

Antidiabetic effect of *Punica Granatum* Peel Extract, *Spilanthes Paniculata* Flower Extract and Selenium in Streptozotocin Induced Diabetes

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Abstract

The present study was aimed to evaluate the antidiabetic potential of combination of *punica granatum* peel extract, *spilanthes paniculata* flower extract and selenium in streptozotocin (STZ) induced diabetes in mice. Group A was used as control and Group B was used as diabetic control (STZ, 40mg/kg for 3 days, i.p.). A placebo was given to both the group orally. Metformin (120mg/kg) was given intraperitoneally as standard drug to Group C. Ethanolic peel extract of *punica granatum* (EPP 600mg/kg, p.o.) was administered to group D, (EPP) in combination with ethyl acetate extract of *spilanthes paniculata* flower (EAS) was administered to group E (EPP 300mg/kg, p.o. + EAS 200mg/kg, p.o.), Sodium selenite was given with combination of herbal extract (EPP 300 mg/kg, p.o.+ EAS 200mg/kg, p.o. + Se 0.3mg/kg, i.p.) to group F. STZ was given in multiple low doses of 40mg/kg, i.p. for 3 consecutive days & hyperglycemia was observed on 7th day. Blood glucose was determined on 0th, 7th, 14th, and 21st day. Diabetic mice in all group showed significant (P<0.01) increase in serum blood glucose as compared to control animals. Blood glucose level of animals treated with metformin & herbo-mineral combination lowers the glucose level significantly (P<0.01) as compared to diabetic control. The herbo-mineral combination shown significant (P<0.01) increase in the antioxidant enzymes Catalase, SOD, GSH and reduction in MDA & NO levels as compared to diabetic control. Thus, the present study indicates decreased blood glucose level & significant improvement in the biomarkers of oxidative stress by using the combination of sodium selenite with herbal extracts. The results confirm that the herbo-mineral combination is not only useful in controlling blood glucose level but also helps to prevent oxidative stress induced damage.

Key words: *Punica granatum*, *Spilanthes paniculata*, Selenium Streptozotocin

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by increased blood glucose levels. This disease is characterized by either lack of insulin production or deficient activity in the presence of normal or even elevated levels of insulin.[1] High amounts of free fatty acids (FFAs) and glucose badly affects β cell function through various pathways such as, defects of metabolic mechanisms, generation of reactive oxygen species (ROS), elevation of intracellular calcium levels and interaction with membrane potassium channels.[2] Beyond glucose, ROS formation is also increased by FFAs, through direct effects on mitochondria. [3] It has been proposed that over expression and activity of a

mitochondrial inner membrane uncoupling proteins (UCPs) contribute to an increase in superoxide formation under diabetic conditions.[4] Chronic hyperglycemia produces multiple biochemical impairments and oxidative stress especially an increased susceptibility to lipid peroxidation that play role in the progression of the symptoms of diabetes.[5] Despite advances in understanding the disorder and management, the mortality and morbidity of the disease is ever increasing.[6] Many studies have shown that anti diabetic herbs with antioxidant property have proven to be efficient in reducing the risk of complication of diabetes. [7] Antioxidant like SOD, CAT, glutathione reductase and some minerals like Mn, Cu, Zn, selenium comes under first line defense. [8] Pomegranate is a widely

used plant that has high nutritional value. [9] It was found that *Punica granatum* peel contain tannins, flavonoids, pectin, 3-estrogen compounds luetolin, quercetin & kemferol. Antioxidant & antidiabetic activity shown by the peel extract stimulation of beta-cells, protection of pancreas, increase the no of beta-cells & subsequent release of insulin it also reduce the blood sugar through regeneration of beta-cells.[10] Genus *Spilanthes* is a plant of choice for many health related disorder. The genus *Spilanthes* (Asteraceae) comprises 30 species and 9 additional intraspecific taxa. [11] The petroleum ether and ethyl acetate extract of *Spilantes paniculata* flowers have shown the hepatoprotective effect and possible mechanism is due to the reduction of oxidative stress and showed protection against increased lipid peroxidation and maintained the glutathione contents. [12] Selenium (Se) is an essential dietary trace element which plays an important role in a number of biological processes.[8] Evidence from in vivo & vitro studies suggest that inorganic selenium can enhance insulin sensitivity by mediating insulin like action.[13] Specially in animal models selenite has been shown to decrease the activity of protein tyrosine phosphate a negative regulator of insulin signal transmission & therefore potentially reduced insulin resistance.[14] Although, *Punica granatum* and selenium is well recognized for possessing the antidiabetic activity but no scientific data have been published supporting the antidiabetic activity of *Spilanthes paniculata* flowers and also the combination study of these drugs. Hence, the objective is to evaluate the antidiabetic effect of combination of *Punica granatum* peel extract, *Spilanthes paniculata* flowers extract and selenium in streptozotocin induced diabetes in mice.

Materials and methods

A) Collection of Fruits of *Punica granatum*: The fruits were purchased locally and authenticated from Dept. of Botany, Dr. Babasaheb Ambedkar Marathwada University Aurangabad. A voucher specimen no. 0733 has been deposited in the same department.

B) Collection of flowers of *Spilanthes paniculata*: The flowers of *Spilanthes paniculata* used for the present studies were collected from Manjlegaon in a location known as Beed (Maharashtra). The plant was identified, confirmed and authenticated by comparing with voucher specimen available at survey of medicinal plants & collection unit, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University,

Aurangabad where voucher specimens have been deposited and accession no.0730.

Preparation of the extracts

A) Ethanolic extract of *Punica granatum*: The powder obtained of *Punica granatum* peel (1000 g) was defatted using pet ether (60-80°C). The marc was extracted with 95% ethanol using soxhlet apparatus. The filtrate was air dried and concentrated under reduced pressure to obtain 37.40g, corresponding to a yield of 24.10% w/w.

B) Ethyl acetate extract of *Spilanthes paniculata*: The flowers of *Spilanthes paniculata* plant were removed and dried under the shade condition, powdered with the help of grinder and stored in an airtight container. The powder of flowers was weighed (300gm). An extract was prepared by soxhlet extraction method. The dried powdered flowers of *Spilanthes paniculata* were extracted with ethyl acetate for 36 hrs using soxhlet extractor. The extracts were concentrated at 60°C under reduced pressure, to obtain dark brownish residue the remaining extract was freeze dried. The yield obtained from the extraction process was found to be 2.60% w/w.

Animals

Swiss albino mice of either sex weighing between (22-30 g respectively) were procured from Wockhardt Ltd, Aurangabad. They were maintained at temperature of $25 \pm 2^\circ\text{C}$ and relative humidity of 45 to 55% and under standard environmental conditions. Animals were housed under standard laboratory conditions with free access to food (Amrut rat and mice feed, Sangli, India.) and water. The mice used in the present study were maintained in accordance with guidelines of the CPCSEA, India and the study was approved by Institutional Ethics committee, Y.B. Chavan College of pharmacy, Aurangabad.

Induction of Experimental diabetes

Overnight fasted experimental mice were injected with STZ at a multiple dose of 40 mg/kg bodyweight for 3 consecutive days.[15]The solution was injected intraperitoneally (i.p.) within 5 min after dissolving in citrate buffer pH 4.5[25] The animals were allowed to drink 5% glucose solution overnight to overcome the drug induce hypoglycemia. Fasting blood glucose (FBG) was estimated before the time of induction of diabetes using commercially available kit (Accu-Chek Active Test Meter) and on the 7th day of STZ administration. The mice with moderate diabetes having glycosuria and hyperglycemia (blood glucose levels of 250 mg/dl) were included in the study.

Groups and treatment

(Each group containing 6 animals)

Group A: Normal control (Distilled water, 1ml/100gm p. o).

Group B: Diabetic control (Distilled water, STZ 40 mg/kg for 3 days, i.p).

Group C: Diabetic (STZ) + Metformin (120mg/kg, i.p).

Group D: Diabetic (STZ) + Ethanolic peel extract of *Punica granatum* (600mg/kg, p.o).

Group E: Diabetic (STZ) + Ethanolic peel extract of *Punica granatum* (300mg/kg, p.o) + Ethyl acetate extract of *Spilanthes paniculata* flowers (200mg/kg, p.o).

Group F: Diabetic (STZ) + Ethanolic peel extract of *Punica granatum* (300mg/kg, p.o)+ Ethyl acetate extract of *Spilanthes paniculata* flowers (200mg/kg, p.o) + Sodium selenite (0.3 mg/kg, i.p).

Preparation of drug solution

Preparation of STZ solution: Streptozotocin was dissolved in cold 0.01 M citrate buffer, pH 4.5 and always prepared freshly for immediate use within 30 min. STZ injections were given intraperitoneally and the doses were determined according to the body weight of animals. [16]

Oxidative stress

At the end of study animals were sacrificed and liver was isolated & used for the estimation of SOD [17], Catalase [18], MDA [19], GSH [20] & NO [21] levels.

Statistical analyses

It was carried out by ANNOVA followed by Dunnett's t-test at level of significance $P < 0.05$. All data were shown as the mean \pm SEM. Statistical analysis was performed using Graph Pad statistical software.

Table 1: Antidiabetic effect of herbo-mineral combination of (EPP + EAS + Sn) on blood glucose levels in streptozotocin induced diabetes in mice.

GROUPS	Fasting blood glucose level (mg/dl)			
	Initial week	First week	Second week	Third week
A. D.W.	82.83 \pm 3.31	85.50 \pm 0.763	86.1 \pm 1.06	82.0 \pm 2.47
B. STZ + D.W.	86.83 \pm 1.302	296 \pm 13.297**	401 \pm 10.80**	437 \pm 18.9**
C. STZ + Metformin	86.16 \pm 1.851	268 \pm 10.24	191 \pm 7.34**	103 \pm 2.98**
D. STZ + EPP	84.50 \pm 4.087	262 \pm 14.70	257 \pm 14.18**	143 \pm 5.03**
E. STZ + (EPP + EAS)	85.16 \pm 1.046	268 \pm 8.41	205 \pm 9.5**	128 \pm 4.42**
F. STZ + (EPP + EAS + Se)	82.33 \pm 3.93	283 \pm 15.13	197 \pm 5.34**	116 \pm 2.88**

Data is presented as Mean+ SEM (n=6); One way Analysis of Variance (ANOVA) followed by Dunnett's test
**p<0.01 vs. Diabetic control.

Table 2: Antioxidant effect of herbo-mineral combination of (EPP + EAS + Sn) on liver tissue Catalase, SOD, MDA, GSH and NO levels in streptozotocin induced diabetes in mice.

Groups	CATALASE nmoles of H ₂ O ₂ consumed/min/ mg protein	SOD Unit/ml of sample	MDA nmole/gm of tissue	GSH mmole /gm of tissue	NO mmole/gm of tissue
A. D.W.	6.07 ± 0.386	7.00 ± 0.37	2.46 ± 0.20	10.36± 0.420	8.21 ± 0.33
B. STZ + D.W.	2.00 ± 0.242**	3.32 ± 0.395**	4.96 ± 0.33**	4.56 ± 0.52**	17.59 ± 1.14**
C. STZ + Metformin	5.34 ± 0.506 **	7.25 ± 0.407 **	1.85 ± 0.20**	9.93 ± 0.23**	7.09 ± 0.82**
D. STZ + EPP	4.34 ± 0.32 *	4.79 ± 0.415	3.88 ± 0.27*	7.64 ± 0.43**	7.74 ± 0.31**
E. STZ + (EPP + EAS)	6.28 ± 0.78 **	5.85 ± 0.28 *	2.55 ± 0.29**	8.18 ± 0.57 **	9.17 ± 0.44**
F. STZ + (EPP +EAS + Se)	6.22 ± 1.25 **	8.07 ± 0.51 *	2.76 ± 0.18**	9.93 ± 0.30**	7.49 ± 0.73**

Data is presented as Mean+ SEM (n=6); One way Analysis of Variance (ANOVA) followed by Dunnett's test
*p<0.05, **p<0.01 vs. Diabetic control.

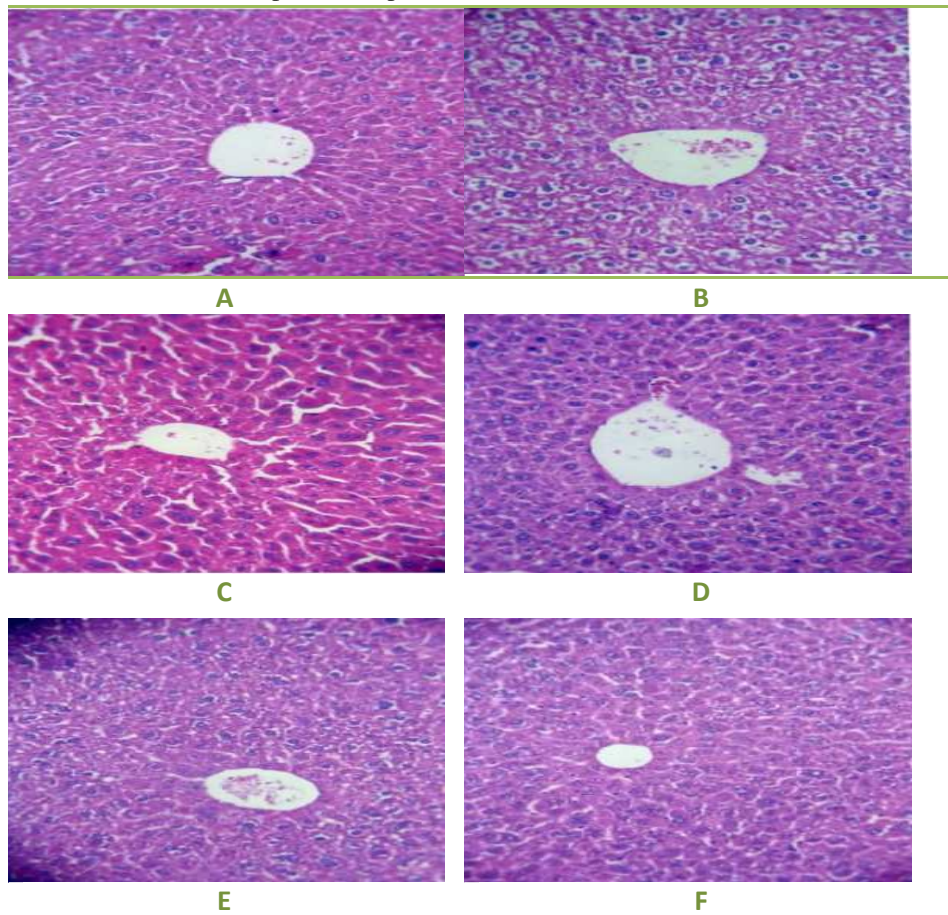


Fig. 1: Photomicrographs of mice liver (H & E stain) Respective treatment showing following changes in liver section (A) Distilled water showing the normal histology, (B) Streptozotocin showing congested liver tissue with congested sinusoids and centrilobular necrosis with infiltration of lymphocytes and neutrophils, (C) metformin showing normal architecture with absence of fatty change and necrosis, (D) *Punica granatum* peel extract showing the normal histology, (E) *Punica granatum* peel extract + *S. paniculata* flower extract showing the normal histology and repaired hepatocytes, (F) *Punica granatum* peel extract + *S. paniculata* flower extract + Selenium showing the normal histology with repairing of hepatocytes. Magnification ×40

Results

Diabetic mice in all group showed significant ($p < 0.01$) increase in serum blood glucose as compared to control animals. Vehicle treated diabetic control group showed significant ($p < 0.01$) increase in serum glucose level as compared to normal control animals. Blood glucose level of animals treated with metformin & herbo-mineral combination lowers the glucose level significantly ($p < 0.01$) as compared to diabetic control. The combination of drugs was found to be more effective in reducing the blood glucose level. (Table 1).

The present study showed decrease in the activity of SOD enzymes in diabetic control group & administration of EPP has not shown any significant improvement in the activity of enzyme whereas group D (EPP + EAS) & group E (EPP + EAS + Se) have shown significant increase in the activity of this enzyme. The present study showed decreased in the activity of catalase enzymes in diabetic control group group C (EPP) showed protective effect whereas group D (EPP + EAS) & group E (EPP + EAS + Se) has normalized the activity of these enzyme. Diabetic control group showed depletion of GSH levels & the diabetic mice treated with the herbal extract (EPP) & (EPP+EAS) showed a marked protective effect. Whereas the diabetic mice treated with (EPP+EAS + Se) normalized the hepatic GSH levels. (Table 2).

Group E (EPP+EAS) have shown marked protective effect whereas group C (EPP) & group E (EPP+EAS + Se) normalized elevated total nitrite level in the liver tissues induced by STZ. A massive increase in lipid peroxidation was observed in diabetic control group whereas treatment with (EPP + EAS) in group E & (EPP + EAS + Se) in group F decreased the level of MDA, thus preventing the lipid peroxidation whereas treatment with (EPP) in group D did not show any significant decrease in the MDA levels. (Table 2).

Histopathological evaluation

Group A: Liver shows normal architecture. The central vein, portal tract and sinusoids appear normal (Figure A). Group B: Liver shows congested liver tissue with congested sinusoids and centrilobular necrosis with infiltration of lymphocytes and neutrophils (Figure B). Group C: Liver shows normal architecture with absence of fatty change and necrosis (Figure C). Group D: Liver shows the normal histology (Figure D). Group E: Liver shows normal hepatic architecture with regeneration of hepatocytes and absence of fatty changes and necrosis (Figure E). Group F: Liver shows normal hepatic architecture with regeneration of hepatocytes and absence of fatty change and necrosis (Figure F). (Figure 1).

Discussion

In Diabetes mellitus chronic hyperglycemia produces multiple biochemical impairments and oxidative stress especially an increased susceptibility to lipid peroxidation that play role in the progression of the symptoms of diabetes. [15] Multiple low doses of STZ 40 mg/kg, i.p. to mice for three consecutive days have been found suitable model to study Type 2 DM. [16] Rise in blood glucose was observed on the 7th day and the animals showed symptoms like polyuria & weight loss which assures that diabetes was successfully induced.

Oxidative stress induced by hyperglycemia leads to liver cell damage because liver is subject to ROS-mediated injury in diabetes. [22] The histopathological evaluation of diabetic mice liver showed degeneration of hepatocytes, centrilobular necrosis with hydropic changes in surrounding and diabetic mice treated with the herbal extract (EPP) & (EPP+EAS) showed mild to moderate degenerative changes while treatment with EPP+EAS with sodium selenite showed regeneration of hepatocytes & normal cord like pattern.

Many studies have shown that antidiabetic herbs with natural antioxidant constituents such as tannins, alkaloids, flavonoids, phenols etc. enhances free radical scavenging activities & have proven to be efficient in reducing the risk of complication of diabetes. Antioxidants like SOD, CAT, GPx, glutathione reductase and some minerals like Mn, Cu, and Zn selenium comes under first line defense. Second line defense antioxidants include glutathione (GSH), Vit C, albumin, bilirubin, carotenoids, Vitamin E, flavonoids, etc. Flavonoids are the phenolic compounds that inhibit the lipid peroxidation & lipoxygenase. [8] Tannins contain several hexahydroxyl diphenoyl groups or galloyl groups both groups provide protons to stabilize the free radicals. [23] Ellagic acid reacts with the free radical & is a potent antioxidant against the lipid peroxidation in mitochondria & microsomes. [24] It has been observed that plasma level of β -sitosterol is lower among Type 2 diabetes mellitus with hypoglycemic effects of β -sitosterol being observed in other studies. [25, 26] β -sitosterol has been shown to be an AMPK agonist, with the beneficial effects of β -sitosterol on glucose metabolism being mediated, in part, through this mechanism. [27] In animal models selenite has been shown to decrease the activity of protein tyrosine phosphatase, a negative regulator of insulin signal transmission, and can therefore potentially reduce insulin resistance. [14]

In the present study, the phyto-chemical screening of the plant extracts was performed and it was found to contain flavonoids, tannins, phenolic

compounds and steroids and the present data suggest that the antidiabetic activity and protective action of herbo-selenium combination against oxidative stress may be due to its synergistic action of these constituents thus, synergistic action of the different chemical constituents appears to be superior to that of single constituents and the mechanism underlying such protection may be mediated by prevention & restoration of antioxidant defense systems. Restoration of antioxidant status led to normalization of the insulin release, decrease in the insulin resistance & hence maintaining glucose serum levels within the normal range.

Conclusion

The present study indicates decrease in blood glucose level & significant improvement in the biomarkers of oxidative stress by using the combination of sodium selenite with herbal extract. Herbal extract showed a marked protective effect whereas herbo-selenium combination normalized these levels. Protection may be mediated by prevention & restoration of antioxidant defense systems. Restoration of antioxidant status led to normalization of the insulin release, decrease in the insulin resistance & hence maintaining glucose serum levels within the normal range. This indicates that *Punica granatum*, *Spilanthes paniculata* & sodium selenite are the three powerful antioxidant which may prove to be beneficial in diabetes and this supplement could attenuate diabetic complications.

This finding also indicated beneficial interaction of selenium at insulin-receptor site resulting in decrease in insulin resistance & hyperglycemia. Although the proof of efficient delivery of selenium at receptor site would need further study

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