



# *Pueraria tuberosa* as Dipeptidyl-Peptidase-IV Inhibitor Prevents Streptozotocin-Induced Intestinal Stress

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## Abstract

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**BACKGROUND:** Enhanced expression of dipeptidyl-peptidase-IV (DPP-IV) has been found in various intestinal diseases. *Pueraria tuberosa* tuber water extract (PTY-2) is already known to have DPP-IV inhibitory potential. At the mRNA level, this inhibition has not yet been investigated. Increased incretin secretion due to inhibition of DPP-IV could lead to the suppression of stress and apoptosis of intestinal cells.

**AIM:** In this research, we tried to study the antioxidant, anti-apoptotic, and DPP-IV inhibitory effect of PTY 2 against intestinal damage induced by STZ.

**METHODS:** We found morphological damage to the intestine following streptozotocin (STZ) injection (65 mg/kg bw) in male Charles foster rats through histological examination. Superoxide dismutase (SOD) and DPP-IV mRNA expressions were analyzed by polymerase chain reaction and cell apoptosis was examined by tunnel assay and Bcl 2 immunoexpression.

**RESULTS:** In STZ-induced diabetic control, the number and length of villi were decreased, but these damages were reversed by 10 days of PTY-2 treatment. SOD expression was found to be decreased whereas DPP-IV expression was enhanced with significant intestinal cell apoptosis in the diabetic control group. Treatment with PTY-2 decreases stress by upregulating SOD expression and by downregulating the expression of DPP-IV. These PTY-2 recoveries contribute to the suppression of apoptosis in the intestinal cells.

**CONCLUSION:** The protective action of PTY-2 against STZ mediated intestinal damage is demonstrated by these short studies. Therefore, PTY-2 may be taken as a herbal remedy for diabetes-induced intestinal damages.

## Introduction

Streptozotocin (STZ) has been used to induce diabetic experimental models since 1963. The STZ enters through glucose transporter 2 (GLUT 2) transporter and harms  $\beta$  cells by methylation of deoxyribonucleic acid (DNA) and by acting as a donor of nitric oxides. GLUT 2 transporters are also responsible for STZ uptake into the epitheliocytes of the intestinal mucosa, renal tubule cells, and hepatocytes, in addition to pancreatic  $\beta$ -cells. It means STZ is also toxic to the cells of other organ, which expresses the GLUT 2 transporter [1]. Several herbals have been studied to improve intestinal deformities against STZ-induced diabetes, such as *Azadirachta indica* [1], green tea extract, and ginseng root [2].

A cell-surface protease, dipeptidyl-peptidase IV (DPP IV)/CD26 belongs to the prolyl oligopeptidase family. By removing the N-terminal dipeptide with proline or alanine in the second position, it makes several bioactive peptides (Glucagon-like peptides [GLP-1, GLP-2], GIP, etc.) inactive [3]. Within the gastrointestinal tract, glucagon like peptides plays an important role

towards gut adaptation. After their synthesis, they get released into the intestine from the enteroendocrine cells. GLP-1 efficiently engages the enteric nervous system, thereby regulates gut motility and activates gut-brain axis that controls insulin secretion, glucose disposal in peripheral tissues, and blood flow [4], [5]. GLP-2 cosecreted from the L cells together with GLP-1 and acting more proximally in the gut to increase the absorption of the nutrient [5], [6]. The valine-pyrrolidide inhibition of DPP-IV decreases GLP-2 degradation and improves its intestinotrophic effect such as growth of the intestines [7].

The differentiation-dependent expression of DPP-IV in the crypt-villus axis of rat jejunum is primarily controlled at the level of messenger ribonucleic acid (mRNA) [8], according to a previous report. It is also reported that the promotion and formation of intestinal ulcers are prevented by DPP-IV inhibition [9]. Thus, DPP-IV inhibitors and glucagon like peptides play an important role in maintaining intestinal health. We have already reported the DPP-IV inhibitory role of *Pueraria tuberosa* tuber water extract (PTY 2) in blood and intestinal homogenate [10], [11], [12]. Here, we further studied the DPP-IV inhibitory property of PTY2 in the

intestinal duodenum at the mRNA level and used this property to focus on intestinal damage caused by STZ. *P. tuberosa* possesses many medicinal properties as an antioxidant [13], [14] and an anti-inflammatory agent [15]. It is highly effective in the treatment of diabetes [10], [11], [16], nephropathy [17], anxiolytic [18], anti-hypertension [19], etc. The key constituents of *P. tuberosa* are steroids, glycosides, triterpenoids, flavonoids, carbohydrates, tannins, proteins, alkaloids, and amino acids, such as puerarin 4',6'-diacetate, tuberosin, daidzin, genistein, puerarin, puerarone, puerarone, and tuberostan [10], [20], [21]. We have hypothesized that treatment with PTY 2 may be responsible for improving intestinal health. In this research, we tried to study the antioxidant, anti-apoptotic, and DPP-IV inhibitory effect of PTY 2 against intestinal damage induced by STZ.

## Materials and Methods

### Materials

Rabbit polyclonal Bcl 2 (26 KDa) (N-19): sc-492, EZ Retrieval System V.3 (Bio Genex), Zeiss LSM510 Meta confocal microscope, and Zen Black (2012) software was used for immunohistochemistry (IHC). For histology analysis, Hematoxylin-Eosin (HE), Nikon microscope (Eclipse 50i, loaded with imaging software-NIS Elements Basic research), and Leica RM2125 room temperature (RT) rotator microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany) were used "TACS® 2 TdT Fluorescein Kit - Trevigen" for the analysis of apoptosis. For reverse transcription polymerase chain reaction (RT-PCR), Trizol (HiMedia, Pvt. Ltd, Kolkata, India), cDNA Kit (Fermentas), and Taq-polymerase (GenaxysScientific Pvt.Ltd) were used.

### Sample preparation

*P. tuberosa* had been purchased from Banaras Hindu University's Ayurvedic Pharmacy. Our previous laboratory research [14] has already proven its authenticity. With 8 volumes of distilled water, 30 g of tuber powder have been extracted. When the volume decreased to one-fourth, the extract obtained was then filtered with cloth. Through this procedure, the total yield was 30% [16].

### Animals design

After acclimatization, Charles foster male rats of the same age group and a weight range of 120–130 g were injected STZ (65 mg/kg bw), prepared in fresh and chilled citrate buffer (pH 4.5), after overnight fasting for 8 h. The diabetic condition was checked on the 5<sup>th</sup> day with the use of glucometer strips (Dr. Morepen). Rats with blood glucose levels above 200 mg/dL were considered

diabetic and kept for 60 days to induce severe diabetes (three rats per cage). On day 61, the rats were divided into three groups (n = 6): Group-1 (STZ untreated rats, i.e., age-matched normal control), Group-2 (diabetic control), and Group-3 (PTY-2 at 50 mg/100 g bw treatment for 10 days to diabetic rats). After 10 days, the rats were sacrificed and intestinal duodenum was isolated. Each intestinal sample was divided into two parts; one for histology and IHC (preserved in 10% formaldehyde) and the other was first crushed in liquid nitrogen and then stored in –80°C freezer for molecular analysis [16].

### Hematoxylin and eosin (HE) staining

Intestinal tissues fixed with formalin were embedded in paraffin wax. Using Leica RM2125 RT rotator microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany), the tissues were cut into 4 µm thick sections. Every section was stained with HE and then imaged and observed using Nikon microscope (Eclipse 50i, loaded with imaging software-NIS Elements Basic research).

### Tunnel assay

Identification and quantification of apoptosis were done using a tunnel assay "TACS® 2 TdT Fluorescein Kit - Trevigen."

### Immunohistochemical staining

Dewaxed the intestinal tissues using xylene for 10 min and through 90%, 70% alcohol and water, tissues were then rehydrated serially in each for 5 min. Then after, each slide get dipped in citrate buffer and proceeded for antigen retrieval using EZ Retrieval System V.3 (Bio Genex). Sections were washed 2 times with phosphate buffered saline (PBS) for 10 min each, and then blocked with 0.1% Triton X-100, 0.1% bovine serum albumin, 10% fetal bovine serum, 0.1% sodium deoxycholate, and 0.02% Thiomersal (anti-fungal agent) in 1X PBS for 2 h at RT. All sections were then incubated overnight with the primary antibodies at 4°C and then washed with phosphate-buffered saline with tween 20 (PBST) thrice for 10 min each, followed by incubating each section with anti-rabbit-AF 546 (Red) (Invitrogen, USA) secondary antibodies at RT for 2 h. Again washed thrice in PBST for 10 min each. Finally, counterstained with DAPI (1 µg/ml DAPI in 1X PBS) and mounted on DABCO. Using Zeiss LSM510 Meta confocal microscope, all the slides were examined. Zen Black (2012) software was used for image analysis.

### RT-PCR

Using trizol, we have homogenized 50 mg of the intestinal tissue. With random hexamers and superscript II RNase H-RT, 5 µg of total RNA was

reverse-transcribed. For DPP-IV, 2  $\mu$ l cDNA, 0.2 mmol/L dNTPs, 1.5 mmol/L MgCl<sub>2</sub>, 0.5  $\mu$ mol/L of each primer, 2.5  $\mu$ l 10 $\times$  PCR buffer, and 1U Taq DNA polymerase were used. The reaction mix for Sod included 2  $\mu$ l c-DNA, 0.2mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 0.5  $\mu$ M of each primer, 2.5  $\mu$ l 10 $\times$  PCR buffer, and 1UTaq DNA polymerase. For glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 0.1  $\mu$ mol/L of each primer was used. Expressions optical density was determined and presented as a ratio against GAPDH with the help of an alpha imager (Bio-Rad). All the expressions were checked in triplicate (Table 1).

## Results

### Histological examination

STZ induces a hazardous effect on the intestinal morphology by reducing the overall length and number of intestinal villi. The PTY 2 significantly reversed the intestinal damage caused by STZ in 10 days of treatment. Therefore, PTY 2 enhances the surface area of the intestine, leading to enhanced absorption of nutrients and minerals (Figure 1).

### Apoptosis

The sections of STZ control groups have shown significant intestinal damage accompanied by cell apoptosis. However, PTY 2 treatment for 10 days significantly prevented this damage (Figure 2).

IHC analysis also proved the anti-apoptotic effect of PTY 2 on intestinal cells. It significantly reversed the downregulation of Bcl 2 expression induced by STZ (Figure 3).

### mRNA expressions

PTY 2 recovers the STZ induced stress; as a result, the expression of SOD was significantly enhanced. In the other hand, we have also found the reduced mRNA expression of stress marker DPP-IV in PTY 2 treated group. This clearly shows the potential of PTY 2 against STZ-induced intestinal damage (Figure 4).

## Discussion

During diabetes, the morphologies and functions of the small intestine get highly altered.

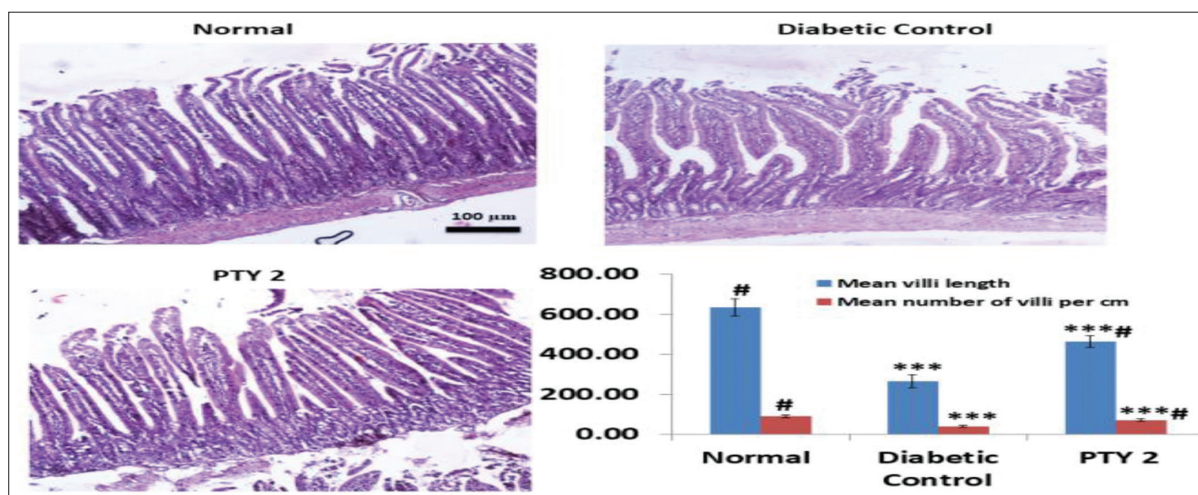


Figure 1: Hematoxylin-eosin staining to measure the morphological changes of intestinal villi. PTY-2 treatment showed a recovery of streptozotocin-induced villi damage. The image was taken at 20 $\times$ . Scale bar 100  $\mu$ m

Table 1: Details of PCR primer sequences, product size, and thermal steps for expressions of DPP-IV, SOD, and GAPDH

Primers	Sequence	Product Size (bp)	RT PCR Thermal steps					
			Initial denaturation	Denature	Anneal	Extension	Final Extension	
DPP-IV FORW	5'- ACTACTACTACGATTCCCATG -3'	546	No. of Cycle Temp. (°C) Time	30 95 2 min	Denature 1 min	Anneal 55 30 s	Extension 72 1.3 min	Final Extension 1 72 5 min
DPP-IV REV	5'- TGTACAGACCTGTTCCGGG -3'							
SOD FORW	5'-TCTAAGAAACATGGC GGTCC-3'	387	No. of Cycle Temp.(°C) Time	1 94 3 min	35 94 45 s	55 55 30 s	72 72 1.3 min	1 72 10 min
SOD REV	5'-CAGTTAGCAGGCCAGC AGAT-3'							
GAPDH FORW	5'-CACGGCAAGTTCAAT GGCACA-3'	244	No. of Cycle Temp.(°C) Time	1 94 3 min	35 94 30 s	58 58 30 s	72 72 45 s	1 75 5 min
GAPDH REV	5'-GAATTGTGAGGGAGAG TGCTC-3'							

RT-PCR: Reverse transcription polymerase chain reaction; SOD: Superoxide dismutase; DPP-IV: Dipeptidyl-peptidase IV; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

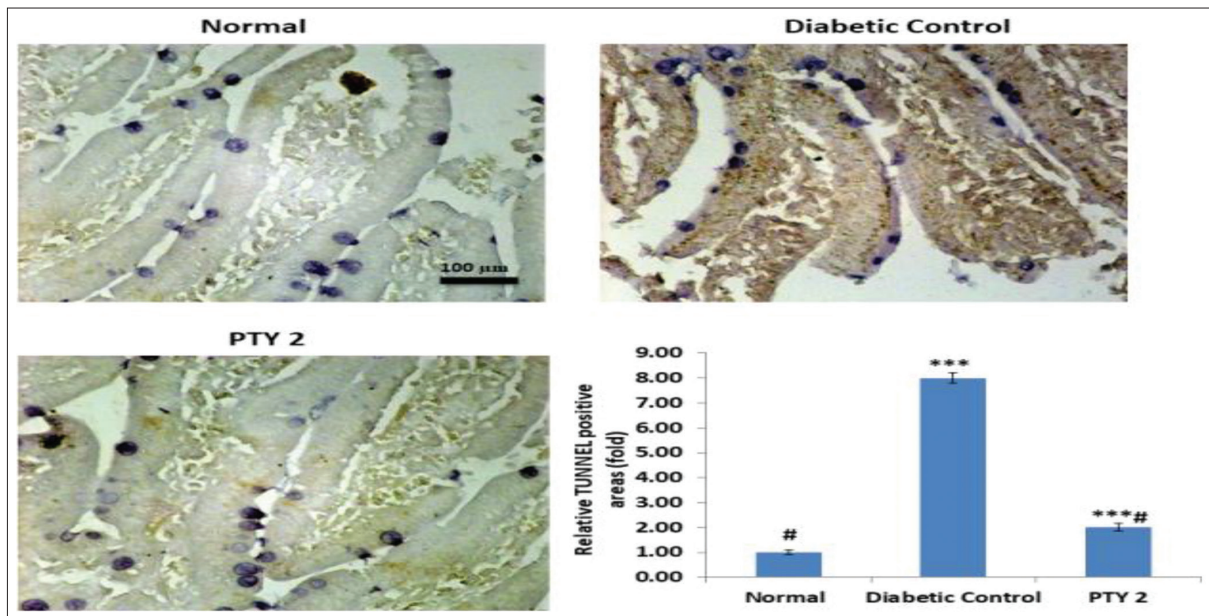


Figure 2: Tunnel assay analysis showed the effect of PTY-2 on intestinal cells apoptosis. PTY-2 recovers the apoptosis induced by streptozotocin. The image was taken at 40x. Scale bar 100  $\mu$ m. The brownish color indicates the TUNEL-positive area

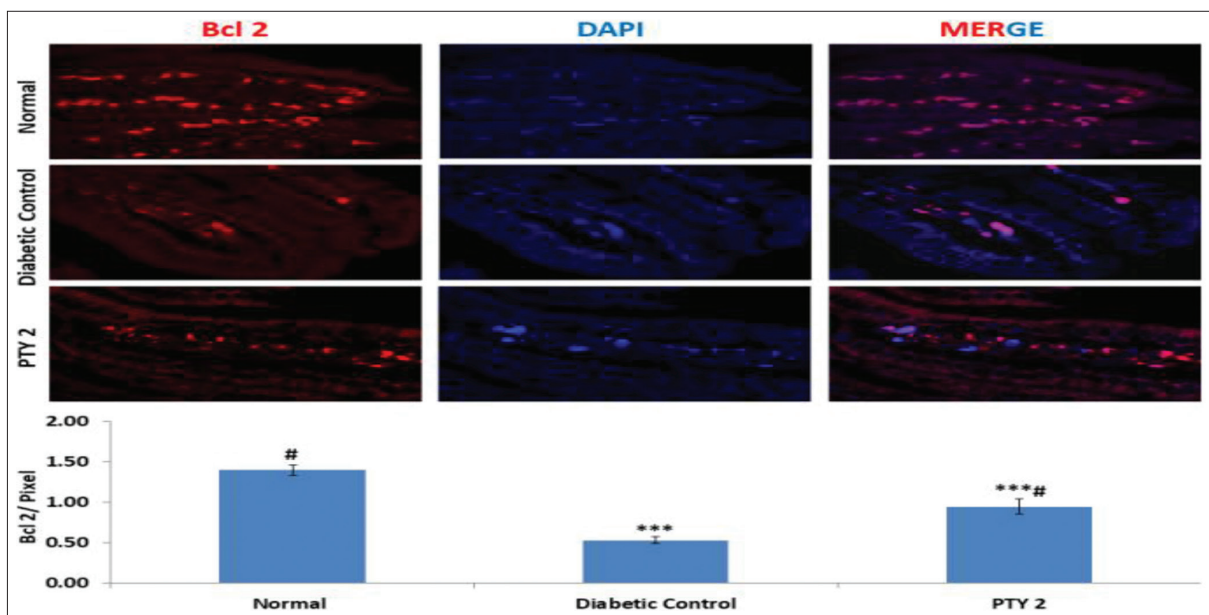


Figure 3: Immunohistochemistry analysis in the intestine of normal, diabetic control, and PTY-2 treated rats to show the effect of PTY-2 on the intestinal apoptosis. The expression was merged with DAPI (blue). In comparison to diabetic control, PTY-2 upregulated the expression of Bcl 2. The image was taken at 63x. The scale bar was 20  $\mu$ m. The intensity was measured in pixel values. Each value represent the mean  $\pm$  SD (n = 6); \*\*\*p < 0.05, compared with normal, # p < 0.05, compared with diabetic control

Some of these alterations are caused by the oxidative stress raised during diabetic complications, studied in STZ-induced diabetic rats [22]. An increase in the intestinal DPP-IV activity is associated with diabetes development [23]. In our case, the enhanced expression of DPP-IV, reduced expression of SOD, apoptosis, and deformities of intestinal morphology clearly indicates the high uptake of STZ by intestinal mucosa as discussed above. PTY 2 recovers this damages by upregulating the antioxidant enzyme SOD and downregulating DPP-IV mRNA expressions (Figure 4). Thus, reduced stress

leads to a significant increase in the number and length of villi as compared to STZ treated group (Figure 1). The apoptosis assay and Bcl 2 protein expression also showed the anti-apoptotic and protective effect on intestinal cells by PTY 2 (Figures 2 and 3).

GLP-1 assimilates nutrients while GLP 2 acts as a regulator of energy absorption by means of mucosal permeability, nutrient absorption, nutrient intake, gastric emptying, and gastric acid secretion [24]. Both GLP-1 and GLP 2 have been proved to play

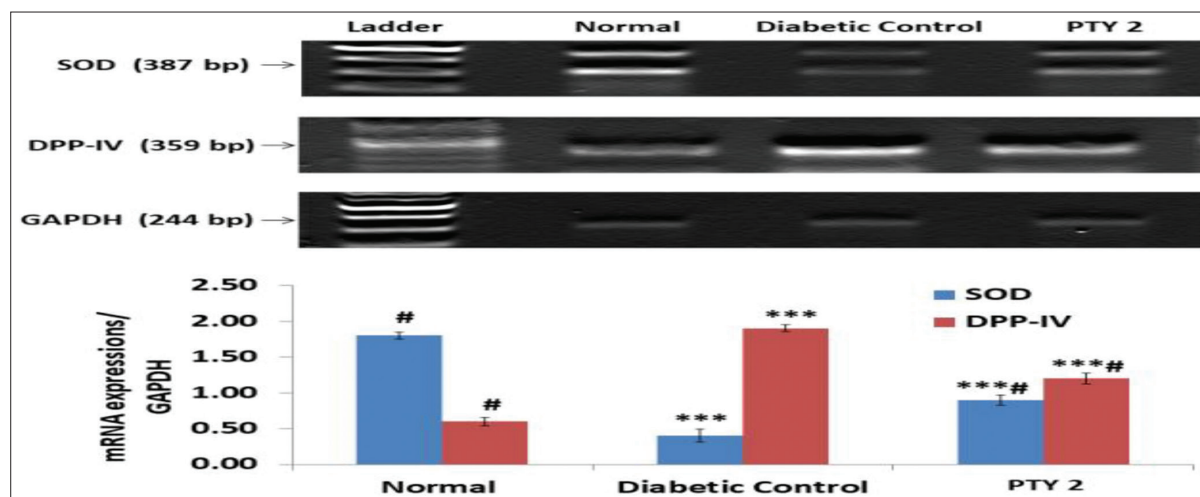


Figure 4: Messenger ribonucleic acid expression of superoxide dismutase and dipeptidyl-peptidase IV on the intestinal tissues of normal, diabetic control, and PTY-2 treated rats to investigate the effect of PTY-2 on stress. Each value represent the mean  $\pm$  SD ( $n = 6$ ); \*\*\* $p < 0.05$ , compared with normal, # $p < 0.05$ , compared with diabetic control

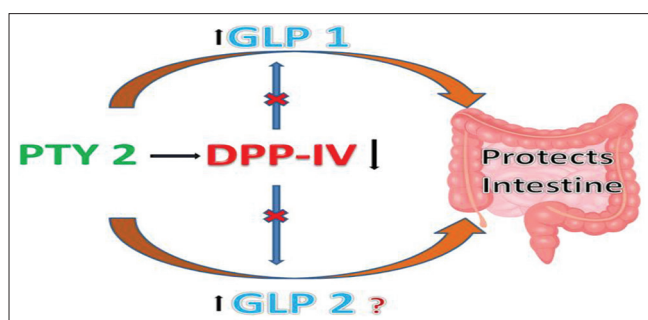


Figure 5: Signaling pathway of Pueraria tuberosa tuber water extract acting against diabetes-induced intestinal damage

the essential regenerative and healing role against intestinal injury in mice [25]. Through the mechanism involving Fgf 7, GLP-1R improves both small and large bowel growth [26]. GLP-2 administration in mice has been shown to regulate both cell proliferation and apoptosis as well as promote the increase in height of the small intestinal villous [27]. In our previous works, we have already proved PTY 2 as an incretin hormones (GLP-1 and GIP) enhancing agent. It significantly inhibits DPP-IV and enhances the levels of GLP-1 and GIP. In addition to DPP-IV inhibition, it also acts as an incretin receptor (GLP-1R and GIP-R) agonist [10], [11], [12], [16]. As DPP-IV inhibitor, PTY 2 must also enhance the intestinotrophic effect of GLP 2.

In Sprague Dawley diabetic rat model, STZ altered the microbiota compositions and decreased microbial diversity with time [28]. PTY 2 improves the villi count and length, thus enhances the surface area to assimilate more nutrients from diet and could also provide the maximum space for the colonization of positive bacteria useful for intestinal health. A detail of the study is needed to reveal the role of PTY 2 and its individual active constituents in the future at molecular,

microbial as well as clinical levels against diabetes-induced intestinal complications.

## Conclusion

PTY 2 recovers the STZ-induced stress, improves the intestinal morphology, increases the villi number and length as well as prevents apoptosis. As a DPP-IV inhibitor, PTY 2 increases the glucagon-like peptides which have positive intestinal health effects, thus, it must act as an effective herbal agent for the treatment of intestinal diseases (Figure 5). Overall, PTY 2 has multiple medicinal roles if taken with proper knowledge of preparation, dose, and duration.

## Authors' Contributions

S. Srivastava conceived, designed, implemented, and analyzed the data for all the experiments and wrote the manuscript. H and E was done by H. Pandey. Y.B. Tripathi, and S.K. Singh provided guidance for all levels from conception to manuscript writing.

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## Ethics Approval and Consent to Participate

Institute Ethical Committee (Dean/2015/CAEC/1266), Institute of Medical Sciences, Banaras Hindu University has approved the overall protocol.

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