Research Article

In Vitro Investigation and Evaluation of Novel Drug Based on Polyherbal Extract against Type 2 Diabetes

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Background/Aim. Type 2 diabetes is the most common form of diabetes mellitus. The aim of this study was to develop and standardize the polyherbal formulation (granule) and check its efficacy with regard to type 2 diabetes.

Methods. 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 (α-amylase and α-glucosidase), glucose uptake activity in yeast cells, and antioxidant activity.

Results. IR spectra indicated the presence of active compounds by showing the characteristic peaks of phenols and amines. The FTIR results also showed no interaction between drug and excipients. The prepared granules exhibited excellent flow properties according to USP 30. The dissolution profile of active pharmaceutical ingredient (API) from granules showed 72–80% release in 2 hrs. Granules exhibited better inhibition of α-amylase and α-glucosidase as in comparison with the standard drug and found to be dose-dependent. The enhanced uptake of glucose was observed with a decrease in drug concentration. Moreover, the DPPH scavenging activity was high (98%) at 1 mg/ml. Conclusion. The stabilized formulation (granules) was formed and the presence of active compounds is responsible for better antidiabetic activity by inhibiting carbohydrate-digesting enzymes. Hence, it could lower the postprandial hyperglycemia and has the potential to be used for the treatment of type II diabetes after determining the dose regime.

1. Introduction

Diabetes mellitus (DM) is a cluster of metabolic conditions identified and characterized by the occurrence of elevated levels of glucose in the 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 629 million by 2045. [3]. According to a recent survey [2nd National Diabetes Survey Pakistan 2016–17], around 27.4 million people (26.3%) aged 20 years are living with diabetes in Pakistan [4]. The proportion of type 2 diabetes is high as 90–95% is stated under this category in Pakistan [5].

Many synthetic drugs such as meglitinides, biguanides, sulfonylureas, and thiazolidinediones have been used 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 play an important role in managing several diseases including hypertension, cardiovascular disorder, and diabetes [8].

Holarrhena antidysenterica (L.) is a well-known plant in Asia that has been traditionally used to treat diarrhea, dysen-
tery, 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 (Indrayava). Prunus dulcis (sweet almonds) has been used in many forms as a nutrient source. It contains several active phytochemicals such as tannins, flavonoids, and vitamin E, which has been identified to have anti-inflammatory, anti-oxidant, anti-carcinogenic, 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 including isoflavonoids and diphenolic phytoestrogens in Cicer arietinum L. (chickpea) have 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 the holistic approach against type 2 diabetes by considering the mechanism of actions of the above-mentioned plant extracts. In vitro approaches were used to investigate the potential antidiabetic effect of our intended formulation.

2. Materials and Methods

2.1. Materials. The Holarrhena antidysenterica seeds were purchased locally, washed, dried, and were ground into powder. Roasted Cicer arietinum and almonds were obtained from a local market and were ground 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.

2.2. Methods

2.2.1. Preparation of Plant Extracts. The 90% ethanolic extract Holarrhena antidysenterica (HA) seeds powder was prepared under stirring overnight at 37°C [16]. The flour of Cicer arietinum 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 the same conditions, and both supernatants were mixed. The almond seeds powder was extracted with 70% ethanol for 48 hrs at 37°C. The respective solvents were removed separately using the BUCHI rotary evaporator and then vacuum dried at 37°C for 24 hrs. The obtained extracts were stored at 0–4°C.

2.2.2. 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.

2.2.3. Preparation of Granules Formulation. The granules of HA, chickpea, almonds, and oleic acid mixture were prepared by wet granulation method [17] according to formulation mentioned in Table 1. The required amount of freeze dried aerosil was mixed with an active pharmaceutical ingredient (API) to overcome the hygroscopicity of the plant extracts. PVP K30 in isopropyl alcohol was used as a 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 magnesium stearate, talcum, and primogel (2.5% of total formulation). Finally, the granule blend was properly mixed for 15 minutes.

2.3. 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.

2.4. Evaluation of Granules. The physicochemical properties of granules were evaluated using standard methods [18].

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

\[ \theta = \tan^{-1}\left(\frac{h}{r}\right). \] (1)

2.4.2. Bulk Density (δb). Bulk density was estimated by pouring the correctly weighed amount (M) of the granule
bated with 1 ml of 5 mM glucose for 10 min at 37°C. 100 μl yeast suspension was added in the reaction mixture to start the reaction. After 60 min of incubation at 37°C, centrifugation was done at 3800 rpm for 5 min and the amount of glucose in the supernatant was measured at 520 nm by UV-Vis spectrophotometer (JASCOV-530, JAPAN). The effective concentration (EC50) was obtained from the percentage activity curve. The (%) increase in glucose uptake was calculated according to the following formula [21]:

\[
\% \text{ increase in glucose uptake} = \left[ \frac{(\text{Abs.of control} - \text{Abs.of sample})}{\text{Abs.of control}} \right] \times 100.
\]

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

2.8. α-Amylase Inhibition Assay. α-amylase inhibition activity was measured according to Akoro et al. (2017) with slight modifications using a microplate reader (BioTek instruments, USA) [22]. 15 μl PBS (pH 6.8) was added in all wells of a 96-well plate. 25 μl of 0.14 U/ml enzyme and 1-4 mg/ml samples were pre-incubated at 37°C for 10 min. The reaction was started with the addition of 40 μl starch (2 mg/ml) and incubated for 30 min at 37°C. 20 μl 1 M HCL was added to stop the reaction, and 90 μl iodine reagent was added. The change in color was observed, and absorbance was measured at 620 nm after incubation. The control contained all the reagents except 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 the following formula [23]:

\[
\% \text{ inhibition} = \left[ \frac{(A_c - A_s)}{A_c} \right] \times 100,
\]

where \(A_c\) was the absorbance of control without inhibitor and \(A_s\) was the absorbance of the sample.

2.9. α-Glucosidase Inhibition. The inhibition activity of α-glucosidase was optimized using the method described by Sundar et al. (2015) [24]. The reaction mixture contained 25 μl of 20 mM 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 37° 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 a microplate reader (BioTek instruments, USA). The inhibition activity was measured by using the following formula:

\[
\% \text{ inhibition} = \left[ \frac{(A_c - A_s)}{A_c} \right] \times 100,
\]

where \(A_c\) was the absorbance of control without inhibitor and \(A_s\) was the absorbance of the sample. Acarbose was used as a positive control.
2.10. Antioxidant Activity. The DPPH radical-scavenging method was used to quantify the antioxidant activity of granules [25]. The freshly prepared 2 ml DPPH methanolic solution (0.1 mM) was incubated with 2 mL of each sample (0.001–1 mg/mL) for 30 min in dark conditions. UV spectrophotometer (JASCOV-530, JAPAN) was used to analyze the absorbance at 517 nm. Ascorbic acid was used as a standard. The scavenging capability was calculated by

\[
\text{% of radical scavenging activity} = \left( \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \right) \times 100.
\]

(9)

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

3. Results and Discussion

3.1. FTIR. The FTIR spectra of pure extracts (H. antidysenterica, almonds, and chickpea), oleic acid, mixture, and granules are shown in Figures 1(a) and 1(b), respectively. The frequency region or bands in each sample manifest the specific functional groups of compounds (Table 2). The peak observed in the 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\(^{-1}\) represent the carbonyl frequencies of amides and ester functional group in almonds [26], oleic acid [27], HA [28], and chickpea [29], respectively. The that 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 relative active compounds (isoﬂavones, amines, and phenols, etc) in all samples [30].

In granules preparation, certain excipients were added to stabilize the formulation. Drug-excipient compatibility can be checked from the spectra of mixture and granule (Figure 1(b)). No modifications in wavenumber are observed, which satisfies the inert behavior of the excipients added.

3.2. Preparation and Evaluation of Granules. Wet granulation technique was used to prepare 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 3). According to USP 30 standard, the angle of repose in 25–30 range shows excellent flow properties whereas an angle >40° indicates poor properties [31]. The angle of repose result (24°) presented 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 to 1.11, respectively. The prepared granules show the excellent flow properties with respect to the deﬁned standard ranges.

3.3. Particle Size Distribution. Sieve method was used to determine the particle size distribution. Size frequency plot shows that 44.6% granules are greater than 246 μm whereas 4.8% are <74 μm in size of the total sample (Figure 2(a)). Cumulative size distribution data represents the percentage number of samples are less than the stated size [32]. Figure 2(b) shows that the median diameter (D50) is 200 μm, which deﬁnes that 50% of particles are less than the 200 μm size. There should be 15% of ﬁnes present in the granular blend to obtain appropriate powder ﬂow and compaction. Particle size distribution poses the signiﬁcant effects on dissolution rate, content uniformity, and stability. Small particle size improves the drug release rate resulting in augmentation of drug solubility [33].

3.4. In vitro Dissolution Result. In vitro dissolution test through the dialysis membrane was designed using standard conditions. The dialysis tube mimics the intestinal membrane which assisted the drug release. The dissolution proﬁle of the polyherbal drug is shown in Figure 3. The result explains the immediate release of drug from the granules as 56–60% drug was released in 60 min and approximately 80% was released in 2 hrs. During the release studies, it was noticed that the drug-carrier system dissolves gradually while 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 [33]. However, the expected in vivo studies will better describe the bioavailability of drug via dissolution proﬁle.

3.5. Glucose Uptake Assay by Yeast Cell. Figure 4 represents the glucose uptake activity of samples (mixture, granules, and metformin) by yeast cell. This method measures the remaining concentration of glucose in medium. The percentage glucose uptake increases with an increase in concentration of metformin were observed. However, an opposite and strange behavior was observed in case of mixture and granules, i.e., percentage glucose uptake decreases with an increase in concentration. At 1 mg/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 shows the EC50 values of granules, mixture, and metformin.

The antidiabetic activity of various natural plants has been evaluated by the 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 [34]. In the present scenario, there are chances that the presence of plant extract-based granules facilitates the high glucose diffusion inside the cell 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 [35]. H. antidysenterica was reported to enhance the glucose diffusion in neighboring cells via sodium-dependent glucose absorption [36].
3.6. *Alpha Amylase*. Starch-iodine method was used to determine percentage inhibition activity of herbal mixture, granules, and acarbose. The results (Figure 5) show that the percentage inhibition increases with dose-dependent concentration. Granules show more inhibition activity ranged from 44% ± 0.008 to 76% ± 0.002 for 1.0–4.0 mg/ml as

![Figure 1: (a) FTIR spectra of almonds, *H. antidysenterica* (HA), oleic acid and chickpea. (b) FTIR spectra of mixture (M) and granules (G).](image-url)
Table 2: 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 alkanes or alkyl groups  |
| 1600–1800 cm⁻¹ | Carbonyl compounds of amides and esters  |
| 1000–1200 cm⁻¹ | Primary amines                            |

Table 3: Evaluation of flow properties of granules and compared it with USP 30 standard.

| 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       |

The data points are representing as mean ± SEM, n = 3.

compared to mixture (34% ± 0.004–67% ± 0.004), which is approximately equivalent to acarbose activity ranged from 53% ± 0.006 to 79% ± 0.0003 at higher concentration. The IC50 was 1.47, 1.86, and 0.83 mg/ml of granules, mixture, and acarbose, respectively, as shown in Table 5. Overall, it can be concluded that the granules containing a mixture of plant extracts exhibited better inhibition activity against amylase as compared to acarbose.

α-amylase produced in saliva begins the digestion of food carbohydrates in the mouth and continued in the intestine. Acarbose is the standard drug possesses greatest α-amylase inhibition activity and delays the hydrolysis of starch into oligosaccharides and disaccharides [37]. 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. H. antidysenterica and oleic acid were reported to exhibit good potency of α-amylase inhibition activity [36, 38, 39].

3.7. α-Glucosidase Inhibition Activity. The ability of active compounds from mixture, granules, and acarbose to inhibit alpha glucosidase was determined. Figure 6 shows the graphical representation of α-glucosidase inhibition in dose-dependent way from 1.0 to 4.0 mg/ml concentration. At maximum concentration (4 mg/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.

In case of IC50 values (Table 5), 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. antidysenterica and oleic acid against α-glucosidase enzyme [36, 38]. The α-glucosidase inhibition activity of H. antidysenterica and oleic acid may be compromised by combining multicomponent plant extracts (H. antidysenterica, almonds, chickpea, and oleic acid) in a single drug (granules). The in vitro enzyme results may be different from in vivo, hence the designed polyherbal-based granules permits the further evaluation of enzyme inhibition through in vivo studies. Though, the presence of α-glucosidase inhibition activity in these study samples, which retards the digestion of polysaccharides and oligosaccharides, suggested the effective therapeutical 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.

3.8. Antioxidant Activity. Radical scavenging activity of mixture and granules was assessed by DPPH radical scavenging method (Figure 7). A dose-response pattern (0.001–1 mg/ml) of DPPH confirms the free radical scavenging activity. At higher concentration granules showed a 98% scavenging effect equal to standard (ascorbic acid). DPPH accepts an electron from antioxidant agents and changes its color from violet to yellow which was measured spectrophotometry at 517 nm. Sujan et al. (2011) has reported the antioxidant activity of alcoholic extract of H. antidysenterica due to the presence of phenolic content [40]. In addition, it has been investigated that the phenolic compounds in alcoholic extracts of chickpea seeds and almond shells are responsible for their radical scavenging properties [41, 42]. Oxidative stress is one of the major abnormalities that would trigger other complications. So, the presence of this scavenging activity may be able to tackle the reactive oxygen species (ROS) in the cells and tissues of diabetics.

4. Conclusion

Herbal formulations are considered more effective as compared to allopathic medicines due to their fewer 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 the crude mixture. The present study validates the antidiabetic activity of the formulation (granules) based on the selected plants (H. antidysenterica, 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 an antidiabetic regimen. Hence, further in vitro and in vivo studies must be conducted for the 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

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**Figure 2:** (a) Size frequency distribution of granules and (b) cumulative frequency plot.

**Figure 3:** In vitro drug release profile of polyherbal-based granules.

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

**Figure 5:** Percentage inhibition of α-amylase activity of plant extracts mixture, granules, and acarbose.

**Table 4:** EC50 values (mg/ml) of granules, mixture, and metformin.

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

The data points are representing as mean ± SEM, n = 3.
activity. Besides, the prescreening analysis for identification and characterization of extracted active compounds is recommended.

**Data Availability**

The underlying data is available, will be provided if required.

**Conflicts of Interest**

The author(s) declare(s) that they have no conflicts of interest.

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**Table 5: IC50 values (mg/ml) of granules, mixture and acarbose.**

| Sample      | α-amylase IC50 (mg/ml) | α-glucosidase IC50 (mg/ml) |
|-------------|------------------------|---------------------------|
| 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             |

The data points are representing as mean ± SEM, n = 3.

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