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Evaluation of pancreatic regeneration activity of *Tephrosia purpurea* leaves in rats with streptozotocin-induced diabetes

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ABSTRACT

Background and aim: Flavonoid rich plant *Tephrosia purpurea* (*T. purpurea*), commonly known as Sarpunkha has been used in traditional systems of medicine to treat diabetes mellitus. However, its effectiveness in promoting regeneration of pancreas in diabetes has not been investigated. Therefore, the present study was undertaken to evaluate pancreatic β-cells regeneration, antioxidant and antihyperlipidemic potentials of *T. purpurea* leaves extract, its fractions and main constituent Rutin in diabetic rats.
Experimental procedure: The leaves extract and its fractions were first screened for acute and sub-chronic antidiabetic activity in a dose range of 250–500 mg/kg orally. Further, fractions with potent antidiabetic activity were screened for pancreatic β-cells regeneration activity using histopathological studies and morphometric analysis, which was followed by estimation of biochemical parameters.
Results and conclusion: The most significant antidiabetic, pancreatic regeneration and antihyperlipidemic activity was exhibited by *n*-butanol soluble fraction of ethanol extract at the dose level of 500 mg/kg. Histopathology revealed that treatment with this fraction improved the β-cell granulation of islets and prevented the β-cells damage which was further confirmed by morphometric analysis. Thus, the present study validated the traditional use of *T. purpurea* plant in the treatment of diabetes, which might be attributed to pancreatic β-cells regeneration potential of its active constituent Rutin.
Taxonomy (classification by EVISE): Traditional Medicine; Metabolic Disorder; Experimental Design; Cell Regeneration and Histopathology.
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1. Introduction

Diabetes mellitus (DM) is a multifactorial chronic disorder of carbohydrate, protein and lipid metabolisms. It is characterized by persistent hyperglycemia, hypercholesterolemia and hypertriglyceridemia. Chronic hyperglycemia results in micro-vascular complications to the organs like eyes and kidneys, lower-limb amputations, and increased risk of cardiovascular diseases such as hypertension and atherosclerosis, which contribute to diabetes-associated morbidity and mortality.[1–3] Defects in insulin gene expression in the islets of β cell and consequent decrease in insulin secretion are the major causes of glucose toxicity. Decreased levels of insulin gene transcription stimulatory proteins such as pancreas homeobox protein 1 (PDX-1) and musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) are responsible for declined insulin gene expression.[4] The hyperglycemia is controlled by multiple injections of insulin in type I diabetic patients, while type II diabetes is controlled by administration of oral hypoglycemic agents. Currently available treatments are expensive and have serious adverse effects.[5] Likewise, commonly prescribed

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List of abbreviations

DM	Diabetes mellitus
PDX-1	Pancreas homeobox protein 1
MafA	Musculoaponeurotic fibrosarcoma oncogene homolog A
WHO	World Health Organization
TPLE	Ethanolic extract of *Tephrosia purpurea*
TPLCS	Chloroform soluble fraction of *Tephrosia purpurea*
TPLBS	Butanol soluble fraction of *Tephrosia purpurea*
TPLBIS	Butanol insoluble fraction of *Tephrosia purpurea*
DPPH	2, 2-diphenyl-1-picrylhydrazyl
FC	Folin-Ciocalteu's
GAE	Gallic acid equivalents
NE	Naringin equivalents
RE	Rutin equivalents
TF	Total Flavonoid
TFA	Flavonoid content
TFO	Flavanone content
TP	Total polyphenol
HPTLC	High Performance Thin Layer Chromatography
TLC	Thin Layer Chromatography
IC	Inhibitory concentration
NO	Nitric oxide
OECD	Organization of Economic Cooperation and Development
STZ	Streptozotocin
µg	Microgram
p.o.	*Per oral*
CAT	Catalase
LPO	Lipid peroxidase
GSH	Glutathione peroxidase
SOD	Superoxide dismutase
HDL	High-density lipoproteins
TC	Total cholesterol
TG	Triglycerides
VLDL	Very low-density lipoproteins
LDL	Low-density lipoproteins
ANOVA	One-way analysis of variance
ROS	Reactive Oxygen Species.
TBARS	Thiobarbituric acid reactive substances

antidiabetic agents are contraindicated in DM patients suffered with renal, hepatic and cardiac failure.[6–8] Therefore, the use of safe, effective and inexpensive plant-derived bioactive compounds are appear to be improved alternatives for the treatment of DM.[9] Indeed, the World Health Organization (WHO) has also recommended traditional medicinal plant-based treatments for the patients suffering with DM.[10]

Numerous plant extracts and herbal biomolecule are being used or evaluated for their potential pancreatic regeneration properties.[11] Chard extract (*Beta vulgaris* L. *var. cicla*) has been extensively used in Turkey as a hypoglycemic agent due to its capacity to increase the number of β-cells and secretary granules during experimental study.[12] Likewise, the morphometric analyses revealed that *Aloe vera* extract has the potential to increase number as well as area of the pancreatic islets in diabetic rats.[13] Similarly, phytoconstituents such as epicatechin, nymphayol and flavonoid extracts of *Pterocarpus marsupium* possess β-cells regeneration capacity.[14–16] The flavonoid rich fraction of *Oreocnide integrifolia* demonstrated prominent newly formed islets of β-cells.[17] Flavonoids present in the aqueous stem bark extract of *Annickia polycarpa* produced hypoglycemic action due to pancreatic regeneration of β-cells.[18] Altogether, from the above survey it seems that the flavonoid rich plants/extracts have the potential to induce pancreatic regeneration.

Tephrosia purpurea L. (*T. purpurea,* Leguminosae), commonly called as Sarpunkha in Sanskrit is a copiously branched, sub-erect, herbaceous perennial plant, which occurs through the Indian subcontinent.[19] Phytochemical screening of *T. purpurea* revealed the presence of flavonoids as purpurin, purpuritenin, lanceolatin A, B, C, purpurenone, flavonoid glycosides like rutin and osyritin; sterols such as β-sitosterol and retenoids as deguelin, elliptone, rotenone and tephrosin.[19] An isoflavone, 7,4′-dihydroxy-3′,5′-dimethoxyisoflavone, and a chalcone, (+)-tephropurpurin, were also isolated from *T. purpurea* by activity guided bioassay method.[20]

Traditionally, *T. purpurea* has been utilized for treating DM and considered beneficial for disorders of kidney, liver and spleen.[21] Its extracts possess wide range of pharmacological activities such as antioxidant, anti-inflammatory,[22] hepato-protective,[23] wound healing,[24] immunomodulatory,[25] *in-vitro* anticancer against human cell lines (MCF-7)[26] and *anti-helicobacter pylori*.[27] By virtue of its antioxidant, anti-inflammatory, cytoprotective and wound healing activities, the extract seems to be effective in promoting regeneration of pancreas in diabetic condition.[24] However, experimental evidences in this regard are lacking. Thus, keeping the above context into consideration, the present study was designed to explore the pancreatic regeneration potential of flavonoid rich plant *T. purpurea* along with its antidiabetic, antioxidant and anti-hyperlipidemic activities.

2. Materials and methods

2.1. Chemicals and standard drugs

Streptozotocin (STZ), Folin-Ciocalteu's reagent and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. Moreover, the analytical grade solvents and chemicals were used in the present study.

2.2. Plant material, preparation of extract and its fractionation

The leaves of *T. purpurea* were collected locally and taxonomic authentication was done by the Botany Department, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur (India). A voucher specimen of the plant material has been preserved and deposited with specimen number RA 9779 in the Herbarium of Botany Department for future reference. One kg of leaves was dried, milled into a coarse powder and defatted with petroleum ether. Defatted material was then extracted with ethanol and concentrated to yield ethanol extract (TPLE; 28.07% w/w) in a rotary vacuum evaporator. TPLE was subjected to fractionation with chloroform and *n*-butanol to yield chloroform soluble fraction (TPLCS; yield: 20.46% w/w), *n*-butanol soluble fraction (TPLBS; yield: 57.83% w/w) and n-butanol insoluble fraction (TPLBIS; yield: 21.70% w/w) respectively.[28] Phytochemical and pharmacological evaluation of TPLE, TPLCS, TPLBS and TPLBIS was undertaken.

2.3. Phytochemical screening

The TPLE extract and its broad fractions TPLCS, TPLBS and TPLBIS were assessed for the presence of different phytochemicals viz.

tannins, saponins, flavonoid, alkaloids, carbohydrates, proteins, etc. employing standard methods.[29] After that, the extract and its fractions were subjected to quantification of flavonoids, total polyphenol and flavonone contents.

2.4. Determination of total polyphenol (TP), flavonoids (TFA) and total flavanones (TFO) contents

2.4.1. Total polyphenol (TP)

Folin-Ciocalteu's (FC) colorimetric method was used to determine total polyphenol content with Gallic acid as a reference standard.[30] Test sample (0.1 ml, n = 3) was mixed with 1 ml of FC reagent (1:10 with distilled water). After an interval of 3 min, 1 ml of saturated sodium carbonate (75 g/l) solution was added with distilled water to make it 10 ml. Reaction was allowed to take place for 90 min, the absorbance was measured at 725 nm (Shimadzu UV-VIS spectrophotometer 1600) compared with standard blanks prepared without the FC reagent. The concentration of total polyphenol content was reported as mg of Gallic acid equivalents (GAE)/g of extract. Linear regression equation of Gallic acid ($y = 0.009x + 0.099$, $r2 = 0.996$) was utilized for the determination.

2.4.2. Total flavonoids (TFA)

Aluminium chloride method was used for flavonoid content determination.[31] Rutin was used as a reference standard. Briefly, 1 ml of test solution (1 mg/ml) in triplicate were mixed with 0.1 ml of 10% aluminum chloride hexahydrate ($AlCl_3.6H_2O$), 1.5 ml of alcohol (95%), 0.1 ml of 1 M sodium acetate and 2.3 ml of distilled water in reaction flask. After incubating the reaction mixture at room temperature for 40 min, its absorbance was read at 435 nm against corresponding blanks prepared without adding $AlCl_3.6H_2O$. The results were expressed as mg of Rutin equivalents (RE)/g of extract. Linear regression equation of Rutin ($y = 0.014x - 0.017$, $r2 = 0.996$) was utilized for the determination.

2.4.3. Total flavanones (TFO)

Modified 2, 4-dinitrophenylhydrazine (DNPH) method was used to estimate Flavanone content by UV–Visible spectrophotometry, with Naringin as reference standard.[32] Serial dilutions of Naringin (500, 1000 and 2000 μg/ml) were prepared in methanol. Each dilution (1 ml) mentioned was mixed with homogenous mixture of 2 ml 2,4-DNPH (1% v/v) and 2 ml methanol. The reaction was allowed to complete at 50 °C for 50 min. At room temperature, reaction mixture was mixed with 5 ml potassium hydroxide (1% w/v) in 70% v/v methanol and allowed to react at room temperature for 2 min. The resulting mixture was diluted with 1:5 ratio of methanol and centrifuged at 1000 rpm for 10 min. Supernatant was collected, adjusted to 25 ml and its absorbance was measured at 495 nm. All the extracts (n = 3) were reacted with 2, 4-DNPH for determination of flavonone as described previously. The results were noted as milligrams of flavonone Naringin Equivalents (NE) per gram of extract. Flavanone content (TFO) was reported using linear regression equation of Naringin ($y = 0.156x + 0.564$, $r^2 = 0.976$). Since the flavonols, flavones and isoflavones formed complexes only with aluminium chloride, 2,4-dinitrophenylhydrazine was needed for reaction with flavanones.[33] Accordingly, total flavonoid content was obtained by adding the flavonoid and flavonone content (Table 1).

2.5. Quantitative estimation of Rutin by HPTLC[34]

Crude extracts and its fractions were standardized with respect to their Rutin content by using HPTLC (Camag, Switzerland). Ten μL of test and standard solution were applied on a precoated silica gel G 60 F_{254} TLC plate (E. Merck). Chromatogram was developed in solvent system containing Ethyl acetate:Formic Acid:Ethanol:-Water (100:11:11:26)[35], visualized at 366 nm and compared with standard Rutin. Quantitation was done using area under the curve method.

2.6. In vitro antioxidant studies

2.6.1. DPPH radical scavenging method

Free radical scavenging activity of extracts was determined using the DPPH method described previously.[36] Ascorbic acid was used as reference standard. Briefly, test samples and standard solutions were diluted to a series of concentration (0.001–2 mg/ml). Then, 0.1 ml aliquot of sample and standard in triplicate was mixed with 4.9 ml of 50 M DPPH solution (buffered at pH 5.5). After incubation for 30 min in dark, the absorbance was read at 517 nm. The activity was expressed in the form of IC50 values (concentration of extract that inhibited DPPH % formation by 50%) calculated by linear regression method.

2.6.2. Nitric oxide (NO) radical scavenging method

NO free radical scavenging activity of extracts was determined using Griess reagent (sulphanilic acid (0.33% w/v) + 0.1% w/v N-(1-Naphthyl) ethylenediamine in 1:1 ratio).[37] Ascorbic acid was used as a reference standard. Briefly, test samples and standard solutions were diluted to a series of concentrations (0.001–2 mg/ml). Then, 1 ml aliquot of sample and standard in triplicate was mixed with 4 ml sodium nitroprusside (5 mM) and 1 ml of pH 7.4 phosphate buffer saline. The reaction mixture was incubated at 25 °C for 2.5 h. After incubation, 4 ml of Greiss reagent was added into 1 ml of the reaction mixture and allowed to stand for 30 min. The reaction produced a red-violet colored chromophore with a maximum absorbance at 540 nm. IC50 values were calculated by linear regression method to report free radical scavenging activity.

2.7. Evaluation of antidiabetic activity in rats

2.7.1. Experimental animals

Adult Sprague-Dawley rats (~200g) were used for experimental studies in conformity with the protocols approved by Institutional Animal Ethics Committee (Permission ID: 23/2009/CPCSEA). The animals were kept at the animal house in a controlled environment and temperature (22 ± 5 °C with 12-h of light/dark cycle) and fed with a standard pellet chow diet and water *ad libitum*. Animals were acclimatized before the commencement of protocol.

2.7.2. Acute toxicity studies[38]

The acute toxicity studies comprised of test and control groups of female rats (n = 6). TPLE extract, TPLCS, TPLBS and TPLBIS fractions were administered in an increasing oral dose of 1, 3 or 5 g/kg in overnight fasted rats. The animals were kept under regular observation for behavioral changes, signs of adverse effects and mortality for 48 h following administration of extract and fractions. During observation period, the animals were on fed state.

2.7.3. Acute antidiabetic studies

2.7.3.1. Induction of experimental diabetes. The diabetes was induced in rats by STZ (65 mg/kg, i.p.).[39] STZ dissolved in freshly prepared citrate buffer (0.1 mol/L, pH 4.5) was injected in overnight fasted rats. Induction of diabetes (hyperglycemia) was confirmed following 72 h by measuring fasting blood glucose ≥250 mg/dl using hand-held glucometer.

2.7.3.2. Experimental design and treatment schedule. Overnight fasted diabetic rats were divided randomly into eleven groups (I–XI, n = 6) and treated as follows; Group I: Treated with vehicle

Table 1
Total Polyphenol, Flavonoid, Flavanone, Total flavonoid, Rutin content, Degree of polymerization and Antioxidant potential of Tephrosia purpurea extract and its fractions.

Sr. No.	Extract/ Fractions	Total Polyphenol Content (TP) (GAE mg/g of extract)	Flavonoid Content (TFA) (RE mg/g of extract)	Flavanone Content (TFO) (NE mg/g of extract)	Total Flavonoid Content (TF)#	Rutin content (% w/w of extract)	Degree of polymerization	Antioxidant activity (IC 50 values in μg/ml) DPPH NO
1	TPLE	140.59 ± 0.036**	24.07 ± 0.037**	2.46 ± 0.021**	26.53	22.88 ± 1.2802	5.84	1.65 23.94
2	TPLCS	20.5 ± 0.001	12.56 ± 0.020*	0.713 ± 0.002*	16.273	Nil	1.31	13.16 100.81
3	TPLBS	192.48 ± 0.017**	28.857 ± 0.38**	3.446 ± 0.005*	33.303	33.458 ± 1.2486	6.67	1.26 18.19
4	TPLBIS	14.25 ± 0.023*	10.92 ± 0.017	0.226 ± 0.0034	11.146	0.09 ± 0.0004	1.30	23.89 158.69

Results are represented as mean ± S.E.M. of three replicates: GAE, RE and NE: Gallic acid, Rutin and Naringin equivalents, respectively.
Total flavonoid content is determined by adding flavonoid content with flavanone content.
TPLE, ethanolic extract of *Tephrosia purpurea;* TPLCS, Chloroform soluble fraction of TPLE*;* TPLBS, n-butanol soluble fraction of TPLE; TPLBIS, n-butanol insoluble fraction of TPLE.
*Represents statistical significance ($p < 0.05$). **Represents statistical significance ($p < 0.001$).

(distilled water 10 ml/kg; *p.o.*); Group II: Treated with Insulin (4 IU/kg; *s.c.*); Group III: Treated with Rutin (100 mg/kg *p.o.*); Group IV–XI: treated with TPLE extract and its fractions at 250 and 500 mg/kg, *p.o.* doses, respectively. Blood samples were withdrawn for assessing antidiabetic potential at 1-, 3-, 5- and 24-h time-points following the administration of extract and fractions. The results were reported as mg/dl.[28]

2.7.4. Sub-chronic antidiabetic studies

Experimental animals were randomly divided into six groups ($n = 6$) as control and treatment groups. The diabetic control group treated with vehicle (Group I), the positive control group (Group II) received standard i.e. 4 IU/kg/day insulin (Torrent Pharmaceuticals Ltd., India). Group III received Rutin (100 mg/kg; *p.o.*). The remaining Groups IV-VI were treated with 500 mg/kg/day (*p.o.*) of TPLE extract, TPLCS fraction, and TPLBS for 21 days respectively. Body weight and fasting blood glucose levels (8 h fasting) using glucometer were measured on day 7, 14 and 21 of the study.[40] Finally, on day 21, the blood was withdrawn from retro-orbital plexus for various biochemical estimations. Animals were sacrificed under chloroform anaesthesia, and the pancreas was removed for biochemical estimations and histomorphometric studies.

2.8. Estimation of biochemical parameters

The biochemical parameters such as catalase (CAT), lipid peroxidase (LPO), glutathione peroxidase (GSH) and superoxide dismutase (SOD) were calculated in erythrocyte and pancreas by previously described method.[41] High-density lipoproteins (HDL), total cholesterol (TC) and triglycerides (TG) were determined[42] using standard kit (Span Diagnostics, India). Serum very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) level were determined using the following formula:

$$VLDL = TG/5$$

$$LDL = TC - (HDL + VLDL)$$

2.9. Pancreatic regeneration study

2.9.1. Tissue processing and histomorphometric analysis

Excised pancreas tissue from all groups were cleared, weighed and fixed for 1 h in 10% formalin and dehydrated with alcohol. Embedding of tissue was done in paraffin and sectioned at 4 μm on a rotary microtome (SHANDON, S325, England) throughout its length to avoid any bias due to changes in islet distribution or cell composition. For every pancreas, ten sections were chosen randomly at a set distance through the block (every 35th section), a procedure that has been shown to make sure the chosen sections are representative of the complete pancreas.[43] Sections were mounted on amino-silane coated glass slides and allowed to dry overnight. These sections were washed with xylene (4 times) and with absolute ethanol for 1 min and finally stained with haematoxylin and eosin (H and E). The slides of respective treatment group were assigned with unique code. Thereafter, a skilled observer, blind to the treatments performed morphometric evaluation and captured the photographs. Morphometric analyses determined the number of islet per section, mean area (μm) of individual islets, measurement of islet size on H- and E-stained section using Motic digital microscope equipped with Motic Image plus 2.0 software (Motic, China).[44] Cross-sectional area of focal tissue was measured using an image analysis system. The measurements were averaged cumulatively for each group and all these data were subjected to statistical analysis.

3. Statistical analysis

The data of fasting blood glucose, biochemical estimations and body weight were expressed as the mean ± S. E. M. Statistical comparison was carried out between the treatments and the control groups with one-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* comparisons test. Differences were considered statistically significant at $P < 0.05$.

4. Results and discussion

4.1. Phytochemical screening and quantitative estimation of Rutin

T, purpurea leaves extract (TPLE) revealed the presence of phytosterol proteins, flavonoids, carbohydrates and tannins. The phytochemical screening of TPLCS fraction showed the presence of flavonoids, sterols and terpenoids while that of TPLBS showed the presence of flavonoids, saponin and tannins. Carbohydrates, proteins, flavonoids and tannins were detected in TPLBIS fraction.

From the HPTLC analysis, Rutin content of TPLE and TPLBS was found to be 22.88% w/w and 33.458% w/w of extract respectively (Table 1). Thus, it is evident that the fractionation results in enrichment of flavonoids in TPLBS.

4.2. Determination of total polyphenol, flavonoid and flavanone contents

Flavonoids belong to polyphenol group and are known for health benefits such as antioxidant, antimicrobial, anti-allergic, anti-inflammatory, anticancer and antidiabetic properties.[45,46] Correspondingly, the extract and its fraction were estimated for

the presence of polyphenol; flavonoid and flavonone contents. The data obtained from the above analysis revealed the complex nature of extract as the content of flavonoid and flavanone represented only 17.12% (w/w) and 1.75% (w/w) of the TP in TPLE extract, and similar results were observed in all its fractions respectively. This finding suggested the high degree of polymerization or presence of other polyphenols such as tannins, flavanones, phenolic acids and isoflavones in the extracts. Degree of polymerization can be determined by the dividing TP with TFA contents.[47] The highest polymerization was observed in TPLBS fraction and it varies from 5.84, 1.31, 6.67 and 1.30 for TPLE, TPLCS and TPLBIS respectively (Table 1). Thus, TPLE fractionation resulted in an enrichment of phytoconstituents. Sterol and moderately hydrophobic polyphenol were separated in TPLCS fraction, while TPLBS fraction were enriched with majority of flavonoid and saponin.

4.3. *In vitro antioxidant studies*

An antioxidant is considered to be "any substance that when present at low concentrations compared to that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate".[48] Therefore, it is significant to determine the free radical scavenging activity using DPPH and NO methods altering in their initiators and targets so as to understand the biological doings of an antioxidant. Based on studies and clinical data, increased production of ROS in both types of diabetes and oxidative stress is mainly accountable for onset of diabetes through nonenzymatic protein glycation and its oxidative degradation[49,50] and for that reasons, the antioxidant potential of extract and its fraction were measured. The results of DPPH scavenging activity showed that the IC50 values of TPLBS fraction (1.26 μg/ml) were significantly lower amongst other fraction and extract (Table 1). This may be recognized due to strong hydrogen donating capacity of polyphenol present in TPLBS fraction to reduce DPPH and the weak abilities of other extracts.[51] Similarly, TPLBS fraction showed significant antioxidant potential against NO radical due to its high polyphenol and flavonoid contents. Thus, polyphenols are noteworthy contributors to measured activities. This is in agreement with previous reports suggesting strong correlation between polyphenol and antioxidant potential of plants.[52,53]

4.4. *Anti-antidiabetic activity*

The results of acute toxicity studies demonstrate no lethality or toxic reactions at any selected dose. Comparing with the diabetic control, the TPLE extract, TPLCS and TPLBS fractions and Rutin showed significant reduction in blood glucose levels in acute antidiabetic study (Fig. 1). The insulin at dose level of 4 IU/kg showed 80.98% reduction in plasma glucose after 24 h (529.25 ± 14.5 mg/dl at 0 h to 103.25 ± 3.95 mg/dl; $P < 0.001$). The 80.49% reduction in blood glucose by TPLBS fraction was as potent as standard drug insulin. Similarly, TPLCS fraction also showed significant blood glucose lowering activity (73.49% at 24 h at 500 mg/kg, p.o.). Biphasic response was observed in extract-and fractions-treated animals at 1-, 3- and 5-h of treatment. TPLCS fraction showed reduction in blood glucose at 1- and 3-h, while both TPLE and TPLBS fraction showed significant reduction only after 5 h of treatment. TPLBIS did not show antidiabetic activity at both the selected doses. On the basis of above results, TPLE extract, TPLBS and TPLCS fractions showing significant antidiabetic activity were screened for sub-chronic activity along with Rutin as flavonoid standard and insulin as positive control.

In sub-chronic antidiabetic study, TPLE (58.55%) and TPLBS (79.31%; compared with day 0 blood glucose) extracts at dose of 500 mg/kg (Fig. 2) caused a significant reduction in fasting blood glucose of experimental rats as compared to TPLCS (46.21%) on day 21 of treatment. The fall was marked even in the 1st week and went on progressively increasing at the end of 3rd week in contrast with the blood glucose of untreated diabetic rats which remained elevated throughout the experimental period. Rutin and insulin showed prominent reduction in blood glucose levels at their selected doses. The increased antidiabetic potential of TPLBS fraction over TPLE extract may encounter due to improved antioxidant potential, high percentage of polyphenol, Rutin content and presence of additional phytoconstituents, synergism of which may have resulted in significant antidiabetic activity in the later weeks of treatment. Earlier findings also suggested that, synergistic effects of bioactive compounds such as flavonoids, saponins and glycosides are responsible for beneficial effect in diabetes.[54] Additionally, previous findings suggested that Rutin showed prominent antidiabetic potential in STZ induced diabetes via different mechanisms.[55,56] Furthermore, the fractionation of extract results in increase degree of polymerization and segregation of phytoconstituents such as steroids, complex polyphenols and increases degree of polymerization. These results are in conformity with previous studies that cytotoxic, anti-diabetic, anti-inflammatory and immunomodulatory activity increases with the increase in degree of polymerization of extract.[28,57] These results clearly demonstrated the antidiabetic efficacy of TPLE and its fraction.

4.5. *Effects on body weight and lipid profile*

During experimental study, it was observed that, as the disease progressed, the changes in body weight of animals also occurred. After 21 days of treatment, the body weight of normal-, Insulin-, Rutin-, TPLE- and TPLBS-treated groups increased significantly by 22.5%, 18.83%, 16.11%, 19.22% and 30.48% respectively. Whereas, the body weight of diabetic control group was decreased by 22.5%. The decrease in body weight with diabetes mellitus has been attributed to the gluconeogenesis i.e., metabolism of proteins and fats, which is associated with the characteristic loss of body weight due to increased muscle wasting and loss of tissue proteins.[58] Treatment with fractions showed improvement in body weight may be due to reversal of gluconeogenesis.

Hypertriglyceridemia and hypercholesterolemia are the most commonly observed defects in diabetes due to abnormality in lipid metabolism. STZ administration had resulted in significant ($p < 0.001$) elevation of TG, TC, VLDL, LDL, and reduction of HDL levels in rats, compared to normal controls. Most prominent reduction in plasma LDL, TG and total cholesterol was found to be 65.14%, 52.23%, 72.25% and 68.66%, 77.98%, 53.80% in insulin treated group and TPLBS fraction respectively as compared to diabetic control. The HDL, TC:HDL and LDL:HDL ratios were significantly improved as compared to diabetic group by +197.30%, −75.88%, −82.40% and +221.05%, −79.14%, −85.87% in insulin treated group and TPLBS fraction respectively (Fig. 3). The lipid level in diabetic rats was brought near to normal level after 3 weeks treatment with TPLBS fraction.

4.6. *In-vivo antioxidant activities in erythrocytes and pancreas*

Significant changes in antioxidant parameters (SOD, CAT, GSH and LPO) in the erythrocytes and pancreas of experimental animal groups were observed and recorded. SOD, CAT and GSH levels were decreased, while LPO level was increased significantly in diabetic rats as compared to normal rats. However, the extract and its fractions were not successful in fully improving the altered level as noticed in normal groups. The clinical findings have shown that there is a strong correlation between free radicals, lipid peroxidation (LPO) and blood glucose level during the progression of

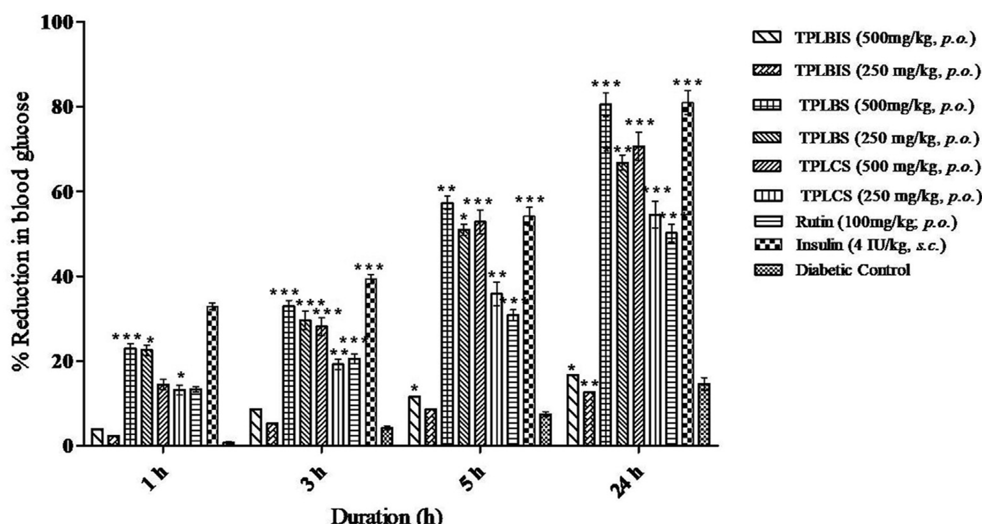

Fig. 1. Effect of acute treatment with *T. purpurea* leaves extract and its fractions on blood glucose levels in diabetic rats. Extract and its fractions were administered to diabetic rats and the blood samples were withdrawn at 1-, 3-, 5- and 24-h time-points for assessing antidiabetic potential. The bars values represent the mean ± S.E.M. The data were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 vs. diabetic control. TPLE, ethanolic extract; TPLCS, chloroform soluble fraction of ethanolic extract; TPLBS, *n*-butane soluble fraction of ethanolic extract, TPLBIS, *n*-butane insoluble fraction of ethanolic extract.

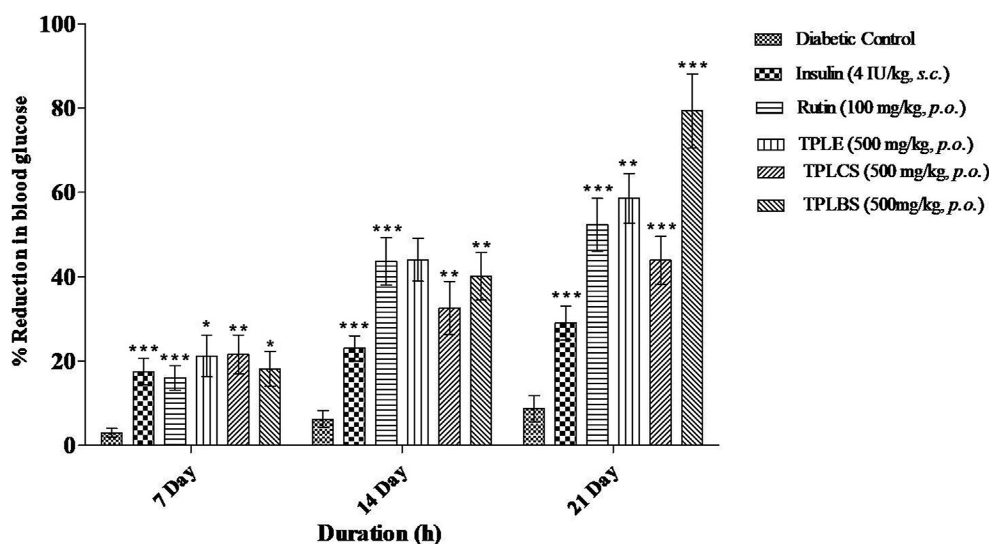

Fig. 2. Effect of sub-acute treatment with *T. purpurea* leaves extract and its fractions on blood glucose levels in diabetic rats. Extract and its fractions were administered to diabetic rats daily for 21 days and the blood samples were withdrawn on day 7, 14 and 21 for assessing antidiabetic potential. The bars values represent the mean ± S.E.M. The data were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001vs. diabetic control. TPLE, ethanolic extract; TPLCS, chloroform soluble fraction of ethanolic extract; TPLBS, *n*-butane soluble fraction of ethanolic extract.

diabetes.[59] Increased LPO impairs membrane function which includes reduced erythrocyte survival, increased membrane rigidity, lipid fluidity and decreased cellular deformability. Lipid peroxidation levels significantly increased in the blood and pancreas as evidence by thiobarbituric acid reactive substances (TBARS) as compared to the control group during the experiment period. Treatment with TPLBS extract (500 mg/kg, *p.o.*) significantly inhibited the increased TBARS in pancreas and blood on 21st day to 80.22% and 50.99% respectively. However, Insulin and Rutin significantly inhibited the increased in LPO level (78.29, 75.01% and 54.49, 41.93%; $p < 0.001$) and normalized it to near normal level in pancreatic tissue and hemoglobin. Whereas, treatments with TPLE and TPLCS group had also shown 41.76 and 67.95% increase in LPO level in pancreas, and 28.75 and 41.23% increase in blood (Table 2).

SOD and CAT are considered as first line of antioxidant defense mechanism since they are involved in the direct removal of ROS and protect the damage caused by them.[60] It was observed that SOD, CAT and GSH activities were decreased in pancreas and blood in STZ-induced diabetic rats compared to normal rats. Treatment with TPLBS resulted in increased SOD, CAT and GSH levels by 65.43, 130.68, 129.33% in pancreatic tissue and by 93.33, 110.76, 110.08% in blood (Table 2). These findings suggested that TPLE and TPLBS elicit *in-vivo* antioxidant potential by attenuating the lipid peroxidation brings out by a range of free radicals. The observed increase in antioxidant status and decline in TBARS concentration proposed its potent antioxidative and antilipidperoxidative effects. This is in

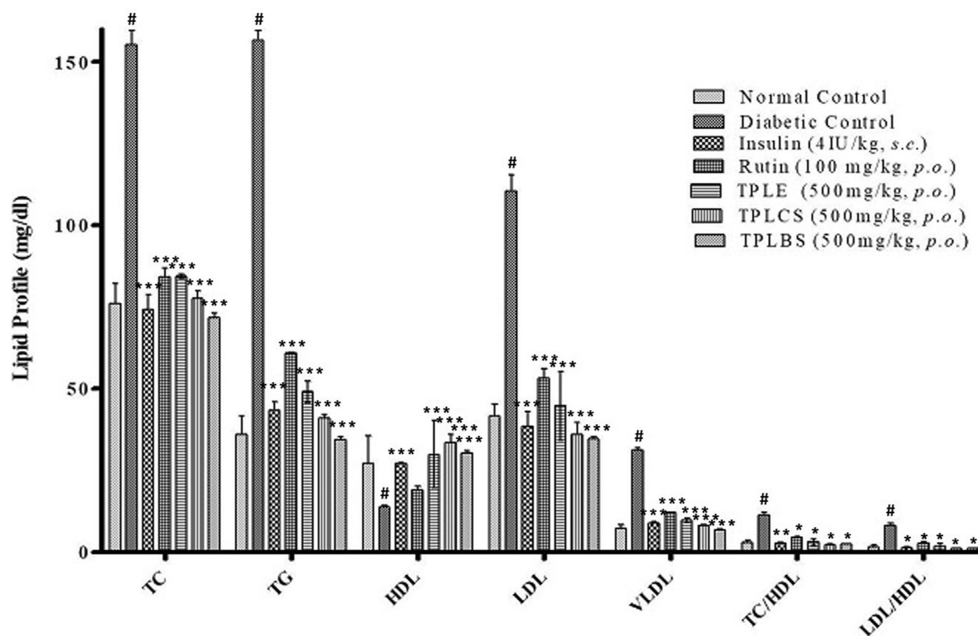

Fig. 3. Effect of *T. purpurea* leaves extract and its fractions on lipid parameters in diabetic rats. Extract and its fractions were administered to diabetic rats daily for 21 days and the blood samples were withdrawn on 21 for assessing lipid parameters. The bars values represent the mean ± SEM. The data were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* comparisons test. #P < 0.001 vs normal control; *P < 0.05, **P < 0.01, ***P < 0.001 vs. diabetic control. TPLE, ethanolic extract; TPLCS, chloroform soluble fraction of ethanolic extract; TPLBS, *n*-butane soluble fraction of ethanolic extract.

Table 2
Effect of *T. purpurea* extract and its fractions on SOD, GSH, CAT and LPO in STZ-induced diabetic rats in pancreatic tissue and erythrocytes.

Treatment	Pancreatic Tissue				Erythrocyte lysate			
	SOD (nM/g of tissue)	CAT (U/mg of protein)	GSH (nM/mg of protein)	LPO (nM/g of protein)	SOD (U/mg of protein)	CAT (U/mg of protein)	GSH (nM/mg of protein)	LPO (nM/g of protein)
Normal Control	67.58 ± 2.56	85.28 ± 3.25**	52.31 ± 1.26	13.2 ± 1.1**	6.32 ± 0.255	111 ± 2**	15.024 ± 0.21	14.2 ± 0.356*
Diabetic Control	40.65 ± 3.1*	35.2 ± 2 .31*	22.36 ± 0.99	70.25 ± 1.1	3.30 ± 0.28*	52.5 ± 1.38	7.14 ± 0.44**	28.59 ± 1.53*
Insulin (4 IU/kg, *s.c.*)	63.89 ± 1.65**	79.56 ± 5.54	47.38 ± 2.4***	15.25 ± 1.1***	6.18 ± 0.04	106 ± 1.24	14.1 ± 1.01*	13.0 ± 0.8***
Rutin (100 mg/kg, *p.o.*)	60.23 ± 3.78*	79.2 ± 1.25*	49.7 ± 1.87 **	17.5 ± 0.42***	4.51 ± 0.02	87.85 ± 3.14*	14 ± 1.6**	16.6 ± 0.21***
TPLE (500 mg/kg, *p.o.*)	58.45 ± 4.1*	74.2 ± 2.47	39 ± 1*	40.91 ± 1.26*	4.35 ± 0.27*	87.45 ± 2.23*	9.0 ± 1**	20.37 ± 1.89
TPLCS (500 mg/kg, *p.o.*)	60.26 ± 2.46**	78.9 ± 2.35**	45.29 ± 4.3	22.51 ± 1.45	5.9 ± 0.22	99.85 ± 2.4	12.1 ± 0.56	16.8 ± 0.45**
TPLBS (500 mg/kg, *p.o.*)	67.25 ± 3.78	81.2 ± 2.25*	51.28 ± 1.45**	13.89 ± 1.78*	6.38 ± 0.61	110.65 ± 1.28*	15 ± 0.23*	14.01 ± 0.27

TPLE, methanolic extract; TPLCS, chloroform soluble fraction of methanolic extract; TPLBS, *n*-butane soluble fraction of ethanol extract.
The data are expressed in mean ± S.E.M.
* Represents statistical significance vs. diabetic control ($p < 0.05$).
** Represents statistical significance vs. Diabetic control ($p < 0.01$).
*** Represents statistical significance vs. Diabetic control ($p < 0.001$).
# Represents statistical significance vs. Normal control ($p < 0.01$).

agreement with previously published report.[58]

4.7. Pancreatic regeneration activity

4.7.1. Histological examination and morphometric analysis of islet

Histological studies were performed by morphometric analysis to verify the restoration of pancreatic cells. Treatments with TPLBS fraction and TPLE extract resulted in improvement in the β-cell granulation of islets as compared with control group. The islets were apparently normal in the architecture of the nucleus, which revealed that TPLE and TPLBS fraction prevented the β-cells damage compared to control rats. However, treatments with TPLCS fraction for 21 days resulted in partial improvement in pancreatic tissue integrity. The number of T-infiltrated and degranulated islet cells were reduced in TPLCS fraction compared to diabetic control group (Fig. 4A–G).

Morphometric analysis confirmed the pancreatic regeneration, where the size distribution pattern reflected a greater number of smaller islets in TPLBS and TPLE (25 ± 1.2 and 17.2 ± 1.56; $p < 0.001$ respectively) in contrast to normal and control group, thus, representing the development of neo-islets. In normal group of animals, islets were more of larger in size (2000–2500 μm perimeter; $n = 6$). The larger islet were also clear in control group but these islets were mostly degranulated having central hyalinization with T-cell infiltration.

The average number of islets were increased in TPLBS and TPLE

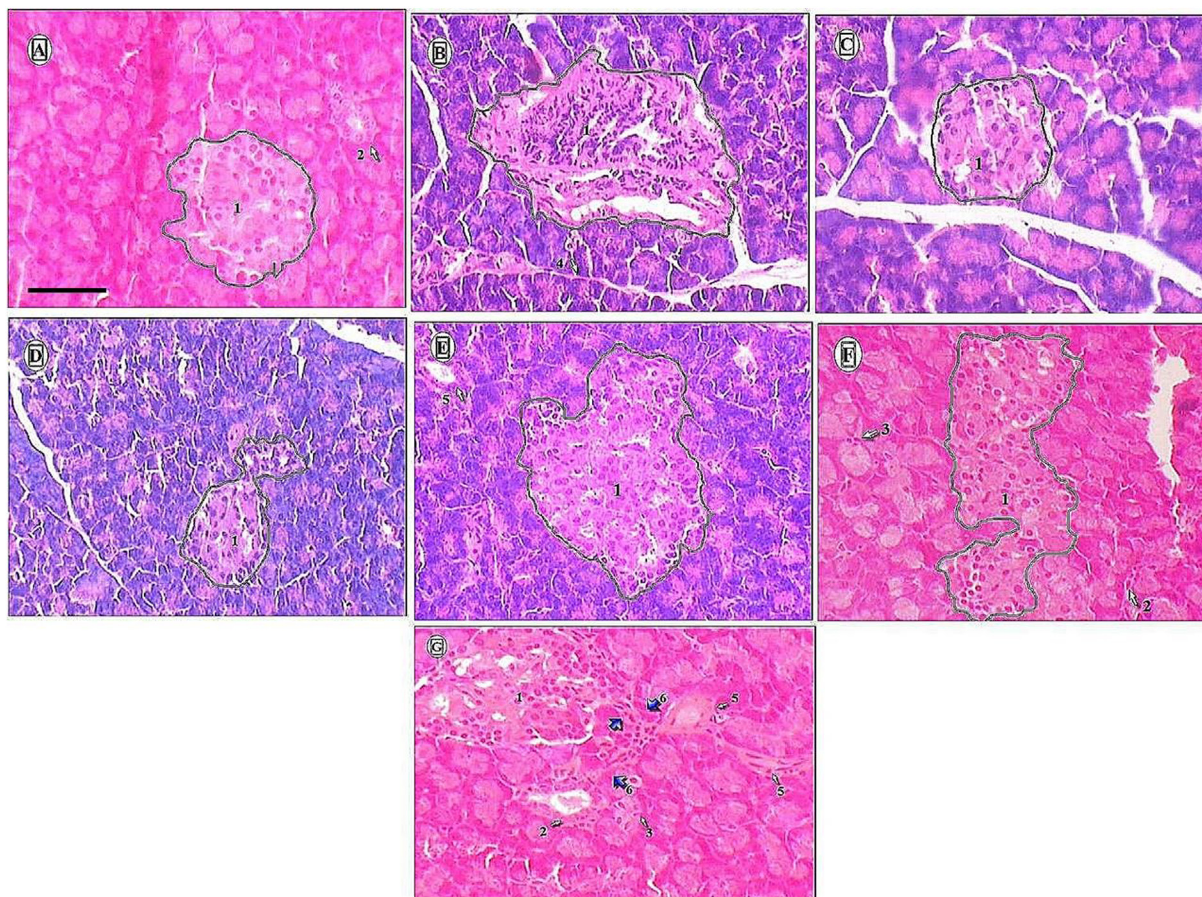

Fig. 4. Light photomicrographs of pancreatic sections from different experimental groups. Haematoxylin and eosin stained histopathological sections of pancreas after 21 days treatment (A) Normal control: Native architecture of islet and pancreatic tissue (B) Diabetic control: Islet of Langerhans displaying degenerative and necrotic changes in diabetic control rats (C) Insulin group: Islet of Langerhans displaying degenerative changes and less β cell granulation compare to normal rats (D) Rutin group: Islet of langerhans displaying increase in size with degenerative changes comparing to normal control group (E) TPLE group: dumbbell shaped pancrease representing the replication of islet on treatments with TPLE (F) TPLCS group: Islet of langerhans displaying increase in size and very light degenerative changes comparing to diabetic control group (G) TPLBS group: Enlarged islet of langerhans with architecture of cells very near to normal control group rats. Figs. A-C are taken from our previous study.[29] (Magnification × 200, scale bar = 4 μm) 1: newly formed islet. 2: Intralobular duct from which the islet sprouted. 3: Glandular Acinus. 4: Intralobular connective tissue septa. **5**: Blood vessels **6**: Connection between the duct and the islet.

by 11.18 and 9.75 fold (0.0868 ± 0.0021 vs. 0.97 ± 0.029 and 0.846 ± 0.035 in TPLBS and TPLE respective group) and this effect was more pronounced than Insulin and Rutin treated groups. Similarly, 540.27% and 293.40% increase in the average islet area (per sq. μm) was observed in TPLBS- and TPLE-treated groups compared to diabetic control respectively. These findings were in accordance with previous studies, where a significant reduction in the islet diameter, area, number and volume was observed in STZ-induced rats.[44,61] However, the average islet area of TPLBS- and TPLE-treated groups was only 73.63% and 56.72% of islets area of normal group. However, the average size of islet was increased only in insulin treated diabetic rats (184.54 and 116.78 μm^2 X 10^{-3}, respectively) compared to normal control rats (76.95 μm^2 X 10^{-3}). While there was a marginal improvement in distribution pattern and islet area on treatment with TPLCS fraction. The pancreatic regeneration potential exhibited by Rutin treated group was less pronounced than TPLBS and TPLE fraction (Table 3).

Thus, it is confirmed that induction of islet neogenesis is possible after regenerating pancreas in STZ-induced diabetic animals. These neo-islets, most likely by islet β-cell replication were further grow into larger mature islets. These effects could rationalize the enhanced pancreatic size and islet number therefore improved insulin secretion and superior glycemic control. This result further supported the hypothesis of possible restoration of islets, thus accomplishing the normal levels of insulin.[13] The current study is the first one that showed improvement in the total number of islets, islet diameter, islet area, and islet volume with reference to the distribution, area, volume, diameter, and number of islets in each group after treatment with *T. purpurea* extract and its fractions. Our findings did suggest that, TPLE and TPLBS fractions showed a preventive effect in diabetes by regenerating pancreatic cells as they contain a polyphenolic flavonoid, Rutin and its protective role was already established. Also, histopathological study of the pancreas of Rutin-treated diabetic rats showed islets expansion and decreased fatty infiltrate of the islets.[62] Furthermore, some studies also reported that Rutin could prompt the intact functional β cells and/or protect them from further deterioration, which is necessary for insulin production.[14,15,63] The effects of Rutin were also tested by using it as a supplement with regular medications for diabetes. It was observed that Rutin supplementation decreases the fasting blood glucose level significantly and its withdrawal reverses the levels back to pathologic levels which reflect its preventive effect.[64]

5. Conclusions

In spite of the introduction of hypoglycaemic agents, diabetes and the related complications continue to be a major medical

Table 3
Effect of *T. purpurea* leaves extract and its selected fractions on islet size and there distribution pattern in STZ-induced diabetic rats after treatment for 21 days.

Islet size (μm^2)	Treatment					
	Distribution of islet as per size					
	Normal Control	Diabetic Control	Insulin (4 IU/kg *s.c.*)	TPLE (500 mg/kg; *p.o.*)	TPLCS (500 mg/kg; *p.o.*)	TPLBS (500 mg/kg; p.o.)
<50	5 ± 1.2	2.5 ± 0.12	2 ± 0.15	7.66 ± 0.58*	17.2 ± 1.2***	25 ± 1.2***
51–100	4 ± 0.3	5 ± 0.136	4 ± 0.12	3 ± 0.24	3 ± 0	5 ± 0.57
100–150	1.5 ± 0	3 ± 0.21	2 ± 0.6	0.33 ± 0	2 ± 0.37	7 ± 0.82**
150–200	1 ± 0.01	2 ± 0.08	0	1 ± 0	1 ± 0.1	0
200–250	0.5 ± 0.21	0.5 ± 0.001	2 ± 0.11	0	0	0
Average no. of islet per sq. μm of pancreas (X 10^{-3})	0.51 ± 0.016	0.28 ± 0.002	0.0868 ± 0.0021	0.106 ± 0.001	0.846 ± 0.035***	0.97 ± 0.029***
Average size of islet (μm^2 X 10^{-3})	76.9 ± 1.29	116.78 ± 2.37$^{\#}$	184.54 ± 8.98$^{\#}$	56.69 ± 1.28	36.67 ± 1	47.79 ± 1.3
Islet area per sq. μm of pancreas	3.285 ± 0.24	2.4883 ± 0.186	1.6018 ± 0.012	0.499 ± 0.002	0.845 ± 0.08	1.256 ± 0.012

TPLE, ethanolic extract; TPLCS, chloroform soluble fraction of ethanolic extract; TPLBS, *n*-butanol soluble fraction of ethanol extract.
The data are expressed in mean ± S.E.M.
* Represents statistical significance vs. diabetic control ($p < 0.05$).
** Represents statistical significance vs. Diabetic control ($p < 0.01$).
*** Represents statistical significance vs. Diabetic control ($p < 0.001$).
$^{\#}$ Represents statistical significance vs. Normal control ($p < 0.01$).

problem as the current therapies provide only symptomatic relief and does not cure the diabetes. *T. purpurea* plant extracts exhibited significant antidiabetic potential and improved associated complications. Fractionation of extract resulted in segregation of flavonoid and polyphenol in the *n*-butanol fraction of plant extract, which in turn increased *in-vitro* antioxidant potential. Administration of flavonoid rich TPLBS fraction resulted in reduction in fasting blood glucose level near to normoglycemia either due to replication or expansion of the existing residual islets or formation of neo-islets as evident in histological examination and morphometric analysis of pancreatic tissue. This was further supported by reversal of *in-vivo* antioxidative parameters, reversal of body weight loss and antihyperlipidemic activity. These results unite the observation that pancreatic regeneration is a beneficial approach in the treatment of diabetes and this effect is attributed to the presence of higher percentage of flavonoid and active constituent Rutin. Many previous findings revealed the cytoprotective role of Rutin by down-regulating the over-expression of nuclear factor kappa B,[65] cytokines[66] and cyclooxygenase[67] signaling pathways. Thus, various underlying mechanisms suggest that flavonoids are responsible for preserving pancreatic beta cells survival and their function.[68] Further research is needed to reveal the exact molecular mechanisms involved.

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Authors' contribution

SA and PV contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the data and draft of the paper. SP and KTN contributed in conducting *in vitro* antioxidant and antidiabetic activities respectively. The concept of the study was designed and supervised by PI. All the authors have read and agreed the manuscript for submission.

Footnotes

Pancreatic regeneration potential of *Tephrosia purpurea* in diabetes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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