbal extract*

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CHAPTER 1: INTRODUCTION

Diabetes mellitus (DM) is a cluster of metabolic conditions identified and characterized by the occurrence of elevated level of glucose in blood caused by defects in insulin secretion and insulin resistance (1). The gradual progression of this disease affects other organs of the body and serious complications appear after the onset of diabetes. It is the most prevalent and rapid-growing worldwide problem and arise as a huge health and socioeconomic burden (2). In 2017, 425 million adults were diabetic globally and these figures are predicted to reach to 629 million by 2045 (3). According to recent survey [2nd National Diabetes Survey Pakistan 2016–17], around 27.4 million people (26.3%) people aged 20 years are living with diabetes in Pakistan (4). The proportion of type 2 diabetes is high as 90-95% are stated under this category in Pakistan (5).

Many synthetic drugs such as meglitinides, biguanides, sulfonylureas, and hiazolidinediones, have been use for treating diabetes. But these showed some side effects like weight gain, toxicity, hypoglycemia and drug resistance (6). This influences researchers to focus towards natural products for less or no side effects and low-cost future drug development strategies (7). Plants are the natural source of various bioactive phytochemicals like terpenes, flavonoids peptides, oils, phenolic acids and carbohydrates which plays an important role in managing several diseases including hypertension, cardiovascular disorder and diabetes (8).

Holarrhena antidysenterica (L.) is a well-known plant in Asia has been traditionally used to treat diarrhea, dysentery, inflammation and hemorrhoids. Recently, some properties such as anti-oxidant, anti- malarial, anti-urolithic and anti-diabetic have been revealed in HA extracts (9). The bioactive compounds of HA may have the potential to act on pancreatic beta cells, inhibiting carbohydrate metabolizing enzymes and increasing blood glucose uptake by neighboring tissues. *Prunus dulcis* (sweet almonds) has been used in many forms as a nutrient source. It contains several active phytochemicals such as tannins, flavonoids, vitamin E which have been identified to have anti-carcinogenic, anti-inflammatory, anti-oxidant and anti-diabetic properties (10). It has been reported that the alcoholic extract of almond skin reduces diabetes by inhibiting PTP1B enzyme. Protein tyrosine phosphatase 1B causes dysregulation of insulin signaling pathways so blood glucose level can be regulated by inhibiting this enzyme (11).

The presence of bioactive compounds and diphenolic phytoestrogens including isoflavanoids, *Cicer arietinum* L. (chickpea) has been investigated to be effective against diabetes

and other hormone dependent disorders (12). The proposed mechanisms in literature by which chickpea reduces insulin resistance involve inhibiting adipogenesis, enhancing glucose transporter-4 (GLUT-4) levels and positively affecting adipokines (13). Oleic acid, monounsaturated fatty acid (MUFA's), as compared to palmitic acid has the potential to improve β- cell survival, inhibit endoplasmic reticulum stress and prevent inflammation by inhibiting proinflammatory cytokines (TNF-α). It also reduces insulin resistance and provides a beneficial impact against diabetes in humans (14, 15).

The aim of this research was to design the selected plant extracts into pharmaceutically suitable formulation using holistic approach against type 2 diabetes by considering the mechanism of actions of above-mentioned plant extracts. In-vitro approaches were used to investigate the potential antidiabetic effect of our intended formulation.

CHAPTER 2: Literature Review

Diabetes mellitus is the oldest common multifactorial disease characterized by chronic hyperglycemia, insulin deficiency and insulin resistance. Type 2 diabetes (T2DM) is the most communal type among all the types of diabetes. In T2DM (also called non-insulin dependent diabetes), level of glucose remains high in blood due to the insulin production is not enough or body cells are unable to use or respond insulin efficiently. There are many factors such as sedentary life style, poor diet, genetic predisposition, obesity, age, history of gestational diabetes and other physical and environmental conditions lies behind the development of type 2 diabetes (16, 17).

It is estimated in 2017 that around 451 million individuals of 18-99 years of age were suffering with diabetes worldwide and this figure was expected to increase up to 693 million (48%) by 2045. However, 49.7% people are lies in the undiagnosed category. About 5 million people were died of this disease in 2017 (18). Type 2 diabetes is the most prevalent (85-90% of whole population) among all the types of diabetes. According to the study based on the available data, it was found that the average prevalence of T2DM in Pakistan is 11.77%. However, the prevalence ratio was different in different regions of Pakistan. The incidence of this disease is high in males (11.20%) than females (9.19%) and more frequent in urban areas as compared to rural areas (19).

Practically, it is sometimes hard to differentiate the type 2 diabetes with the other forms of diabetes. The diagnosis of T2DM is based on the glycaemia index (regular blood glucose or fating blood glucose). In addition, the amount of glycated hemoglobin (HA_{A1c}) is an optional test for identifying diabetes. In most cases, T2DM is diagnosed after the appearance of severe symptoms and the onset of complications. Polyuria, constant hunger, polydipsia, frequent infections and delay in wound healing are included in the first symptoms of type 2 diabetes (20, 21).

2.1 Insulin and insulin signaling pathway

Insulin is hormone (51 amino acid peptide) produced by the β- cell islets of pancreas as proinsulin precursor containing B- chain, c-peptide and A-chain. Insulin is formed by enzymatic cleavage of proinsulin precursor and the final form contains the A-chain (21 amino acid) and B-chain (30 amino acids) linked by disulfide bond (22). The main function of insulin is regulating blood glucose by suppressing hepatic glucose synthesis, enhancing glucose uptake in skeletal

muscles and liver cells. It is also involved in increasing lipid production in adipocytes and liver tissue by suppressing the secretion of fatty acids (23).

Insulin binds to its receptor located on the cell membrane of the target tissue in response to increased blood glucose level secreted by pancreas. This key-receptor binding initiates the insulin signal pathway by first autophosphorylate the tyrosine kinase residues leads to the phosphorylation of proteins of insulin receptor (IR1 and IR2) and different intracellular enzymes. The activation of phosphorylated mitogenic protein kinase (MAPK) and phosphatidylinosito 3-kinase (PI3-kinase) involves in the regulation of activities of insulin such as cell growth and proliferation, GLUT4 translocation to cell membrane and synthesis of proteins and lipids. The glucose enters into the cell by facilitated diffusion through glucose transporters (GLUTs) and used accordingly (24). The insulin signaling multicellular effects are shown in figure 2.1.

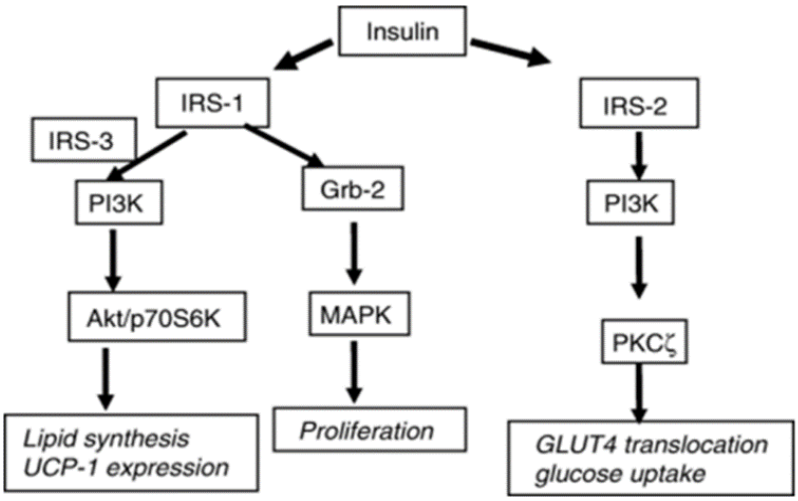

Figure 2-1: Insulin signaling multicellular effects

2.2 Pathophysiology

After intake of meal, glucose from carbohydrates hydrolysis absorbs into the blood which stimulates the insulin secretion from β-cells of pancreas. During fasting, brain tissue uses stored glucose produced by the liver cells. In addition to glucose storage, insulin also suppresses glucagon production and decreases the fatty acid concentration resulting in lowering the hepatic glucose secretion. The uptake of glucose is reduced when insulin production is insufficient or insulin is resistant to body cells receptors resulting in extracellular hyperglycemia and intracellular hypoglycemia. The process of gluconeogenesis starts due to intracellular low level of glucose that

directs fat metabolism (diabetic ketoacidosis) and lowering synthesis of proteins whereas, hyperglycemia causes osmotic dieresis and coma (figure 2-2) (25). In order to keep the normal blood glucose level or to balance the impact of insulin resistance, pancreatic β-cells continue to secrete insulin. However, the efficiency of β-cells lost with the time and firstly the postprandial glucose level and then fasting blood glucose level increases, begin to manifest diabetes (26).

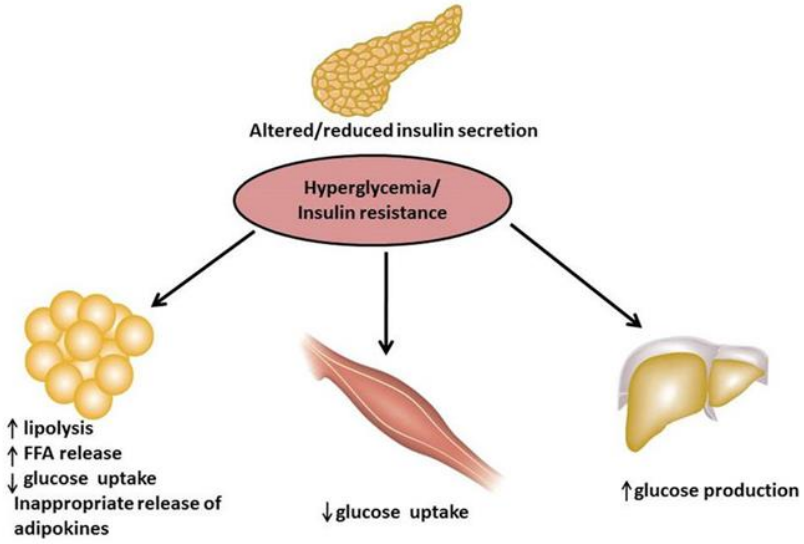

Figure 2-2: Pathophysiology of type 2 diabetes

2.3 T2DM and Insulin resistance

Insulin resistance occurs when body does not respond to the effects of insulin. The major sites of insulin resistance are muscles, liver and adipocytes as they are involved in glucose homeostasis. Insulin resistance disables insulin to inhibit glucose secretion from liver and to fuel glucose uptake by skeletal muscles and adipose tissues. Consequently, hyperglycemia, dyslipidaemia and more production of insulin (hyperinsulinaemia) state develops in the body. Defects in insulin signaling, glucose transport and accumulation of metabolic lipid intermediates in the liver and muscle cells could be the reason of insulin resistance (26).

2.4 Skeletal muscle insulin resistance

In T2DM, insulin stimulated glucose uptake is impaired due to insulin resistance in skeletal muscle. According to recent study, defects in glucose transport and glycolytic pathway plays an important role in insulin resistance in skeletal muscle (27). Concisely, the insulin signaling pathway activates by the binding of insulin to insulin receptor (IR) and further stimulation of

insulin receptor substrate (IRS) which propagates glucose uptake, triggers glucose metabolism, fatty acid production and cell growth by different cellular metabolic pathways (28).

Obese individuals are at the increased risk of acquiring type 2 diabetes. In obese state, elevated level of fatty acids and lipid deposition in skeletal muscles and liver tissues causes insulin resistance by disrupting insulin signaling pathway. High levels of circulating fatty acids are known to hinder the activation of phosphoinositide 3-kinase (PI3-kinase) action by inhibiting IRS signal activation. PI3- kinase is a kinase enzyme which is responsible for mediating cellular reactions to insulin. Inspite of increased production of insulin by pancreatic β- cells, inhibition of IRS signaling pathway due to fatty acid impairs glucose uptake in skeletal muscles probably by reducing the expression or translocation of GLUT 4 receptor (figure 2-3) (29).

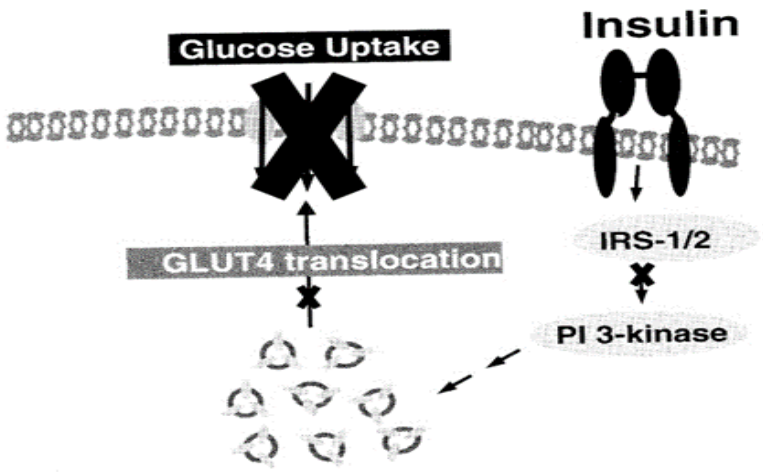

Figure 2-3: Insulin resistance in skeletal muscle

It was deducted from the results of Szendroedi et. al. study that high deposition of fatty acids causes high synthesis of diacylglycerol leading to the activation of protein kinase Cθ which subsequently causes serine phosphorylation of insulin receptor substrate (IRS). This type of phosphorylation hinders the activation of PI3-kinase and inhibits the insulin signaling pathway (30) . It is also proposed that entirely fatty acid accumulation might not be the only reason for insulin resistance in skeletal muscle. But alteration in mitochondrial size and function may also involve in the development of skeletal muscle insulin resistance (31).

2.5 Adipocytes malfunctioning and insulin resistance

The primary function of adipose tissue is to store lipid whereas adipocytes dysfunction impairs the lipid metabolism and insulin signaling pathway in skeletal muscle. In late stages of T2DM, reduced insulin production leads to β-cell damage due to hyperlipidemia. Various biologically active substances such pro-inflammatory and anti-inflammatory cytokines (also called adipokines) are released by adipose tissue. Some adipokines have positive effects and others have detrimental effects. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine which is released by the activation of NF-KB in response to increased blood glucose level in early stages of disease involved in pathogenesis of insulin resistance (32). It has been suggested that adiponectin (adipokine) may have anti-inflammatory response and may use as marker for identifying obesity related metabolic disorders. Therefore, it can be concluded from these effects of adipokines that alteration in adipokines level plays a critical role in adipocytes dysfunction and may eventually β-cell disruption in type 2 diabetes (33).

2.6 Role of oxidative stress in T2DM

During type 2 diabetes, due to continuous hyperglycemia, increase amount of reactive oxygen species (ROS) from glycosylation process are produced in the body which develops many complications. Oxidative stress is the disproportion between the production of ROS and cellular antioxidant system that causes tissue injury or damage. Glucose oxidation, proteins glycation and lipid peroxidation are the processes which are involved in the formation of free radicals (oxidative stress) causing damages to enzymatic cellular machinery and increased insulin resistant (34). Mitochondrial oxidative metabolism converts the excess oxygen to free radical oxygen specie (O.) which later transforms into hydrogen peroxide, nitric oxide and hydroxide species. Excessive ROS or RNS (reactive nitrogen species) alters the insulin signaling pathways. In normal condition, these reactive species are produced in response to insulin to perform its physiological function in different metabolic pathways. However, insulin resistance and beta cell destruction develop in case of impaired efficiency of cellular antioxidants such and negative regulation of ROS/RNS. To prevent the oxidative tissue damage, enzymatic and non-enzymatic antioxidants plays a critical role in scavenging free radical species like superoxide dismutase (SOD), glutathione and vitamin C and E (35).

2.7 Complications

The persistent high blood glucose in diabetic patients causes microvascular (neuropathy, nephropathy, and retinopathy) and macrovascular complications (hypertension, atherosclerosis, cardiovascular disease and diabetic foot disease). About three or more complications are seen in 14.3% of patients of total 57.9% of diabetes cases in USA. Peripheral cells possess load to metabolize the excess glucose from the blood to normal level. Therefore, there is increase in glycolysis and a resultant increase in metabolites like pyruvate. The metabolites overload is then involved in the generation of reactive oxygen species (ROS) and advanced glycation end products following mitochondrial dysfunction which develops diabetic complications (36). There are two groups of diabetic complications associated with type 1 and type 2 diabetes:

- Acute metabolic complications: Short term which includes diabetic ketoacidosis, coma and hyperglycemia.
- Late systemic complications: Long term which includes microangiopathy, diabetic nephropathy and neuropathy.

2.8 Current treatment approaches for T2DM

Multiple conventional treatment options are available for controlling type 2 diabetes. The rationale for drug combination therapy is to avoid the constant fluctuation of glucose level in blood. Synthetic drugs target specific enzymes in insulin/glucose metabolic pathway to improve insulin resistance, insulin deficiency and to avert the β-cell failure. Various challenges such as side effects, high cost and complication issues are still present despite of the great development in these drugs. Some examples of conventional drugs with their mechanism of action and side effects are summarized in Table 2-1 (37).

2.9 Herbal approach: An alternative treatment

Medicinal plants are gaining more interest in future drug development system for the management of type 2 diabetes. In past decades, medicinal plants have been used as therapeutic agent for the treatment of many diseases. The presence of various therapeutically vital phytochemicals, fewer side effects, low cost and ease of availability make the herbal formulations are the cream choice for the pharmaceutical industry. Various plants have been effectively examined against type 2 diabetes in STZ- induces animal models (38).

Table 2-1: Some therapeutic agents for the treatment of type 2 diabetes

Drug class	Drug examples	Action	Possible side effects
Meglitinides	Nateglinide Repaglinide	Increase insulin secretion	Hypoglycaemia
Sulfonylureas	Glyburide Gliclazide Glimepiride	Stimulate insulin secretion by acting on pancreatic β-cell	Weight loss Hypoglycaemia
Biguanides	Metformin	Inhibit hepatic glucose secretion	Lactic acid formation, nausea and vomiting
Thiazolidinediones	Pioglitazone Rosiglitazone	Improve insulin sensitivity and glucose uptake and prevent hepatic glucose release	Liver and cardiovascular disease.
Alpha-glucosidase inhibitors	Acarbose Miglitol	Delays the carbohydrates breakdown and glucose absorption	Gastrointestinal tract infection
Glucagon like peptide-1 agonist	Exenatide Dulaglutide Liraglutide	Increase insulin production and decrease glucose production from liver	Nausea, vomiting and diarrhoea
Dipeptidyl peptidase-4 (DPP-4) inhibitor	Sexagliptine Linagliptine	Prevent hepatic glucose release and increase insulin production	Pancreas inflammation, upper respiratory tract (UTI) infection
Sodium glucose cotransporter 2 (SGLT-2) inhibitor	Depagliflozine	Inhibit glucose reabsorption and excrete glucose through urine	Hypoglycaemia, UTI infection

2.9.1 *Holarrhena antidysentrica* (L.)

Holarrhena antidysentrica (HA) is commonly known as Kutja and is found in tropical and subtropical areas of Asia and Africa. HA seeds (indrayava), leaves and bark have been used in many Ayurvedic preparations for the treatment of diarrhea, dysentery fever, and eczema (9). Recently, certain other pharmacological properties of HA have been identified such as anti-diabetic and anti-hyperlipidemic. The ethanol extract of HA seeds has been reported useful at dosage of 300mg/kg against type 2 diabetes (39). The methanolic extract of *H.antidysentrica* seeds has been proved advantageous against STZ-induced animal model. Quercetin is the main active agent found in the alcoholic extract of HA seeds which has antidiabetic property (40). In another study, the antidiabetic activity of ethanolic extract of HA leaves has been revealed by administering (50mg/kg body weight dose) for 21 successive days in STZ induced rats (41).

The anti-diarrheal property of aqueous extract of HA seeds was studied against diarrheal responsible pathogens like *S.aureus, E.coli, S.typhi* and *Shigella* . According to Yang et al. (2012), five different steroidal alkoiloids (conessine, connesimin, conimin, iso-connesine and conimin) were identified by chromatographic fractionation of alcoholic extract of HA seeds (42). It was depicted that the active ingredient of *H.antidysentrica* seed extract inhibits alpha-glycosidase and delays the carbohydrate conversion and absorption of glucose from intestine. A decrease in glycosylated hemoglobin was observed when administered the HA seed extract for 48 frequent days to an old woman aged 65 years, showed significant effect against type 2 diabetes (43). These properties of *H.antidysentrica* plant have provided the better opportunity for pharmacological industry to develop a drug for the management of diabetes.

2.9.2 *Cicer arietinum* L. (Chickpea)

Cicer arietinum (chickpea) belongs to leguminosae family and most abundantly found in Pakistan and India. It is a rich source of vitamins, and minerals and has become the third most important legume worldwide. In past decades, it has been used in Uighur and Chinese medicine for the treatment of hyperglycemia, hyperlipemia, coprostasis and bronchitis. It is also used as a calcium supplement and blood enrichment diet and considered as the superlative food for diabetic patients. The prevalence of cardiovascular disease and diabetes is far less in Uighur people despite of intake of high fat and calories diet. Later, it was found by WHO that they consume chickpea mixed balanced diet that prevents them from the development of such diseases. It was revealed that legumes isoflavonoids and diphenolic phytoestrogens (dietary based hormones) which may involve in protecting hormone dependent diseases (44).

The methanolic extract of chickpea was investigated to highly effective against alloxan induced diabetes in rats in improving lipid profile, body weight ad regeneration of pancreatic beta cells (45). Biochanin A and genistein are the bioactive isoflavone components in chickpea which are reported to have hypoglycaemic activity by lowering production of serum hepatic glycogen level, serum insulin, ad improving glucose tolerance in type 2 diabetic rat model. In another study, it was reported that better antidiabetic activity was observed with the structural modification of biochanin A and genistein, and formononetin and their combinations as compared to individual compounds (46). Recently, it was found that the isoflavones in chickpea effects the insulin resistance through the prevention of 3T3-L1 adipocytes differentiation (3T3-L1). Bhagyawant et

al purified the lectin from chickpea y ion exchange chromatography and demonstrated that chickpea lectin has the potential activity against diabetes due to its antioxidant character and inhibition of angiotensin converting enzyme (ACE-I), a-amylase and a-glucosidase enzymes (47).

2.9.3 *Prunus dulcis* (Almonds)

Prunus dulcis (sweet almonds) belongs to a Rosaceae family and is the most-trendy tree worldwide. It has been consumed in raw form, used in processed food and considered as the nutrient source in traditional diet. The presence of phenolic compounds (phenols, flavonoids and tannins) and vitamin E in almonds are responsible for its antioxidants and other health promoting activities. Cardiovascular diseases, anticarcinogenic properties, blood pressure, anti-inflammatory issues can be control by the daily moderate use of almond nuts (10). It has been described that the ethanolic extract of almonds acts as PTP-1 B inhibitor and can be the used in drug formulations for type 2 diabetes. Protein tyrosine phosphatase 1B (PTP-1 B) is an enzyme which negative regulates the insulin pathway thus its inhibition plays a beneficial role in treating type 2 diabetes (11).

2.9.4 Oleic acid

Unsaturated fatty acids especially monounsaturated fatty acids (MUFAs) are gaining an increasing interest due to their health benefits as compared to saturated fatty acids (SFAs). Excess of free fatty acids (FFA) causes lipotoxicity and palmitic acid is the rich saturated fatty acid present in serum and diet. Palmitic acid has been investigated to activate lipotoxicity in muscular cells, hepatocytes and pancreatic β-cell. An improvement was found in the condition of type 2 diabetes with the consumption of oleic acid rich food (peanuts or olive oil). It was investigated that oleic acid prevents the negative impact of proinflammatory cytokine by inhibits the TNF-α (14).

CHAPTER 3: Materials and Methods

3.1 Materials

The *Holarrhena antidysenterica* seeds were purchased locally, washed, dried and were grinded into powder. Roasted *Cicer arietimnum* and almonds were obtained from local market and were grinded into fine powder. Aerosil 200 pharma was procured from Brother Enterprises, Islamabad. Methanol and ethanol were obtained from Sigma Aldrich and used as a solvent for extract preparation. All materials used were of analytical grade.

3.2 Methods

3.2.1 Preparation of plant extracts

The 90% ethanolic extract *Holarrhena antidysenterica* (HA) seeds powder was prepared under stirring overnight at 37^0C (39). The flour of *Cicer arietinumm* seeds was extracted in 75% methanol overnight and then was centrifuged at 3000 rpm for 10 min to collect the supernatant. The pellet was extracted again overnight and was centrifuged under same conditions and both supernatants were mixed. The almonds seeds powder was extracted with 70% ethanol for 48 hours at 37^0C. The respective solvents were removed separately using rotary evaporator and then vacuum dried at 37^0C for 24 hrs. The obtained extracts were stored at 0-4^0C.

3.2.2 Preparation of mixture

The mixture of the obtained alcoholic extracts of HA, chickpea, almonds and oleic acid was prepared by amalgamation them into 1:1 ratio. This viscous sticky polyherbal mixture was then lyophilized to get crystalline powder. This highly hygroscopic powder was stored at vacuum desiccator for further use.

3.2.3 Preparation of granules formulation

The granules of HA, chickpea, almonds and oleic acid mixture were prepared by wet granulation method (48) mentioned in Table 3-1. The required amount of preseived aerosil was mixed with active pharmaceutical ingredient (API) to overcome the hygroscopicity of the plant extracts. PVP K30 in isopropyl alcohol was used as binder solution. The granules were obtained by passing the wet mass through sieve # 40. The obtained granules were air dried at room

temperature. The dried granules were lubricated with primogel, Magnesium stearate and talcum (2.5% of total formulation). Finally, the granule blend was properly mixed for 15minutes.

Table 3-1: Formulation of granules

Ingredients	Amount (mg)	%age
API	500	62.18
Aerosil	290	36
PVP-K30	2	0.24
Primogel	3	0.37
Magnesium stearate	3	0.37
Talc	6	0.74
Isopropyl alcohol	Q. S	-
Total	804	99.9

3.2.4 FTIR

Fourier Transform Infrared Spectroscopy of individual plant extracts, mixture and granules was done by using Perkin Elmer 100 FT-IR spectrometer. KBr discs were prepared by hydraulic press and semi-liquid samples were placed over it. The pallet was subjected in light path and spectrum was obtained in the wavelength range of 4000-400 cm-1

3.2.5 Evaluation of granules

Physicochemical properties of granules were evaluated by using standard methods (49)

3.2.6 Angle of repose (θ)

Angle of repose was measured by funnel method to determine the friction forces in granules. Granule formulation was streamed through a funnel fixed at 1cm height (h). The radius (r) of the pile was assessed and applied to calculate the angle of repose using following formula:

$$\theta = tan^{-1}(h/r) \quad (3.1)$$

3.2.7 Bulk density (δ_b)

Bulk density was estimated by flowing the correctly weighed amount (M) of the granule blend into measuring cylinder. The volume obtained is called bulk volume (V_b) and the density was measured by the given formula:

$$\delta_b = M/V_b \quad (3.2)$$

3.2.8 Tapped density (δ_t)

The bulk mass of the granules was tapped 500 times and the tapped density was calculated by:

$$\delta_t = {}^{M}/_{V_t} \tag{3.3}$$

3.2.9 Carr's compressibility index

Carr's index shows the compressibility of granules. It was determined by the given formula:

$$Carr's\ Index = \left[\left({}^{\delta_t - \delta_b}/_{\delta_t}\right)\right] * 100 \tag{3.4}$$

3.2.10 Hausner's ratio (*H*)

Hausner's ratio was calculated by dividing tapped density to bulk density:

$$H = {}^{\delta_t}/_{\delta_b} \tag{3.5}$$

3.2.11 Particle size distribution

Particle size distribution study was performed by sieve method. Particle size has significant effects on content uniformity, dissolution rate and stability. Set of sieves with mesh sizes of 246µm, 175 µm, 147 µm, 125 µm and 74 µm were arranged in sieve shaker. The collector pan was placed under the last smallest sieve. A weighed amount of granules was put on the upper sieve and shaker was agitated for 5 min. After shaking, percentage of the sample retained on each sieve was calculated (50).

3.2.12 In vitro dissolution study

In vitro dissolution study of the prepared granules was performed using dialysis membrane (13000MW) in USP Type 2 paddle apparatus at 50 rpm at 370Cfor 2 hrs. The dialysis tube was filled with 1mg of sample and placed in dissolution beaker containing 500ml PBS (6.8). An aliquot of sample was removed at specific time intervals and the same amount of sample was replaced with fresh prewarmed PBS. The absorbance of each sample was measured by UV-Vis

spectrophotometry at 320nm and concentration was calculated by means of standard curve. All study was done in triplicates (51).

3.2.13 Glucose uptake assay by yeast cell

1% of yeast suspension was prepared by dissolving commercial baker's yeast in distilled water and was incubated overnight at room temperature. The yeast suspension was centrifuged at 4200 rpm for 5min on the next day. The pallet was washed with distilled water repeatedly till the clear supernatant was attained. 10% w//v of the yeast cell suspension was prepared by using clear supernatant. Samples (mixture, granules and metformin) of different concentrations were prepared in DMSO and incubated with 1ml of 5mM glucose for 10min at 370C. 100µl of yeast suspension was added in the reaction mixture to start the reaction. After 60 min of incubation at 370C, centrifugation was done at 3800rpm for 5min and the amount of glucose in supernatant was measured at 520nm by UV-Vis spectrophotometer. The effective concentration (EC50) was obtained from the percentage activity curve. The (%) increase in glucose uptake was calculated according to formula (52):

$$\%\ increase\ in\ glucose\ uptale = \left[\frac{(Abs.of\ control - Abs.of\ sample)}{Abs.of\ control} * 100\right] \qquad (3.6)$$

Metformin was used as the standard drug and control contains all reagents except sample.

3.2.14 α-amylase inhibition assay

Alpha amylase inhibition activity was measured according to Akoro et al (2017) with slight modifications using microplate reader (53). 15µl PBS (pH 6.8) was added in all wells of 96-well plate. 25µl of 0.14 U/ml enzyme and 1-4mg/ml samples were pre-incubated at 370C for 10min. The reaction was started with the addition of 40µl starch (2mg/ml) and incubated for 30min at 370C. 20µl 1M HCL was added to stop the reaction and 90µl iodine reagent was added. The change in color was observed and absorbance was measured at 620nm after incubation. Control contained all the reagents expect inhibitor/sample. Acarbose was used as the standard or positive control. The IC50 values were derived from the percentage inhibition plot. IC50 defines as the concentration at which inhibitor shows 50% of its inhibition activity. The percentage inhibition was calculated according to formula (54):

$$\% \, inhibition = \left[\frac{(A_c - A_s)}{A_c} * 100\right] \quad (3.7)$$

where A_c was the absorbance of control without inhibitor and As was the absorbance of sample.

3.2.15 α -glucosidase inhibition

The inhibition activity of alpha-glucosidase was optimized using the method described by Sundar et. al. (2015) (55).The reaction mixture contained 25 µl of 20mM p-nitrophenyl-α-D-glucopyranoside (pNPG), 69 µl PBS (pH 6.8), 5 µl sample of different concentration and 5 µl enzyme (0.637 U). The reaction was incubated at 370 for 30 minutes. NaHCO3 100 µl (0.5 mM) was used to stop the reaction and change in absorbance was measured at 405 nm by using microplate reader. The inhibition activity was measured by using following formula:

$$\% \, inhibition = \left[\frac{(A_c - A_s)}{A_c} * 100\right] \quad (3.8)$$

where A_c was the absorbance of control without inhibitor and A_s was the absorbance of sample. Acarbose was used as positive control.

3.2.16 Antioxidant activity

DPPH radical-scavenging method was used to quantify the antioxidant activity of granules (56). The freshly prepared 2ml DPPH methanolic solution (0.1 mM) was incubated with 2 mL of each sample (0.001 – 1mg/mL) for 30min in dark conditions. UV spectrophotometer was used analyze the absorbance at 517nm. Ascorbic acid was used as standard. The scavenging capability was calculated by:

$$\% \, radidal \, scavenging \, activity = \left[\frac{(Abs.of \, control - Abs.of \, sample)}{Abs.of \, control} * 100\right] \quad (3.9)$$

3.2.17 Statistical analysis

All experiments were conducted in triplicates and all the results were stated as mean ± standard error of mean (SEM).

CHAPTER 4: Results and Discussion

4.1 FTIR

The FTIR spectra of pure extracts (*H. antidysentrica*, almonds and chickpea), oleic acid, mixture and granules are shown in Figure 4-1 (a) and (b). The frequency region or band in each sample manifests the specific functional groups of compounds (Table 4-1). The peak observed in spectral region between 3400-3200 cm-1 and 2800-3000 cm-1 indicates the presence of OH groups (alcohols and phenols) and methylene CH symmetric/asymmetric vibrations in all samples respectively. In IR range of 1600-1800 cm-1, the specific peaks at 1623 cm-1, 1626 cm-1, 1742 cm-1 and 1710 cm-1 represents the carbonyl frequencies of amides and ester functional group in almonds [26], oleic acid (57) , *H. antidysentrica* (58), and chickpea (59) respectively. The area lies in 1000-1200 cm-1 corresponds to the C-N stretch of primary amine compounds. These characteristic peaks of different functional groups confirm the presence of particular relative active compounds (isoflavones, amines and phenolic etc) in all samples (60),

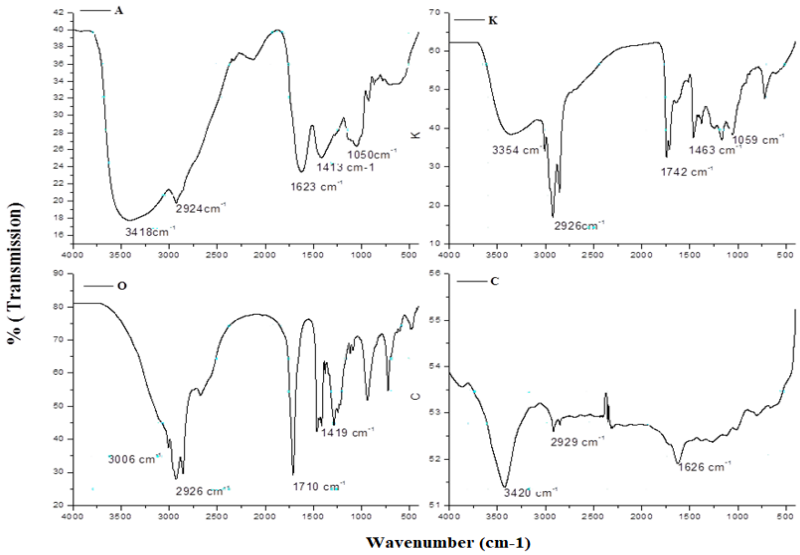

Figure 4-1: FTIR spectra of (a) almonds, *H. antidysentrica*, oleic acid and chickpea; (b) mixture ad granules.

In granule preparation, certain excipients were added to stabilize the formulation. Drug-excipient compatibility can be checked from the spectra of mixture and granule (Fig 5 (b)). No modifications in wavenumber are observed which satisfies the inert behavior of excipients added.

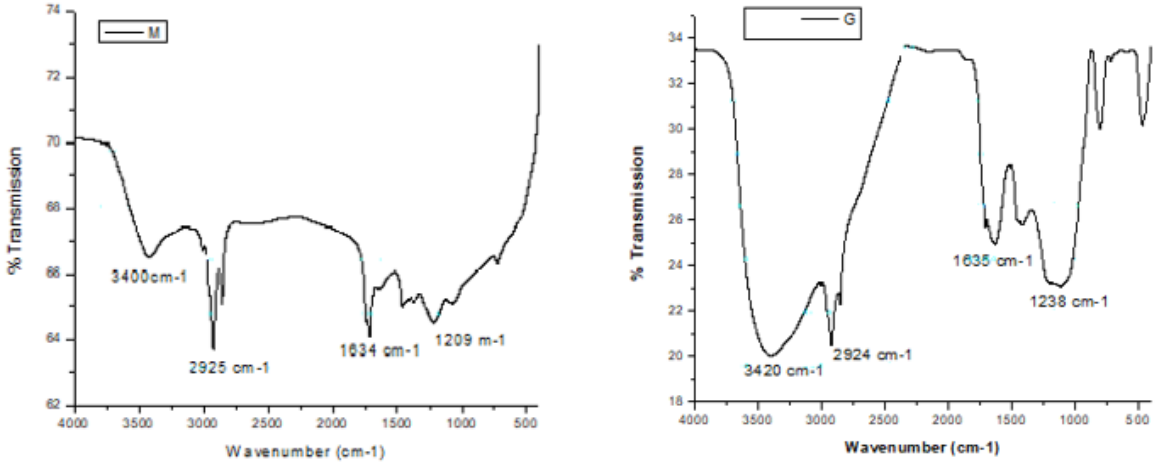

Figure 4-2: FTIR spectra of mixture and granules

Table 4-1: FTIR peak ranges and their respective assigned functional groups of compounds.

IR range	Peak origin
3200-3400 cm^{-1}	O-H group in alcohols and phenols
2800-3000 cm^{-1}	CH stretching of alkane or alkyl groups
1600-1800 cm^{-1}	Carbonyl compounds of amides and esters
1000-1200 cm^{-1}	Primary amines

4.2 Preparation and evaluation of granules

Wet granulation technique was used to formulate the granules. The physicochemical properties of granules were evaluated by angle of repose (θ), bulk density, tapped density, Carr's compressibility index and Hausner's ratio (Table 4-2). According to USP 30 standard, the angle of repose in 25– 30 range shows excellent flow properties whereas an angle > 400 indicates poor properties (61) The angle of repose result (240) showed excellent flow properties of granules. The significantly close values of bulk and tapped densities were used to measure Carr's index and Hausner's ratio. The excellent character of Carr's index and Hausner's ratio ranges from ≤ 10 and 1.00-1.11 respectively. The prepared granules show the excellent flow properties with respect to defined standard ranges.

Table 4-2: Evaluation of flow properties of granules compared it with USP 30 standard. The data points are representing as mean ± SEM, n=3

Flow properties	Sample values	USP 30 Standard
Angle of repose	24±0.03	25-30
Bulk density	0.5±0.006	
Tapped density	0.52±0.002	
Carr's index	2.42±0.04	≤ 10
Hausner's ratio	1.02±0.002	1.00-1.11

4.3 Particle size distribution

Sieve method was used to determine the particle size distribution. Size frequency plot shows that 44.6% granules are greater than 246µm whereas 4.8% are <74 µm in size of total sample (Figure 4-3 (a)). Cumulative size distribution data represents the percentage number of samples are less than the stated size (62). Figure 4-3 (b) shows that the median diameter (D50) is 200□m which defines that 50% of particles are less than the 200µm size. There should be 15% of fines present in granular blend to obtain appropriate powder flow and compaction. Particle size distribution poses the significant effects on dissolution rate, content uniformity and stability. Smaller the particle size improves the drug release rate resulting in augmentation of drug solubility [33].

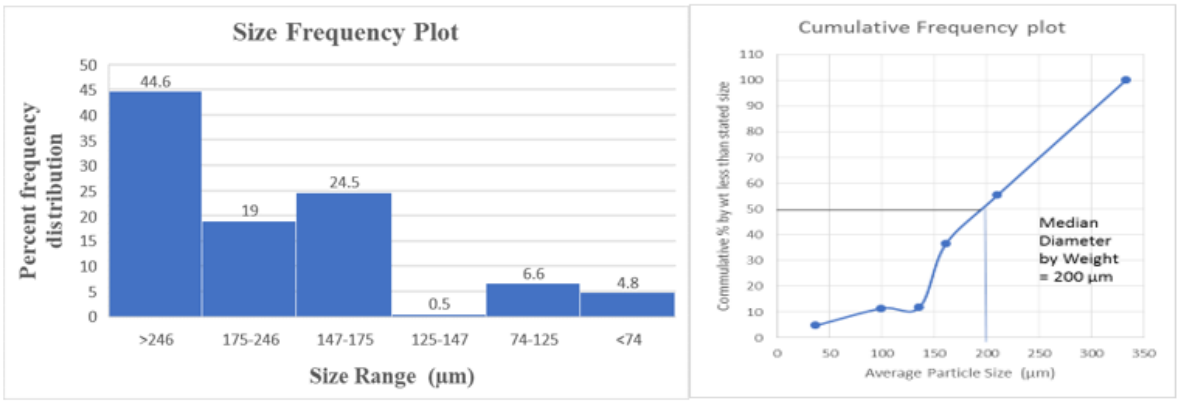

Figure 4-3: (a)Size frequency distribution of granules (b) cumulative frequency plot.

4.4 In-vitro dissolution result

In-vitro dissolution test through dialysis membrane was designed using standard conditions. The dialysis tube mimics the intestinal membrane which assisted the drug release.

Dissolution profile of polyherbal drug is shown in Figure 4-4. The result explains the immediate release of drug from the granules as 56-60% drug was released in 60min and approximately 80% was released in 2hrs. During release studies, it was noticed that the drug-carrier system dissolves gradually when contacting with the phosphate buffer saline medium. Since the smaller particle size provides greater surface area and enhances the drug solubility which results in better dissolution rate (63). However, the expected in-vivo studies describe the bioavailability of drug via dissolution profile.

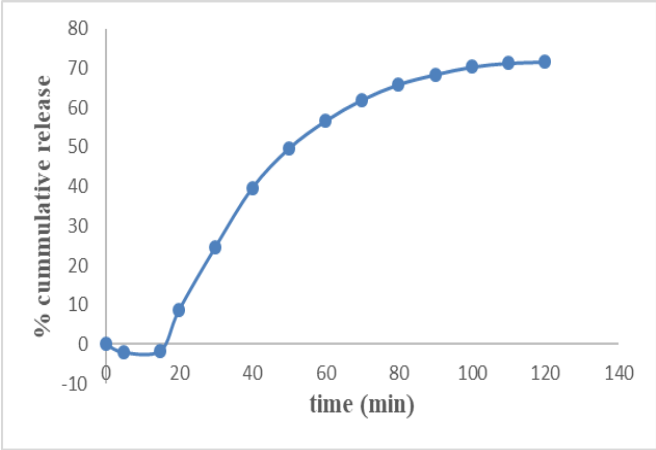

Figure 4-4: In vitro drug release profile of polyherbal based granules

4.5 Glucose uptake assay by yeast cell

Figure 4-5 represents the glucose uptake activity of samples (mixture, granules and metformin) by yeast cell. This method measures the remaining concentration of glucose in medium. A percentage glucose uptake increases with increase in concentration of metformin was observed. However, an opposite and strange behavior was observed in case of mixture and granules i.e. percentage glucose uptake decreases with increase in concentration. At 1mg/ml, mixture and granules showed 53% ± 0.004 and 12% ± 0.007 glucose uptakes respectively. Fifty percent effective concentration (EC50) determines the concentration at which half maximum drug responses. Table 4-3 shows the EC50 values of granules, mixture and metformin.

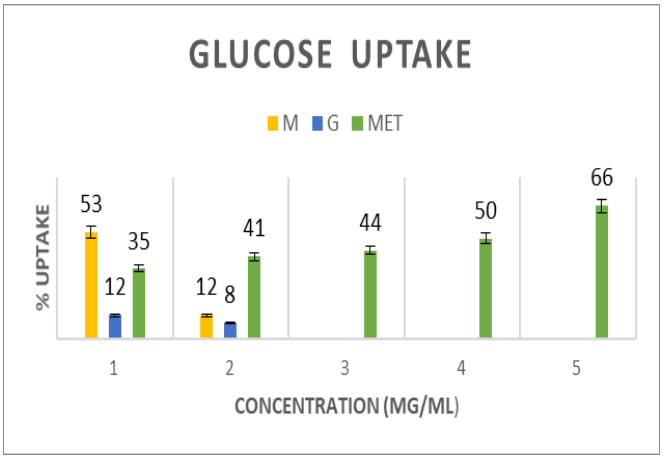

Figure 4-5: Glucose uptake by yeast cell in 5mM concentration of glucose in the presence of mixture, granules and metformin. The data points are representing as mean ± SEM, n=3

Table 4-3: EC50 values (mg/ml) of granules, mixture and metformin. The data points are representing as mean ± SEM, n=3

Sample	EC50 (mg/ml)
Granule	0.41± 0.03
Mixture	0.6 ± 0.003
Metformin	0.08 ± 0.002

The antidiabetic activity of various natural plants has been evaluated by yeast cell glucose uptake method. Facilitated diffusion process may be involved in transporting glucose across the yeast cell. The cellular glucose concentration decreases when most of it is used or converted into other metabolites. This condition favors the high uptake of glucose inside the cell (63). In present scenario, there are chances that the presence of plant extract-based granules facilitates the high glucose diffusion inside the cell with down the concentration gradient. This may be due to the reason that the antimicrobial property of natural extracts at high concentration retards the growth or may destroy the yeast cell (64). *H. antidysentrica* was reported to enhance the glucose diffusion in neighboring cells via sodium dependent glucose absorption (65).

4.6 Alpha amylase

Starch-iodine method was used to determine percentage inhibition activity of herbal mixture, granules and acarbose. The results in figure 4-6 show that the percentage inhibition

increases with dose dependent concentration. Granules show more inhibition activity ranged from 44% ± 0.008 - 76% ± 0.002 for 1.0 - 4.0 mg/ml as compared to mixture (34% ± 0.004 - 67% ± 0.004) which is approximately equivalent to acarbose activity ranged from 53% ± 0.006 - 79% ± 0.0003 at higher concentration. The IC50 was 1.47, 1.86 and 0.83mg/ml of granules, mixture and acarbose respectively as shown in Table 4-4. Overall, it can be concluded that the granules containing different plant extracts mixture exhibited better inhibition activity against amylase as compared to acarbose.

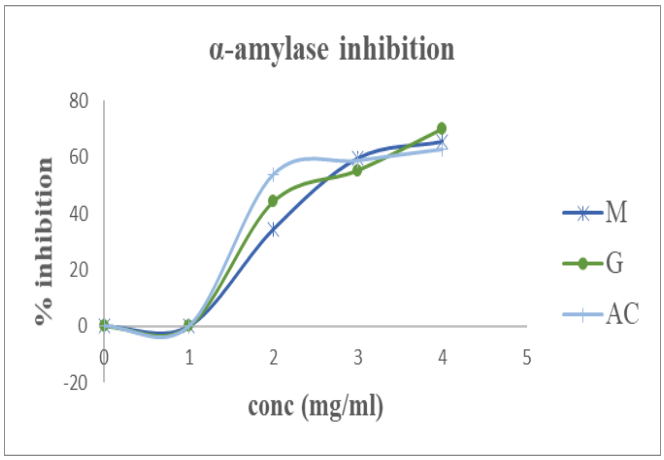

Figure 4-6: Percentage inhibition of α-amylase activity of plant extracts mixture, granules and acarbose.

Table 4-4: IC50 values (mg/ml) of granules, mixture and acarbose. The data points are representing as mean ± SEM,n=3

Sample	IC50 (mg/ml)	
	α-amylase	α-glucosidase
Granule	1.47 ± 0.18	4.69 ± 0.01
Mixture	1.86 ± 0.102	5.34 ± 0.04
Acarbose	0.83 ± 0.007	2.78 ± 0.106

α-amylase produced in saliva and begins the digestion of food carbohydrates in mouth and continued in intestine. Acarbose is the standard drug possess greatest α-amylase inhibition activity and delays the hydrolysis of starch into oligosaccharides and disaccharides (66) Studies have shown that some specific phenols, steroids, isoflavones and other active compounds extracted from

the plant sources were stated to have potential antidiabetic activity. I and oleic acid were reported to exhibit good potency of α-amylase inhibition activity (67, 68).

4.7 α- glucosidase inhibition activity

The ability of active compounds from mixture, granules and acarbose to inhibit alpha glucosidase was determined. Figure 4-7 shows the graphical representation of α-glucosidase inhibition in dose dependent way from 1.0 – 4.0 mg/ml concentration. At maximum concentration (4mg/ml), 34.5% ± 0.014, 31% ± 0.013 and 66% ± 0.008 of enzyme inhibition activity was observed for mixture, granules and acarbose respectively. It was noticed that the percentage inhibition activity of mixture and granules was less potent when compared to acarbose. Acarbose is a strong competitive inhibitor against α-glucosidase which was used as a positive control.

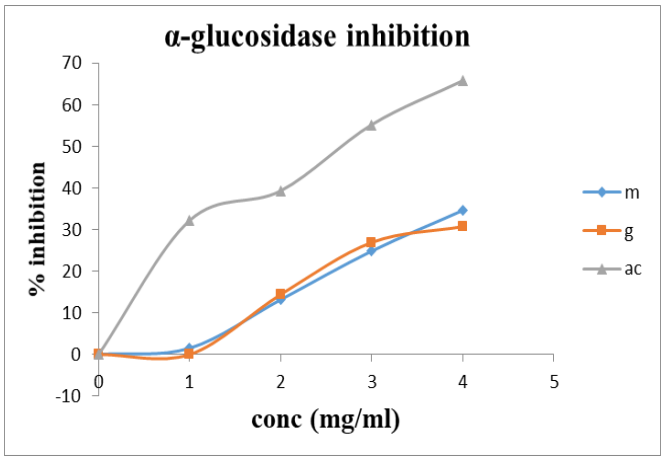

Figure 4-7: Percentage inhibition of α-glucosidase activity of plant extracts mixture, granules and acarbose

In case of IC50 values (Table 4-4), the concentration at which inhibitor shows 50 % of its inhibition response, the mixture and granules show less effective (IC50: 5.34 ± 0.04 and 4.69 ± 0.01 mg/ml) inhibitory α-glucosidase activity as compared to acarbose (IC50: 2.78 ± 0.106 mg/ml). However, Deepak et al (2013) and Chun-Han et al (2013) reported the strongest effect of *H. antidysentrica* and oleic acid against α-glucosidase enzyme (65, 67). The α-glucosidase inhibition activity of *H. antidysentrica* and oleic acid may be compromised by combining multicomponent plant extracts (*H. antidysentrica*, almonds, chickpea and oleic acid) in single drug. The in vitro enzyme results may be different from in vivo hence, the designed polyherbal based granules warrant additional evaluation of enzyme inhibition in in vivo studies. Though, the

presence of α-glucosidase inhibition activity in this study samples, which retards the digestion of polysaccharides and oligosaccharides, suggested the effective therapeutic effect by decreasing the absorption of glucose in the blood from intestine. Thus, the inhibition of α-glucosidase plays an important role to control the hyperglycemia after intake of meal for the treatment of type 2 diabetes.

4.8 Antioxidant activity

Radical scavenging activity of mixture and granules was assessed by DPPH radical scavenging method (figure 4-8). A dose-response pattern (0.001-1 mg/ml) of DPPH confirms the free radical scavenging activity. At higher concentration granules showed 98% scavenging effect equal to standard (ascorbic acid). DPPH accepts electron from antioxidant agents and changes its color from violet to yellow which was measured spectrophotometry at 517nm. Sujan et al., (2011) has reported the antioxidant activity of alcoholic extract of *H. antidysentrica* due to the presence of phenolic content (69). In addition, it has been investigated that the phenolic compounds in alcoholic extracts of chickpea seeds and almond shell are responsible for their radical scavenging properties (70, 71). Oxidative stress is one of the major abnormalities that would trigger the other complications. So, the presence of this scavenging activity may able to tackle the reactive oxygen species (ROS) in the cells and tissues of diabetics.

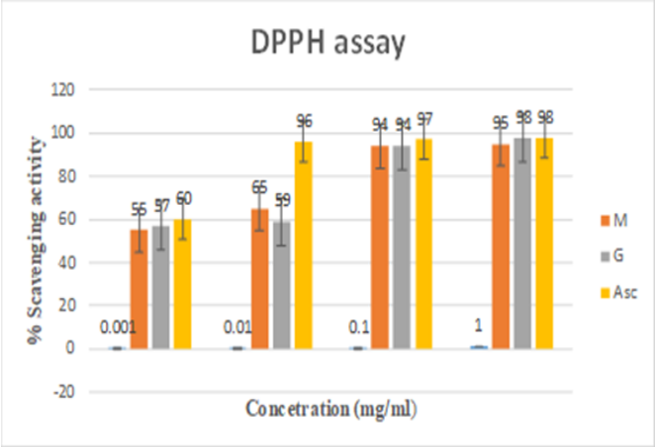

Figure 4-8: DPPH radical scavenging activity of mixture, granules and ascorbic acid. The data points are represented as mean ± SEM, n=3

CHAPTER 5: Conclusion and Future Recommendations

5.1 Conclusion

Herbal formulations are considered more effective as compared to allopathic medicines due to their less side effects, low cost, higher safety and availability. The therapeutic tactics of lowering the postprandial hyperglycemia can be attained through the inhibition of carbohydrate hydrolyzing enzymes (α-amylase and α-glucosidase). It is concluded from the results that the mixture of selected plant extracts is successfully formulated into stabilized form and showed better outcomes as compared to crude mixture. The present study validates the antidiabetic activity of the formulation (granules) based on the selected plants (*H. antidysentrica, Prunus dulcis, Cicer arietinum*) and oleic acid. This study demonstrates the possible mechanisms of regulating blood glucose level by inhibiting α-amylase and α-glucosidase, enhancing glucose uptake and exhibiting good antioxidant activity. Thus, this research concluded that the designed stable formulation has the potential to use as antidiabetic regimen.

5.2 Recommendations

- *In vitro and in vivo* studies must be conducted for determination of its multiple actual targets (PTP 1B inhibition activity by almonds and inhibition of TNF-α activity by oleic acid) and confirming its antidiabetic activity.
- The prescreening analysis for identification and characterization of extracted active compounds are recommended.

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