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ANTIDIABETIC EFFECTS OF PROTEIN ENRICHED BARLEY BASED EXTRUDED SNACKS AGAINST STREPTOZOTOCIN INDUCED DIABETES IN *SPRAGUE DAWLEY* RATS

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ABSTRACT
Several studies revealed that protein and dietary fiber enriched diet has therapeutic effect against hyperglycemia or diabetes mellitus. Diabetes mellitus is one of the major non-communicable diseases and the leading cause of deaths in developing countries and around the globe. This study aimed to evaluate the antidiabetic effect of protein enriched barley based extruded snacks against the diabetic rats. For this purpose, barley based extruded snacks with the incorporation of 20% defatted sunflower meal were used for efficacy trials on *Sprague Dawley* rats. In this context, 40 healthy rats were selected and divided into four groups *i.e.*, negative control (NC), positive control (PC), standard drug (SD) and treatment (EB_{20}) groups. Negative control were fed on normal diet and hyperglycemia was induced by using 2 doses of Streptozotocin injection along with the administration of normal diet (PC). SD group receive metformin per kg body weight for 28 days after the induction of hyperglycemia. While treatment group received treatment diet for 28 days as long as the hyperglycemic condition resulted. Blood glucose was recorded and monitored on regular basis. Blood samples were collected and analyzed for different hyperglycemic parameters, kidney and liver stress biomarkers. Hispathological examination of pancreatic tissues was also conducted. The results of biochemical tests and the examination of histopathological tissues of rats fed on protein enriched barley based diet revealed that the adminitation of protein diet significantly lowered the blood glucose or insulin levels does improve the serum glucose, serum insulin, HbA1c, T_3, T_4, and TSH levels and less likely to affect pancreas.

Keywords: Diabetes, Barley, Sunflower seeds, Protein diet, Beta glucan, Biochemical tests, Histopathology

1. INTRODUCTION
Hyperglycemia known as diabetes mellitus is a crucial health anomaly being one of the four major targeted non-communicable disorders worldwide and major health concern in developing countries. It is strongly subjected to genetic factors, but poor dietary habits and sedentary lifestyles are other main contributors [1]. Diabetes mellitus results in different metabolic disorders related to insulin insensitivity to its receptors, insufficient insulin secretion, and failure of pancreatic beta cells.

International Diabetes Federation depicted on the prevalence of diabetes, diabetic people around the globe were 451 million (8.8% of population worldwide) in 2017 that is likely to rise up-to 693 million (9.9%) by the year 2045 [2, 3]. In USA, the prevalence rate of diabetes among older adults is ~ 10 per 1000 people and overall prevalence among older adults (age ≥ 60 years) is almost 21.5% [4, 5]. Considering the prevalence of diabetes among different countries with respect to their economic status, both high- and middle-income countries showed great prevalence rates such as 22% and 19%, respectively in older adults [2].

Pakistan being a developing country is facing a rapid surge in diabetes prevalence. According to IDF, rate of diabetes in Pakistan has drastically increased as 33 million adults of the country are diabetic depicting 70% increase since 2019. Moreover, the report estimates that diabetes will kill more than 0.4 million people in Pakistan which is the highest figure amongst all Middle East countries. Report also states that every fourth adult (26% of all adults) in Pakistan is diabetic which marks the highest national level around the Globe. In addition to this, about 11 million adults in the country are suffering from the impaired glucose tolerance that leaves them at the greater risk of getting diabetic in next few years [3, 6].

Conventional treatments used for hyperglycemia are insulin and antidiabetic drugs which are expensive and have side effects. Changing dietary patterns, shift towards refined and processed foods along with less consumption of plant-based diet set the seed of many metabolic disorders including hyperlipidemia, diabetes, cardiovascular diseases, hepatotoxicity, and several types of cancer [7]. Researchers have been investigating non-pharmacological treatments such as functional foods and physical activity to mitigate or prevent hyperglycemia [5, 8]. Foods with low glycemic index have been proven helpful in lowering blood glucose concentration especially those which have high soluble fibre *i.e.*, *β*-glucan. *β*-glucan has antidiabetic and anticholesterolemic properties and build a barrier in the small intestine to prevent absorption of nutrients and lower the risk of insulinemia, glycemia, and serum cholesterol level [9]. Diet rich in protein or supplemented with protein not only help in improving growth and maintaining body weight but also helps in lowering blood glucose concentration. Protein-rich diet also stimulates secretion of pancreatic *β*-cells which helps in maintenance of HbA1c and glucose level in the blood [10, 11].

Researches have also reported that *β*-glucans have ability to reduce the postprandial blood glucose content [12]. The possible mechanism behind the control of postprandial blood glucose content could be the presence of phenol amides, phenolic acids, hordatine, and flavonoids in barley [13] which maintains the health of gut microbiota that alters the blood glucose concentration[14]. Protein diet plays an important role to maintain an ideal body weight and good glycemic control [15]. Dietary proteins stimulate release of insulin from *β*-cells and decrease postprandial concentrations of glucose in blood [16]. Now-a-days, the plant proteins are considered economical, easily accessible and a good source of functional components for reformation of food products [17]. Sunflower seeds have shown potent effect in reducing blood glucose levels in diabetic patients. Hypoglycemic effects of these seeds have been observed due to their bioactive components such as chlorogenic acid and secoisolariciresinol diglucoside which helps by increasing the insulin production and by treating the insulin resistance [18].

Sunflower biologically knows as *Helianthus annuus* L. belongs to family Compositae (or Asteraceae). Word ‘Helianthus’ is combination of two Greek words such as ‘Helios’ means Sun and ‘Anthos’ means flower [19]. In developing countries, farmers pay more attention towards the production of sunflower seeds due to presence of multi-nutritional components. Presence of numerous nutritional components such as dietary fiber, flavonoids, phenolic, glutathione reductase, triterpene, glycosides, phytosterols, chlorogenic acid, caffeic acid, peptides, manganese, fat soluble vitamins, saponins and tannins; contributes to the development of functional food products as well as nutraceutical supplements [20]. Sunflower seeds are also well known due to presence of high content of antioxidants. In a pharmacological survey, it is proved that sunflower has potential to cure different kinds of diseases. Furthermore, sunflower is effectively used in treatment of high blood pressure,

controls blood sugar levels, protect skin from infections and helps in maintaining body's metabolism [21].

Barley is extensively implanted and known as one of the important crops of the cereal group after corn, wheat, and rice [22]. The United States Department of Agriculture **(**USDA) estimates that barley production (2019-20) was 156.58 million tons while the world barley production 2020-21 is 157.19 million metric tons, with an increase of around .60 million tons or 0.39% in barley production around the globe [23]. Barley is a good source of starch and protein while apart from the macronutrients found in barley, it is also rich in dietary fiber (11–34%) and classified according to their solubility *i.e.*, soluble dietary fiber (3–20%) and insoluble dietary fiber (8–14%) [24, 25]. The main ingredient present in the hull, endosperm, and barley gem are tocols with the level of 63%, 10% and 95%, 63%, and 10% in each tissue, respectively [26]. Barley has numerous bioactive components such as ferulic acid and phenolic acids as a fundamental component (68%), in addition to coumaric acid with a minor amount of sinapic acid, vanillic acid, syringic acid, *p*-hydroxybenzoic and sinapic acid. Furthermore, flavonoids are also present in barley in good amounts including anthocyanins, proanthocyanidins and flavonols [24, 26]. Several studies have depicted that the incorporation of barley in different food products can significantly enhance the level of bioactive compounds and phenolics [27-29].

It has low glycemic index than other numerous cereals, therefore, the insoluble fibers, beta-glucans, and amylose found in barley are the chief contributors to lower glycemic index, which reduces the risk of diabetes [30]. Another study has depicted that the intake of barley can also normalize the levels of blood sugar due to the presence of *β*-glucan [31]. Barley having low glycemic index, is being used in formulation of various antidiabetic products as functional ingredient. Beta glucans (*β*-glucans) is a major bioactive component of barley that has shown therapeutic effects against hyperlipidemia, obesity, and diabetes [12]. In this era of ever-increasing population and rapidly changing lifestyle, nutrition and health are becoming the most demanding fields and people are more concerned about their diet and health. Poor and imbalanced dietary habits are the major causes of lifestyle-related illnesses, *i.e.*, cardiovascular diseases, diabetes, hypercholesterolemia, obesity, constipation, cancers *etc*. that adversely affect the human health. The current study has been designed to access the antidiabetic effects of barley based protein enriched extruded snacks in streptozotocin induced diabetic and normal diet fed rats. The outcomes of current study provides evidence based antidiabetic effectiveness of protein enriched diet.

2. MATERIAL AND METHODOLOGY

The present study was conducted in Grain Science and Technology Laboratory, Food and Nutrition Laboratory and animal house of National Institute of Food Science and Technology (NIFSAT), University of Agriculture (UAF), Faisalabad.

2.1. Preparation of raw materials

Barley *(Hordeum vulgare)* and sunflower (*Halianthus annuus*) seeds were procured from New Grain Market, Faisalabad. Barley and sunflower seeds were cleaned to remove dirt and other extraneous materials. Barley was pearled, dehulled, milled and sieved to get flour of uniform size. Sunflower seeds were roasted, dehulled, and defatted using cold press extractor to obtain defatted sunflower meal. The barley flour was replaced with defatted sunflower meal to prepare composite flour blends for extrusion cooking. Defatted sunflower meal, non-extruded composites and extruded snacks of barley and defatted sunflower were analyzed for the physicochemical parameters and sensory triats. Based on physicochemical and sensory evaluation score, barley based extrdued snacks containing 20% of defatted sunflower meal (EB_{20}) was used as treatment diet for efficacy study.

2.2. Efficacy study

Efficacy trial was carried out to assess therapeutic effect of EB_{20} against hyperglycaemia. For this purpose, *Sprague Dawley* rats were procured from NIH (National Institute of Health), Islamabad and kept in the animal room at NIFSAT, UAF. Basal diet and water *(ad libitum)* were provided to

experimental rats for two weeks. After acclimatization, they were divided randomly in four groups (n=10). Normal and respective group diets were prepared by adjusting each nutrient content, especially by adjusting the protein content, corn oil, mineral and vitamin mixture and flour. The composition of diets is explained below in table 2.1. The temperature and humidity ((25±2ºC & 50±5%) for animal room was maintained with 12 hr light or dark cycle throughout the bio-efficacy trial.

Table 2.1. Components of experimental diets used in the bio-efficacy study

Diet components	Normal diet	Treatment diet
Casein (g)	10	10
Corn oil (g)	10	10
Mineral mix (g)	1.0	1.0
Vitamin mix (g)	5.0	5.0
Flour (g)	74.0	--
EB_{20} (g)	--	74.0
Total weight (g)	100.00	100.00

EB_{20} = Barley flour (80%) based extruded snack containing 20% defatted sunflower meal

2.3. Treatment plan

This study was conducted for hyperglycaemia / diabetes mellitus that was induced in rats by administering intraperitoneal injection of Streptozotocin (65 mg/Kg body weight) dissolved in normal saline. Blood glucose was checked with commercially available glucometer before and after the induction of diabetes. To avoid fatal hypoglycemia and mortality that was reported after injecting Streptozotocin, the rats were provided with dextrose solution (10%) within 12-24 hours of injection administration. In this study, D_0 served as negative control fed on normal diet, D_1 as positive control was given normal diet along with injection of streptozotocin, D_2 was served as standard drug group fed on normal diet along with streptozotocin injection and antidiabetic drug (metformin) while D_3 was provided with diet in conjunction with extruded snack (EB_{20}) and served as treatment group (Table 2.2).

Table 2.2.: Animal study plan for hyperglycemia

GROUPS	EXPERIMENTAL DIETS
D_0: Negative control	Normal diet
D_1: Positive control	Normal diet + Streptozotocin injection
D_2: Standard drug	Normal diet + Streptozotocin injection + Antidiabetic drug (Glibenclamide)
D_3: Treatment (EB_{20})	Barley flour (80%) containing 20% DSM extruded snack diet + Streptozotocin injection

2.4. Growth study parameters

Feed and water were recorded on regular basis. Net feed and water intake of rats were measured in grams and milliliters. Body weight of rats was measured on first day of trial then weighed on seventh day of week to assess growth performance. Immediately after decapitation of animals, relative organs *i.e.*, heart, kidney, and liver weight were recorded. Likewise, feed efficiency ratio (FER) was calculated using gain in body weight and feed intake by given expression.

$$\text{FER (\%)} = \frac{\text{Gain in body weight (g)}}{\text{Feed intake (g)}} \times 100$$

2.5. *In-vivo* analysis

On the last day of trial, rats were fasted over nightly then anesthetized by chloroform and decapitated. At the termination of trial, blood was collected in EDTA coated tubes and the blood and serum

samples from each group were analysed for different biomarkers *i.e.,* fasting blood glucose was measured using commercially available glucometer (ACCU-Check Instant S Blood Glucose Test Kit Glucometer) following the method outlined by [32]. Serum insulin was assessed using respective ELISA (Enzyme Linked Immunosorbent Assay) kit at the end of the study [33]. HbA1c level was measured before induction of diabetes and at the end of the trial [34]. Thyroid hormones *i.e.,* serum triiodothyronine (T_3), serum thyroxin (T_4) and serum thyroid stimulating hormone (TSH) concentrations were estimated using their commercially available kits *i.e.,* PISHTAZTEB T_3 (PT-T_3-96), T_4 (PT-T_4-96) and TSH (PT-TSH-96) diagnostic ELISA kit. Additionally, safety assessment was performed through liver and kidney function tests. Furthermore, the histopathology of pancreas of experimental rats of each group was also carried out. Creatinine and urea test were done to analyze the kidney functioning following the Jaffe and GLDH methods using their commercially available kits [35, 36]. Liver functioning was tested through its enzymatic assessment *i.e.,* alanine aminotransferase (ALT) and aspartate transaminase (AST) according to the guidelines explained by [36, 37]. After the decapitation of experimental rats' pancreas were stored in 40% formalin solution for tissue examination under microscope. Histopathology of the organs was done according to the procedures of [38] and [39]. A rotary microtome was used for tissue sectioning (thickness: 5-6 µm) then tissue was secured on a glass slide. Haemat-oxylin and eosin dye were used for staining of the tissue slides. After preparation of slides, light microscope (MCX 100, Micros Austria) was used to examine the tissue/specimen.

2.6. Statistical analysis

Experimental units and efficacy trials were randomly allocated, and the obtained results were subjected to factorial under completely randomized design (CRD) to check the effects of multiple variables at level of significance ($\alpha \leq 0.05$). Whereas Tukeys Post-Hoc test was applied to test means differences [57].

3. RESULTS AND DISCUSSION

3.1. Growth performance parameters

The mean squares (Table 3.1 & 3.2) illustrated that the weight gain, feed and water intake, and FER were significantly varied among all experimental groups. Changes in mean values (Figure 3.1) for body weight gain depicted that negative control (D_0) and positive control (D_1) groups had comparable weight gain at the beginning *i.e.,* 236.25±4.15 g and 239.25±3.03 g while standard drug and treatment groups (D_2 and D_3) had 214.25±3.77 g and 219.50±2.96 g, respectively. At 7^{th} day, from mean table of body weight it was observed that in positive control (D_1), significant decrease was observed due to the induction of disease.

Body weight measured after seven days showed significant decrease in the body weight of experimental rats *i.e.,* diabetic group (D_1) has 205.20±5.05 g, standard drug group (D_2) and treatment group (D_3) has 157.75±2.95 g and 165.25±2.86 g, respectively. However, significant increase was observed in the rats of negative control group (D_0) fed on normal diet. A gradual increase was observed in rats' weight at 14^{th}, 21^{st}, and 28^{th} day of the trial by 8%, 8%, and 9% weight gain in D_2 while in D_3 firstly decrease by 10.5% at 14^{th} day but with the intake of standard drug in diet started increasing the body weight by 23% at 21^{st} day and 8.25% at 28^{th} day of the trial. A gradual increase was observed in the body weight of rats of negative control group (D_0) with the intake of normal diet as 8.25, 8.25, and 7%, respectively. Results revealed that after experimental induction of diabetes by streptozotocin, body weight reduced significantly ($P<0.05$) in rats of positive control (diabetic) group, D_1 as compared to those of negative control D_0, standard drug and treatment groups (D_2 and D_3). This reduction in body weight could be attributed to the structural protein degradation as diabetes is associated with different biochemical derangements including increased gluconeogenesis, glycogenosis, and lipolysis leading to tissue protein loss and muscle wasting [36, 40, 41].

Considering the figure 3.2. representing feed intake, feed intake of all the experimental groups showed significant variation during the trial such as at first day, the feed intake recorded for D_0, D_1, D_2 and

D_3 was 39.90±0.59, 31.70±0.0.31, 36.55±0.43, and 37.04±0.35 g/day/rat. Feed intake of all experimental groups (D_0, D_1, D_2 and D_3) has significant variation of the weekly intake of feed among all groups. The feed intake of negative control (D_0) for 4 weeks varied from 39.90±0.59 to 43.56±0.57 g/day/rat while slightly variation with increase/decrease was observed in the rats of group D_1, D_2 and D_3 as from 31.70±0.31 to 32.12±0.52, 36.55±0.43 to 39.61±0.58, and 37.04±0.35 to 41.61±0.35 g/day/rat from 1st day to 28th day of the trial, respectively. However, consistent increase or decrease was observed in feed intake of all rat groups [36, 40].

Water intake from figure 3.3. was observed to be significantly affected during the trial within all groups. A different trend was observed for water intake from weight gain and feed intake. At first day, water intake was recorded as 44.11±0.32, 28.45±0.16, 31.10±0.30, and 31.24±0.65 mL/day/rat in D_0, D_1, D_2, and D_3, respectively. After seven days of trial, water intake was fluctuated among all groups. The water intake of positive control D_1 decreased on 7th day as 21.77±0.37 mL/day/rat and increased in other groups *i.e.*, D_0, D_2 and D_3 as 32.45±0.69, 23.10±0.53, and 23.53±0.51 mL/day/rat. Afterwards, fluctuations (increase/decrease) were observed in the water intake from 14th day to 28th day in all groups, D_0, D_1, D_2, and D_3 from 28.63±0.46 to 20.32±0.57, from 20.09±0.45 to 22.06±0.28, and from 18.45±0.66 to 22.99±0.56, correspondingly.

Feed efficiency ratio (FER) showed in figure 3.4. revealed that positive control group (D_1) taken normal diet with streptozotocin induced diabetes had negative efficiency during the trial due to variation in feed intake and decrease in weight *i.e.*, -4.65, -3.44, -2.95, and -2.13 for 1st, 2nd, 3rd, and 4th week, respectively. However, a positive efficiency was observed in negative control D_0, 5.59, 5.47, 5.18, and 6.29 and negative/positive efficiency was observed in D_2 and D_3 such as -0.66, 4.58, 4.78, and 4.51, and -0.67, -3.80, 1.76, and 5.10 for 1st, 2nd, 3rd, and 4th week, respectively.

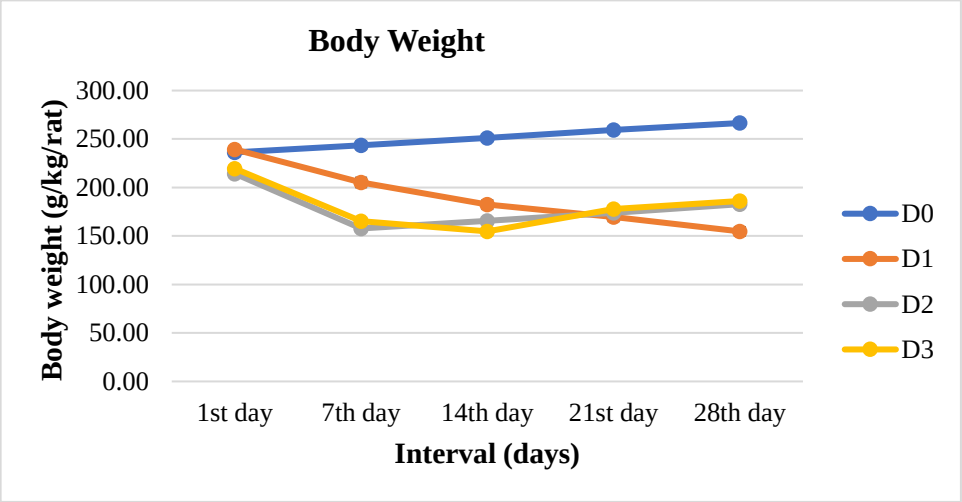

Figure 3.1. Mean±SD for weight gain of experimental rats

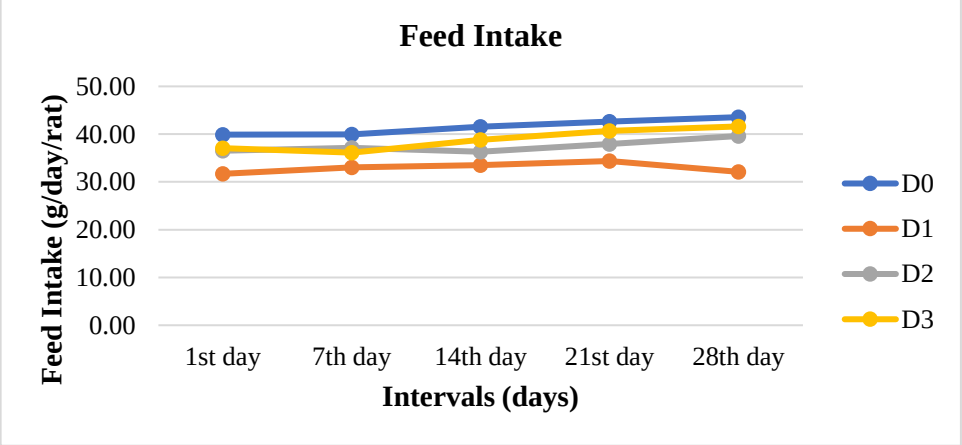

Figure 3.2. Mean±SD for feed intake of experimental rats

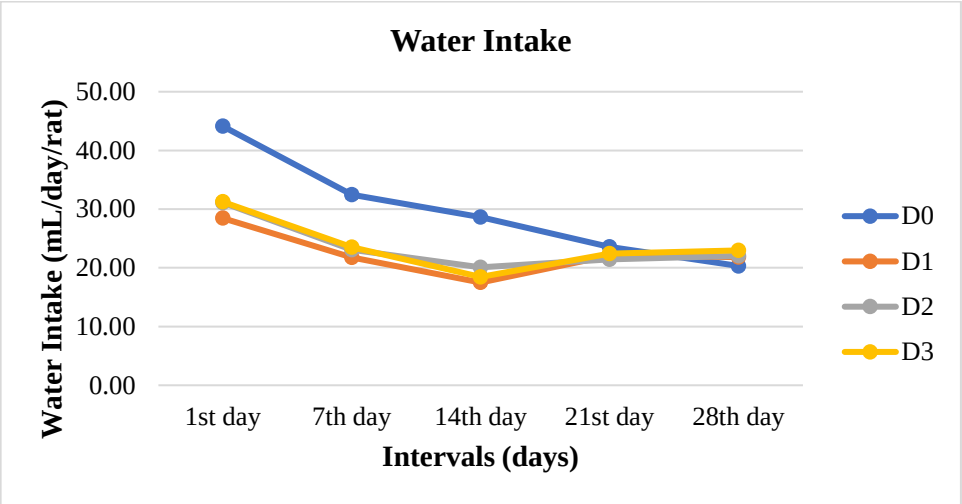

Figure 3.3. Mean±SD for water intake of experimental rats

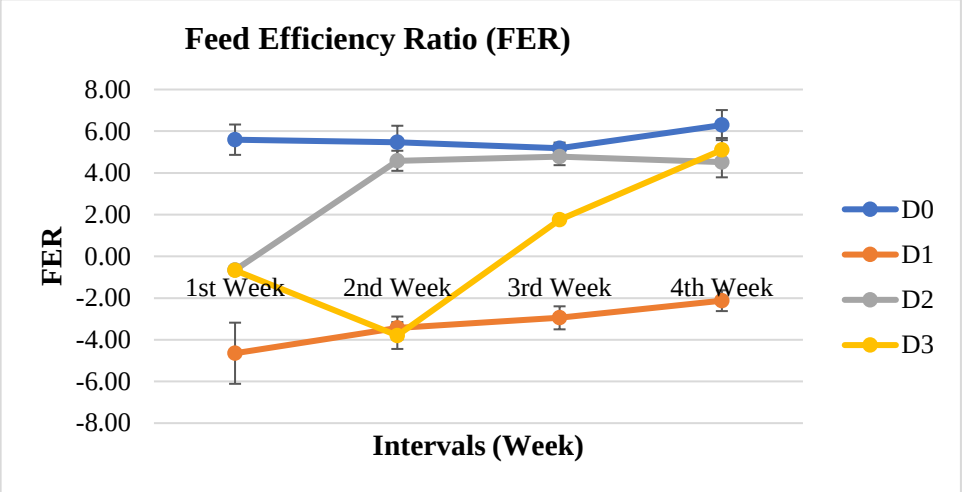

Figure 3.4. Mean±SD for feed efficiency ratio of experimental rats

D_0 = Negative control group fed on normal diet
D_1 = Positive control group fed on normal diet + streptozotocin
D_2 = Standard drug group fed on normal diet + streptozotocin + glibenclamide
D_3 = Treatment group fed on 20% DSM+80% barley containing diet + streptozotocin

Table 3.1. Mean squares for grwoth performance parameters of experimental rats

Source	df	Body weight	Feed intake	Water intake
Groups (G)	3	24247.4**	257.972**	226.641**
Days (D)	4	2079.9**	28.555**	431.058**
Error	72	247.3	1.086	7.701
Total	79			

df = Degree of freedom **Highly significant = P<0.01

Table 3.2. Mean squares for Feed efficiency ratio of experimental rats

Source	df	Feed efficiency ratio
Groups (G)	3	213.671**
Weeks (W)	3	35.236**
Error	57	9.711
Total	63	

df = Degree of freedom **Highly significant = P<0.01

3.2. Fasting blood glucose (mg/dL)

Fasting blood glucose level revealed significant effect of treatments and days among all of experimental rat groups. Mean squares (Table 3.3.) illustrated that the experimental diets revealed significant variation in the fasting blood glucose concentration among the rats of group D_1, D_2, and D_3 while non-momentous variation was observed in the blood glucose concentration of negative control group. The results showed (Figure 3.5.) the blood glucose concentration measured on 1st day before induction of diabetes for negative control group (D_0) 95.81, positive control group (D_1) 96.84, standard drug group (D_2) 93.25, and 94.55 in treatment group (D_3). At 7th day, the mean±SD showed significant ($P<0.01$) increase in the blood glucose concentration due to the induction of diabetes in rats of group D_1, D_2, and D_3 as 480.67, 280.93, and 345.88 mg/dL, respectively. The group treated with standard drug and treatment diet showed a significant decrease in mean fasting blood glucose. The blood glucose concentration for negative control group (D_0) ranged from 88.04 mg/dL to 92.44 mg/dL during the 28 days of study trial. At 28th day, the blood glucose concentration was constant with high concentration for positive control group, D_1 (434.87 mg/dL) while for standard drug group (D_2) and treatment group (D_3), the fasting blood glucose levels decreased to 100.14 mg/dL and 136.58 mg/dL at 28th day, respectively. In current study, it was observed that administration of streptozotocin led to elevation in fasting blood glucose level, which was maintained in positive control group (D_1) throughout the experimental period. Daily treatment with glibenclamide and extruded snacks diet over a period of 28 days significantly decreased ($P<0.05$) fasting blood glucose level[36, 40, 42].

Table 3.3. Mean squares for fasting blood glucose of experimental rats

Source	df	Fasting blood glucose
Groups (G)	3	512298**
Days (D)	4	100435**
Error	72	3623
Total	79	

df = Degree of freedom **Highly significant = $P<0.01$

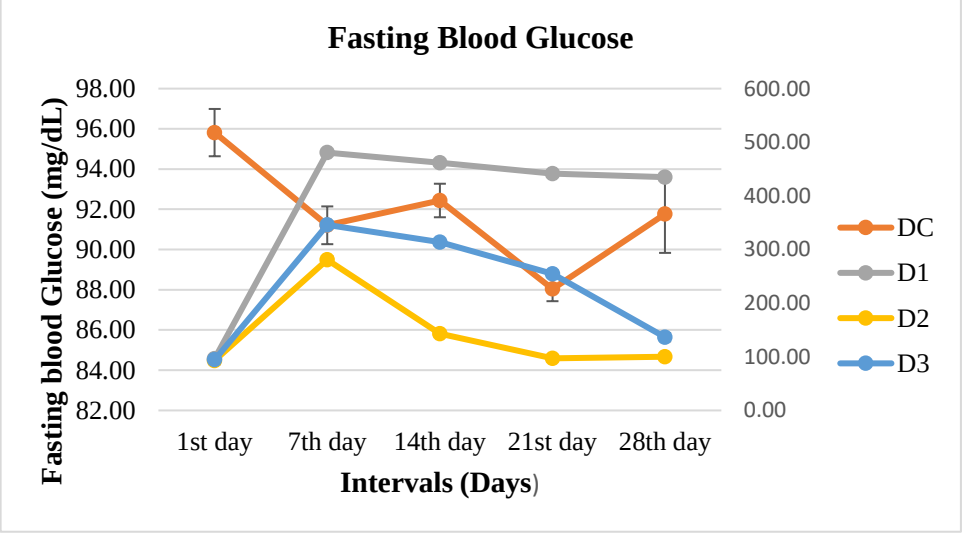

Figure 3.5. Mean±SD for fasting blood glucose of experimental rats

D_0 = Negative control group fed on normal diet
D_1 = Positive control group fed on normal diet + streptozotocin
D_2 = Standard drug group fed on normal diet + streptozotocin + glibenclamide
D_3 = Treatment group fed on 20% DSM+80% barley containing diet + streptozotocin

3.3. Serum glucose (mg/dL) and serum insulin (µIU/mL)

Mean values for serum glucose concentration are presented in table 3.5. Streptozotocin significantly ($P<0.05$) increased the mean serum glucose concentration in positive control/diabetic group (D_1) as

compared to negative control (D_0), standard and treatment group. Groups (D_2 and D_3) treated with glibenclamide, and extruded snacks diet exhibited a significant reduction ($P<0.05$) in concentration of serum glucose as 174.52±2.88 mg/dL and 163.95±2.28 mg/dL. The difference between groups (D_2 and D_3) was statistically non-momentous when compared between each other (table 3.4.). No significant effect was observed on the serum glucose level of negative control group rats (D_0) fed on normal diet (92.35±1.39 mg/dL). Statistical analysis revealed that the mean serum insulin level in positive control (diabetic) group (D_1) was significantly ($P<0.05$) lower (6.12±0.24 µIU/mL) in comparison to negative control group (D_0) standard drug group (D_2) and treatment group (D_3) *i.e.*, 19.53±0.80 µIU/mL, 17.97±1.18 µIU/mL, and 17.27±0.68 µIU/mL, correspondingly. Groups treated with glibenclamide, and extruded snacks diet (treatment group) showed a significant ($P<0.05$) rise in serum insulin level. The overall mean serum level of insulin did not differ significantly between groups treated with glibenclamide and fed on protein diet when compared with each other. A significant decrease ($P<0.05$) in mean serum glucose concentration and increase ($P\leq0.05$) in serum insulin level were also noticed in D_3 and D_2 groups as compared to D_1. This decline in mean insulin level in positive control diabetic rats observed in current experiment has also been described by previous researchers [43-45]. In a previous study, it was observed that serum insulin level was higher in diabetic rats fed on protein rich diet compared to those receiving normal diet [36, 46]. High protein diet might exert its anti-hyperglycemic effect by helping in reduction of oxidative stress of pancreatic β-cell and allow the recovery or regeneration of damaged *β*-cells. Hence, protein enriched barley based extrude snacks diet might have the potential to accelerate the regeneration capacity of pancreatic *β*-cells.

Table 3.4. Mean squares for serum glucose and insulin of rats fed on experimental diets

Source	df	Serum glucose	Serum insulin
Groups	3	198489**	151.018**
Error	12	7	0.855
Total	15		

df = Degree of freedom **Highly significant = $P<0.01$

Table 3.5. Mean±SD for serum glucose and insulin of rats fed on experimental diets

Groups	Serum glucose (mg/dL)	Serum insulin (µIU/mL)
D_0	92.35±1.39^{d}	19.53±0.80^{a}
D_1	583.11±2.45^{a}	6.12±0.24^{c}
D_2	174.52±2.88^{b}	17.97±1.18^{ab}
D_3	163.95±2.28^{c}	17.27±0.68^{b}

D_0 = Negative control group fed on normal diet
D_1 = Positive control group fed on normal diet + streptozotocin
D_2 = Standard drug group fed on normal diet + streptozotocin + glibenclamide
D_3 = Treatment group fed on 20% DSM+80% barley containing diet + streptozotocin

3.4. HbA1c

Mean values for HbA1c concentration are presented in table 3.6. Streptozotocin significantly ($P<0.05$) decrease (2.81±0.50%) the mean HbA1c percentage in positive control/diabetic group (D_1) as compared to negative control (D_0), standard drug (D_2) and treatment (D_3) groups *i.e.*, 6.53±0.42%, 4.68±0.48% and 4.53±0.35%. Groups (D_2 and D_3) treated with standard drug, glibenclamide and treatment diet (high protein diet) exhibited a significant increase ($P<0.05$) in concentration of HbA1c. The difference between treated groups (D_2 and D_3) was statistically non-significant when compared between each other (Table 3.7.). The HbA1c percentage of negative control group (D_0) rats fed on normal diet was 6.53±0.42%. The reason for low HbA1c percentage is the little dietary intake that

results in diseased condition therefore, positive control group (D_1) has low HbA1c percentage as compared to others[36, 40, 46].

Table 3.6. Mean squares for HbA1c of rats fed on experimental diets

Source	df	HbA1c
Groups	3	9.22062**
Error	12	0.26289
Total	15	

df = Degree of freedom **Highly significant = P<0.01

Table 3.7. Mean±SD for HbA1c of rats fed on experimental diets

Groups	HbA1c (%)
D_0	6.53±0.42[a]
D_1	2.81±0.50[c]
D_2	4.68±0.48[b]
D_3	4.53±0.35[b]

D_0 = Negative control group fed on normal diet
D_1 = Positive control group fed on normal diet + streptozotocin
D_2 = Standard drug group fed on normal diet + streptozotocin + glibenclamide
D_3 = Treatment group fed on 20% DSM+80% barley containing diet + streptozotocin

3.5. Thyroid hormones

The statistical analysis (Table 3.8.) for serum T_3 level revealed significant variation among experimental groups. The mean concentration of T_3 in serum was significantly (P<0.05) reduced (2.70±0.05 ng/mL) in positive control group (D_1) as compared to negative control group (D_0) as 6.71±0.15 ng/mL. A significant (P<0.05) increase (6.19±0.09 and 4.89±0.05 ng/mL) was observed in serum T_3 level in groups treated with glibenclamide (D_2) and high protein diet (D_3). The overall mean for serum T_3 level differ significantly between standard drug group and treatment group (Table 3.9.). The mean serum concentration of T_4 was significantly (P<0.05) reduced (2.62±0.08 µg/dL) in D_1 as compared to D_0 (7.20±0.11 µg/dL), D_2 (5.93±0.09 µg/dL) and D_3 (6.19±0.07 µg/dL), respectively. Groups treated with glibenclamide, and treatment diet displayed a significant (P<0.05) increase and restored normal serum T_4 level (Table 3.8.). Mean±SE values for serum TSH levels (Table 3.9) revealed significant difference among different treatment groups. The mean serum TSH concentration was significantly (P<0.05) lower in D_1 (0.19±0.05 µlU/mL) than D_0 (0.55±0.06 µlU/mL), D_2 (0.53±0.03 µlU/mL), and D_3 (0.45±0.06 µlU/mL), correspondingly. Groups treated with glibenclamide, and high protein diet exhibited a significant (P<0.05) rise and restored normal serum TSH level.

In present study, a significant reduction (P<0.05) was observed in serum T_3, T_4 and TSH levels in hyperglycemic rats of positive control group (D_1) as compared to normal rats in negative control group (D_0) while treatment diet used to treat diabetic rats presented a significant improvement in thyroid profile. These findings are supported by the previous studies [47-49] where a significant decrease in level of serum thyroid hormone was observed in experimentally induced hyperglycemic rats. This decline in serum thyroid hormone concentration might be due to inhibition of de-iodination of T_4 and other partially iodinated threonine into T_3 in diabetic rats.

Table 3.8. Mean squares for thyroid hormones of rats fed on experimental diets

Source	df	T_3	T_4	TSH
Groups	3	12.7658**	15.7850**	0.11016**
Error	12	0.0119	0.0102	0.00351
Total	15			

df = Degree of freedom **Highly significant = P<0.01

Table 3.9. Mean±SD for thyroid hormones of rats fed on experimental diets

Groups	T_3 (ng/mL)	T_4 (µg/dL)	TSH (µIU/mL)
D_0	6.71±0.15[a]	7.20±0.11[a]	0.55±0.06[a]
D_1	2.70±0.05[d]	2.62±0.08[d]	0.19±0.05[b]
D_2	6.19±0.09[b]	5.93±0.09[c]	0.53±0.03[a]
D_3	5.89±0.05[c]	6.19±0.07[b]	0.45±0.06[a]

D_0 = Negative control group fed on normal diet
D_1 = Positive control group fed on normal diet + streptozotocin
D_2 = Standard drug group fed on normal diet + streptozotocin + glibenclamide
D_3 = Treatment group fed on 20% DSM+80% barley containing diet + streptozotocin
T_3 = Serum triiodothyronine
T_4 = Serum thyroxin
TSH = Thyroid stimulating hormone

3.6. Renal stress biomarkers

Renal stress biomarkers *i.e.*, serum creatinine and blood urea are conducted to estimate the functioning and nature of kidney injury. Statistical analysis of serum creatinine level (Table 3.10.) revealed a significant difference in creatinine levels of negative control (D_0), positive control (D_1), standard drug group (D_2) and treatment group (D_3). Positive control group exhibited significantly ($P<0.05$) higher concentration (4.37±0.05 mg/dL) of serum creatinine as compared to negative control (0.95±0.05 mg/dL) and other treatment groups. Glibenclamide and extruded snacks diet treated groups exhibited a significant ($P<0.05$) reduction (2.16±0.04 and 2.62±0.11 mg/dL) that showed the improvement in renal function by treatment diet (Table 3.11.). However, the difference between groups treated with glibenclamide and fed on treatment diet was non-significant when compared between each other. The mean concentration of urea was significantly ($P<0.05$) raised in D_1 (70.43±3.74 mg/dL) than D_0 (43.72±3.87 mg/dL), D_2 (59.94±2.19 mg/dL) and D_3 (60.03±1.94 mg/dL), respectively (Table 3.11.). Glibenclamide and treatment diet in streptozotocin induced hyperglycemic rats significantly reduced ($P<0.05$) the urea levels that showed improvement in renal protective effect of protein rich diet (Table 3.10.). However, non-significant difference was observed between groups treated with drug (D_2) and fed on protein diet (D_3). Renal dysfunctioning due to persistent hyperglycemia resulted from high level of serum creatinine and urea levels in the blood. Results of current study have revealed significant increase ($P<0.05$) serum creatinine and urea content in diabetic rats while treatment with protein diet reduced the levels of these renal function markers that might support further the cure of renal system from injury[50, 51].

Table 3.10. Mean squares for renal stress biomarkers of rats fed on experimental diets

Source	df	Serum creatinine	Blood urea
Groups	3	8.03408**	486.744**
Error	12	0.00593	12.500
Total	15		

df = Degree of freedom **Highly significant = $P<0.01$

Table 3.11. Mean±SD for renal stress biomarkers of rats fed on experimental diets

Groups	Serum creatinine (mg/dL)	Blood urea (mg/dL)
D_0	0.95±0.05[d]	43.72±3.87[c]
D_1	4.37±0.05[a]	70.43±3.74[a]
D_2	2.16±0.04[c]	59.94±2.19[b]
D_3	2.62±0.11[b]	60.03±1.94[b]

D_0 = Negative control group fed on normal diet
D_1 = Positive control group fed on normal diet + streptozotocin
D_2 = Standard drug group fed on normal diet + streptozotocin + glibenclamide
D_3 = Treatment group fed on 20% DSM+80% barley containing diet + streptozotocin

3.7. Hepatic stress biomarkers

Liver functioning tests were done to evaluate the functioning and degree of liver damage due to streptozotocin induced diabetic anomaly. For this purpose, measurement of liver enzymes *i.e.*, alanine transaminase (ALT) and aspartate transaminase (AST) concentrations may serve as valuable source to diagnose the liver damage. Diabetes mellitus can cause damage to liver cells leading to release of liver enzymes into systemic circulation [50, 51]. Statistical analysis (Table 3.12.) revealed significant difference in serum AST concentration of negative control D_0, positive control D_1, standard drug D_2, and treatment D_3 groups. The mean concentration of AST was significantly increased ($P<0.05$) in D_1, positive control group (diabetic rats) 139.59±2.93 U/L as compared to negative control group, D_0 (54.33±3.50 U/L) (Table 3.13.). Glibenclamide (D_2) and extruded snacks diet (D_3) treated groups exhibited a significant decrease ($P<0.05$) and restored normal serum AST level. However, the overall mean for AST did not differ significantly between groups treated with drug and fed on protein diet. Statistical analysis (Table 3.12.) revealed that effect of different treatments on serum ALT level was significant. The mean serum concentration of ALT (Table 3.13.) was significantly raised ($P<0.05$) in D_1 (73.33±2.78 U/L) as compared to D_0 (29.72±2.94 U/L), D_2 (31.73±2.60 U/L), and D_3 (40.55±3.66 U/L), respectively. Glibenclamide and treatment diet treated groups for a period of 28 days resulted in significant reduction ($P<0.05$) and restoration in normal level of serum AST. Measurement of liver enzymes' concentration in serum may serve as a valuable mean for diagnosis of liver damage. A very important role is played by liver in carbohydrate metabolism as it stores glucose in the form of glycogen and is also involved in gluconeogenesis by utilizing non carbohydrate sources. Metabolic disorders especially diabetes mellitus can cause damage or injury to hepatocytes leading to release of intracellular elements including liver enzymes into systemic circulation. Results of current study revealed a significant rise ($P<0.05$) in the serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) in streptozotocin induced diabetic rats as compared to negative control group (D_0) possibly due to out-flow of hepatic enzymes from damaged hepatocytes into plasma [52]. However, the treatment with protein diet decreased and restored the normal serum AST and ALT concentrations.

Table 3.12. Mean squares for hepatic stress biomarkers of rats fed on experimental diets

Source	df	ALT	AST
Groups	3	4906.39**	6105.71**
Error	12	145.96	11.38
Total	15		

df = Degree of freedom **Highly significant = $P<0.01$

Table 3.13. Mean±SD for hepatic stress biomarkers of rats fed on experimental diets

Groups	ALT (U/L)	AST (U/L)
D_0	29.72±2.94^c	54.33±3.50^c
D_1	73.33±2.78^a	139.59±2.93^a
D_2	31.73±2.60^c	60.02±2.35^c
D_3	40.55±3.66^b	77.86±2.79^b

D_0 = Negative control group fed on normal diet
D_1 = Positive control group fed on normal diet + streptozotocin
D_2 = Standard drug group fed on normal diet + streptozotocin + glibenclamide
D_3 = Treatment group fed on 20% DSM+80% barley containing diet + streptozotocin
AST = Aspartate Transaminase
ALT = Alanine Transaminase

3.8. Histological examination

Histological examination is usually done to evaluate the tissue volume and cell structures of internal organs in order to manifestation of pathological conditions. In current study, histological examination of pancreas tissues was conducted to evaluate the adverse impacts of Streptozotocin. Moreover, the

therapeutic prospective of treatment diet (EB_{20}) in maintaining the cellular architecture against diabetes mellitus was also observed.

a. Pancreas

Histological investigation of pancreatic cells of negative control rats (D_0) showed normal appearance of islets of Langerhans and no signs of vacuolation of acinar cells were observed. In streptozotocin (STZ) induced diabetic rats (D_1) pathological alterations were observed in both exocrine and endocrine components of the pancreatic tissue. Vacuolation in almost all acinar cells was present and several acinar cells were swollen. Epithelium of interlobular ducts was flattened and almost all islet ß cells were lost. Glibenclamide showed not much protective influence in maintaining the cellular architecture of pancreas of rats receiving glibenclamide + STZ (D_2) however, increase in count of islets cells was observed. Treatment with barley and sunflower based (EB_{20}) extruded snacks (D_3) showed marked improvement in STZ induced pathological alterations as evident by it partially restored architecture of acini, islets of Langerhans, decreased epithelial vacuolation and ß cell damage. Moreover, islet ß cells count was also increased in pancreatic tissue of rats in D_3.

The current outcomes are strongly supported by [53] who observed pancreatic ß cells damage and swollen acinar in STZ induced diabetic rats. Likewise, [54] reported severe pancreatic damage in STZ induced diabetic rats moreover, the also observed no protective role of glibenclamide in restoring the ST induced pathological alterations in pancreatic cells. Barley and sunflower meals based extruded function snacks restored these damages which could be attributes to antioxidant potential of dietary components as several previous studies have shown that dietary components rich in antioxidants prevent the pancreatic cell damage, increases the islets area as well as reduces the STZ induced acini atrophy/swelling [55, 56].

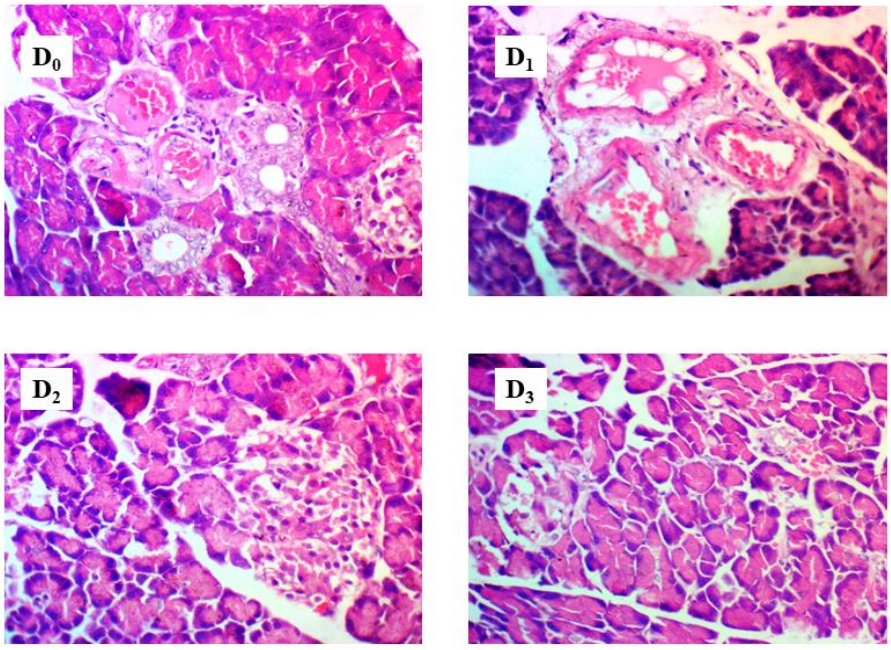

Figure 3.6. Histopathological indications of pancreas parenchyma. (D_0) Normal control rats (D_1) **Streptozotocin induced diabetic rats** (D_2) Glibenclamide treated diabetic rats (D_3) EB_{20} treated diabetic rats

4. CONCLUSION

Conclusively, barley flour enriched with plant protein comprising optimal mix of health benefiting nutrients that can be an ideal substitute for a range of value-added products. From this research study, we concluded that protein enriched barley based extruded snacks proved effective in the modulation

of blood glucose and insulin levels, HbA1c, thyroid hormones, hepatic and renal parameters. Moreover, its regular consumption didn't impart any toxic effect on kidney and liver functions. Concisely, value added foods developed utilizing cereals and by-products of oilseeds have the capacity to curtail the menace of hyperglycemia. Additionally, barley and sunflower seeds are easily available and cost effective. Hence, barley flour and oilseeds waste by-products *i.e.,* oilseeds meal or cake should be encouraged among the masses to ensure optimal health. Increasing the utilization of oilseeds by-products (defatted sunflower meal) stands for specific food applications and can benefit the food industry and economy of the country.

Supplementary Materials: N/A

Author Contributions: A.H: Data curation; Formal analysis; Resources; Writing - original draft. A.R: Methodology, Validation, Visualization, Writing - review & editing, Formal analysis. M.S.B: Conceptualization; Methodology; Supervision; Data curation, Laboratory Resources. Writing - review & editing. M.A: Methodology, Validation, Visualization, Review & Laboratory Resources.

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