

Effect of *Potentilla Fulgens* L. on Selected Enzyme Activities and Altered Tissue Morphology in Diabetic Mice

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J Morphol Sci 2018;35:153–160.

Abstract

Introduction The objective of the present study was to investigate the in vitro inhibitory effect of the *Potentilla fulgens* extract on amylase, α - and β -glucosidase, and lipase, as well as its effect on the ultrastructure of the liver, of the kidneys, and of the eye tissues in alloxan-induced diabetic mice. The present study was designed to get further insight regarding the action of *P. fulgens* from what has been previously known and reported about this plant.

Materials and Methods Roots of *P. fulgens* were extracted with 10 volumes of aqueous-methanol solution (1:4), and the prepared extract was used for in vitro inhibitory activity on amylase, α -glucosidase, β -glucosidase, and lipase. Afterwards, the plant extract was intraperitoneally administered for alternated days (250 mg/kg body weight) to diabetic mice for 4 weeks, and an ultrastructural examination of the liver, the kidneys and the eye tissues was performed using a transmission electron microscope (JEM-100 CX II, Jeol Ltd., Tokyo Japan).

Results The *P. fulgens* extract showed inhibitory activity against all the four enzymes (amylase, α - and β -glucosidase, and lipase), with the highest percentage of inhibition ($94.57\% \pm 0.16$ at 1 mg/mL) being observed against α -glucosidase when compared with the standard. The ultrastructural studies revealed a distortion in the structure of the nuclei and of the mitochondria in the kidneys and liver tissues of diabetic mice. Distortion of cell shape and disturbed orientation was observed in the eye lens of diabetic mice. The *P. fulgens* extract reversed/protected/reduced the ultrastructural alteration observed in the tissues (liver, kidney, and eye lens) of diabetic mice.

Conclusion The inhibitory effect of the *P. fulgens* extract against the aforementioned enzymes and its protective effect on the tissues of diabetic mice against alloxan-induced diabetes add further insight into the antidiabetic properties of this plant.

Keywords

- amylase
- α - and β -glucosidase
- lipase
- *Potentilla fulgens*
- ultrastructural studies

Introduction

Many medicinal plants have been reported to have multiple beneficial biological properties. *Moringa oleifera*,¹ *Phyllanthus amarus*,² *Momordica charantia*,³ *Gymnema sylvestre*,⁴ and

Grewia asiatica,⁵ to name a few, are medicinal plants with various pharmacological properties, including an antidiabetic property, which have been attributed to the presence of different bioactive compounds in them. *Potentilla fulgens* L. is one of these medicinal plants known to possess various

received
October 25, 2017
accepted
July 23, 2018
published online
October 2, 2018

DOI <https://doi.org/10.1055/s-0038-1669934>.
ISSN 2177-0298.

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biological properties. It belongs to the Rosaceae family, commonly found in the Western Himalayas. Pharmacologically, the plant is reported to have hypoglycemic,⁶ antihyperglycemic,⁶ antitumor,⁷ antihypolipidemic,⁸ and antioxidant properties,^{9,10} as well as an antidiarrheal.¹¹ It is a prophylactic agent,¹² with anthelmintic¹³ and wound healing properties,¹⁴ and even improves gum health.¹⁵ Qualitative and quantitative analysis of *P. fulgens* roots by nuclear magnetic resonance spectroscopy, matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MS), electrospray ionization MS/MS and high-performance liquid chromatography/ultraviolet have also been reported.¹⁶ Phytochemical investigations of the root parts of *P. fulgens* have shown the presence of a novel bioflavonoid potifulgene along with epicatechin.¹⁷ It has also been reported that the aerial parts contain two new triterpenes, potentene-A and potentene-B, as well as three known compounds, afzelchin-4 $\alpha \rightarrow 8''$ catechin, epiafzelechin, and rutin.^{15,18,19}

Although the effect of the *P. fulgens* extract on different parameters^{6,8,9,19–22} under diabetic conditions has been reported previously, its inhibitory effect against amylase, β -glucosidase, and lipase under in vitro conditions has not been reported yet. Pancreatic α -amylase and glucosidase are known to lower the level of postprandial hyperglycemia via the control of starch breakdown.²³ The pancreatic lipase plays a key role in the efficient digestion of triglycerides,²⁴ and is responsible for the hydrolysis of between 50 and 70% of the total dietary fats.²⁵ Elevated triglyceride levels are a common dyslipidemic feature accompanying type 2 diabetes.²⁶ Therefore, the inhibition of these digestive enzymes is a suitable target for the treatment of diabetes. Drugs that inhibit these enzymes are already available in the market, and are used for diabetes treatment.^{25,27} However, many of these synthetic hypoglycemic agents have their limitations, are non-specific, produce serious side effects, and fail to elevate diabetic complications.²⁸ This opens an exciting opportunity for the development of new therapeutic drugs, especially from plant sources, which are considered safe.²⁹ Therefore, in the present paper, the in vitro inhibitory effect of *P. fulgens* extract on the aforementioned enzymes was studied. Although α -glucosidase inhibitory activity of *P. fulgens* has been reported previously by Kumar et al.³⁰, we have performed *in vitro* α -glucosidase inhibitory activity of *P. fulgens* extract for comparison with its β -glucosidase inhibitory activity. In addition, the effect of the plant extract on the ultrastructure of the liver, of the kidneys, and of the eye lens tissues in diabetic mice was also investigated. Thus, the present study was designed to provide additional insights regarding the mechanism of action of this antidiabetic plant.

Materials and Methods

Reagents

Butylated hydroxyanisole, bathocuproine, copper nitrate, lecithin, lipase, potassium sodium tartarate, sodium acetate, sodium carbonate, sodium chloride, and triolein were purchased from Hi-Media Laboratories Pvt. Ltd. (Mumbai, Maharashtra, India). Acetic acid, α -amylase, α -glucosidase,

β -glucosidase, dimethyl sulfoxide, dinitrosalicylic acid, dipotassium hydrogen phosphate, disodium hydrogen phosphate, glycine, n-heptane, methanol, p-nitrophenyl- α -D-glucopyranoside (pNDG), p-nitrophenyl- β -D-glucopyranoside, potassium dihydrogen phosphate, sodium dihydrogen phosphate, starch, sodium hydroxide, and triethanolamine were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Alloxan and taurocholic acids were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), acarbose (Glucobay 50) was purchased from Bayer Zydus Pharma (Thane, Maharashtra, India), and orlistat (Reeshape) was purchased from Meyer Organics Pvt. Ltd. (Thane, Maharashtra, India).

Plant Material

The roots of *P. fulgens* were collected from Bara bazaar, Shillong, Meghalaya, India (Voucher no: 464, North-Eastern Hill University [NEHU]). The collected plant materials were washed, shredded, dried and weighed. Afterwards, they were powdered, homogenized and extracted with an aqueous-methanolic solution (1:4). The mixture was filtered, and the filtrate was evaporated at 40° C using a rotary evaporator, (RE300, Stuart, Cole-Parmer, Beacon Road, Stone, Staffordshire, ST15 0SA, UK) and it was further lyophilized until it was totally dry.^{6,31} The obtained dried mass was used for the investigation.

In vitro Enzyme Inhibitory Studies

The evaluation of α -amylase inhibition was determined by the method of Kim et al.³² The plant extract and acarbose (positive control) were prepared in a concentration range between 0.02 and 1.0 mg/mL. A total of 50 μ L of plant extract/acarbose, 250 μ L of porcine pancreatic amylase (1 mg/100 mL), and 250 μ L of sodium phosphate buffer (0.02 M, pH 6.9) were mixed and incubated at 37° C for 10 minutes, and 450 μ L of 0.5% starch solution was added to the reaction mixture and incubated at 37° C for 20 minutes. The reaction was concluded by adding 500 μ L of 3,5-dinitrosalicylic acid, then heating at between 70 and 80° C for 15 minutes, and centrifuging at 650 rpm for 1 minute. The absorbance was measured at λ 540 nm.

The evaluation of α -glucosidase inhibition was performed using pNDG as a substrate.³² A total of 50 μ L of plant extract/acarbose (0.02–1.0 mg/mL) was mixed with 50 μ L of buffer and 50 μ L of α -glucosidase solution (1 mg / 100 mL), followed by incubation at 37° C for 5 minutes. After the incubation period, 100 μ L of pNDG was added and incubated again at 37° C for 15 minutes. The reaction was stopped by adding 750 μ L of sodium carbonate. The absorption of 4-nitrophenol was measured at λ 400 nm. Acarbose was used as a positive control.

The β -glucosidase assay was performed by adopting the method of Sánchez et al.³³ with slight modification. A total of 100 μ L of p-nitrophenyl- β -D-glucopyranoside, 50 μ L of plant extract/acarbose (0.02–1.0 mg/ml), and 100 μ L of acetate buffer were mixed and incubated at 37° C for 10 minutes, followed by the addition of 50 μ L of β -glucosidase solution and incubation at 37°C for 30 minutes. After the incubation period, the reaction was stopped by adding 700 μ L of glycine-NaOH buffer. The absorbance was measured at λ 410 nm. Acarbose was used as a positive control.

A method slightly modified from that of Lin et al³⁴ was utilized to measure the pancreatic lipase inhibitory activity. A total of 50 µL of pancreatic lipase, 100 µL of substrate, and 100 µL of plant extract/orlistat (0.02–1.0 mg/ml) were mixed and incubated at 37° C for 30 minutes, followed by the addition of 3 ml of a chloroform, n-heptane and methanol mixture and centrifugation at 2,000 rpm for 10 minutes. One ml of copper reagent was added to the lower organic phase, followed by vigorous shaking for 10 minutes and centrifugation at 2,000 rpm for 10 minutes. One-half ml of supernatant was transferred to 0.5 ml of chloroform containing 0.1% weight/volume (w/v) of bathocuproine and 0.05% w/v of 3–2-tert-butyl-4 hydroxylanisole. The absorbance was measured at λ 480 nm. Orlistat was used as a positive control.

The reaction mixture without the plant extract was used as a control, and the reaction mixture without the plant sample and the enzyme was used as a blank. The experiments were performed for five sets, and the data were represented as percentage of inhibition (mean ± standard error of mean [SEM]). The percentage of inhibition of α-amylase, α-glucosidase, β-glucosidase, and lipase activities was calculated using the following formula:

Percentage of inhibition =

$$\frac{[\text{Absorbance (control)} - \text{Absorbance (sample)}] \times 100}{\text{Absorbance (control)}}$$

In vivo Studies

Animals and Experimental Designs

Healthy, male Swiss albino mice, weighing between 25 and 30 g, were used for the study. The mice were kept under controlled conditions, with the temperature maintained at 22°C on a 12-hour light/dark cycle in the animal facility room of the NEHU. The mice were fed with balanced mice food obtained from Pranav Agro Industries Ltd. (New Delhi, India). The institutional guidelines were followed during the experimentation. The animal models were divided into three groups. The normal control group (group I) received only 2% ethanol intraperitoneally (ip); the diabetic control group (group II) received only 2% ethanol ip; and the *P. fulgens*-treated diabetic group (group III) received 250 mg/kg body weight (bw) of extract ip. After a period of 4 weeks, the mice were sacrificed, and the dissected tissues (liver, kidneys and eye lens) were studied using a JEOL100 CX II transmission electron microscope (JEM, JEOL Ltd., Tokyo, Japan).

Preparation of Diabetic Mice

The diabetic mice were administered ip with alloxan monohydrate prepared in acetate buffer, as described earlier.⁷ Prior to the administration, the mice fasted overnight but had ad libitum access to water. Mice with more than a three- to four-fold increase in their blood sugar levels were considered diabetic and used for further tests.

Transmission Electron Microscopy Study

The primary fixation of the isolated tissues of the liver, the kidney, and the eye lens from all the three groups were

made in 3% glutaraldehyde prepared in a sodium phosphate buffer (200 mM; pH 7.4) for 3 hours at 4° C. The materials were washed with the same buffer and postfixed in 1% osmium tetroxide and in a sodium phosphate buffer for 1 hour at 4° C. The tissue samples were then washed with the same buffer for 3 hours at 4° C, dehydrated in a graded ethanol series and then embedded in Araldite CY212 (Agar Scientific, Essex, UK) sections ranging between 60 and 90 nm. The embedded tissues were cut on a RMC Ultra-microtome (Powertome-PC, RMC Boeckeler, USA) using a diamond knife (Ultra 45 degree, Diatome, USA) and the sections were mounted on a copper grid. Afterwards, the sections were stained with uranyl acetate and Reynolds lead citrate. The grids were examined using a JEOL100 CX II TEM at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya, India.

Results and Discussion

Diabetes, particularly type 2, is a multifunctional disease.³⁵ Therefore, a treatment with drugs that have multiple targets has great potential for tackling diabetes.³⁶ Medicinal plants are known to contain a complex of phytochemicals and bioactivities that may have multiple benefits by targeting several metabolic pathways and, essentially, “killing several birds with one stone”.³ Drugs with the ability to target more metabolic pathways seem to show more encouraging results than those that target a single pathway.³⁷ The purpose of the present study was to explore the effect of *P. fulgens* root extract on multiple targets other than the known activities as an anti-diabetic plant. Here, the in vitro inhibitory effect of *P. fulgens* extract on α-amylase, α-glucosidase, β-glucosidase, and lipase were explored. In addition, its effect on the ultrastructure of the liver, of the kidney and of the eye lens was studied as no previous reports have been given on this study.

In vitro Enzyme Inhibitory Studies

The in vitro inhibitory activity of the *P. fulgens* extract against α-amylase, α-glucosidase, β-glucosidase, and lipase is shown in ► **Table 1**. The maximum percentage of inhibition of *P. fulgens* against α-amylase was found to be 37.51 ± 0.750 at 1 mg/mL, which was lower than the inhibition percentage of 44.02 ± 0.79 at 1mg/ml showed by the positive control. The percentage of inhibition of *P. fulgens* against α-glucosidase was found to be maximum at 1 mg/mL (94.57 ± 0.16), whereas that of the positive control was found to be 45.08 ± 1.91 at the same dose.

Thus, the *P. fulgens* extract showed an even higher inhibitory activity than the positive control at the same concentration. The highest percentage of inhibition of β-glucosidase by the *P. fulgens* extract was found to be 25.56 ± 1.63 at 0.6 mg/mL, which was lower than that of the positive control. The maximum percentage of inhibition against lipase of *P. fulgens* and positive control was found to be 24.87 ± 0.43 and 24.31 ± 0.75 respectively, at the corresponding concentration of 1 mg/mL. In the present study, the plant extract showed an inhibitory effect on all of the studied

Table 1 Percentage (%) of inhibition of *Potentilla fulgens* extract/positive control against α -amylase, α -glucosidase, β -glucosidase, and lipase

Concentration (mg/mL)	α -amylase		α -glucosidase		β -glucosidase		Lipase	
	Positive control (%)	Plant extract (%)	Positive control (%)	Plant extract (%)	Positive control (%)	Plant extract (%)	Positive control (%)	Plant extract (%)
0.02	15.58 \pm 1.81	7.75 \pm 2.01	12.11 \pm 1.03	24.62 \pm 1.85	16.55 \pm 1.38	6.34 \pm 1.82	10.68 \pm 1.30	5.52 \pm 0.30
0.04	16.11 \pm 1.59	14.95 \pm 2.86	14.86 \pm 0.99	27.56 \pm 1.87	18.19 \pm 1.74	8.73 \pm 1.22	15.22 \pm 1.61	8.27 \pm 0.33
0.06	16.98 \pm 1.12	17.22 \pm 2.33	16.21 \pm 1.51	33.22 \pm 1.20	20.09 \pm 1.42	10.92 \pm 0.78	16.31 \pm 1.43	10.81 \pm 0.33
0.08	17.01 \pm 0.94	19.77 \pm 2.41	17.01 \pm 1.98	38.68 \pm 1.49	21.98 \pm 1.43	13.22 \pm 1.07	18.97 \pm 1.08	13.60 \pm 0.32
0.1	17.55 \pm 1.08	23.46 \pm 2.08	18.85 \pm 2.01	42.78 \pm 0.37	22.12 \pm 1.74	14.82 \pm 1.10	20.70 \pm 1.03	17.94 \pm 0.39
0.2	18.16 \pm 0.58	26.70 \pm 0.89	19.97 \pm 3.06	65.11 \pm 0.55	24.37 \pm 1.70	16.44 \pm 0.58	22.51 \pm 0.81	21.24 \pm 0.52
0.4	24.56 \pm 0.80	29.36 \pm 1.89	26.54 \pm 2.88	90.80 \pm 0.93	30.14 \pm 1.52	18.58 \pm 0.80	24.31 \pm 0.75	24.87 \pm 0.43
0.6	32.18 \pm 0.48	33.59 \pm 0.92	34.06 \pm 2.62	93.38 \pm 0.88	34.35 \pm 1.08	25.56 \pm 1.63	33.96 \pm 2.09	14.30 \pm 0.71
0.8	39.44 \pm 0.48	35.21 \pm 0.85	39.16 \pm 2.20	94.40 \pm 0.28	40.20 \pm 1.30	24.40 \pm 1.12	41.78 \pm 1.58	11.02 \pm 0.86
1.0	44.02 \pm 0.79	37.51 \pm 0.75	45.08 \pm 1.91	94.57 \pm 0.16	45.73 \pm 1.32	23.06 \pm 1.72	52.58 \pm 1.20	7.37 \pm 0.99

Note: Values are represented as mean \pm standard error of mean where $n = 5$.

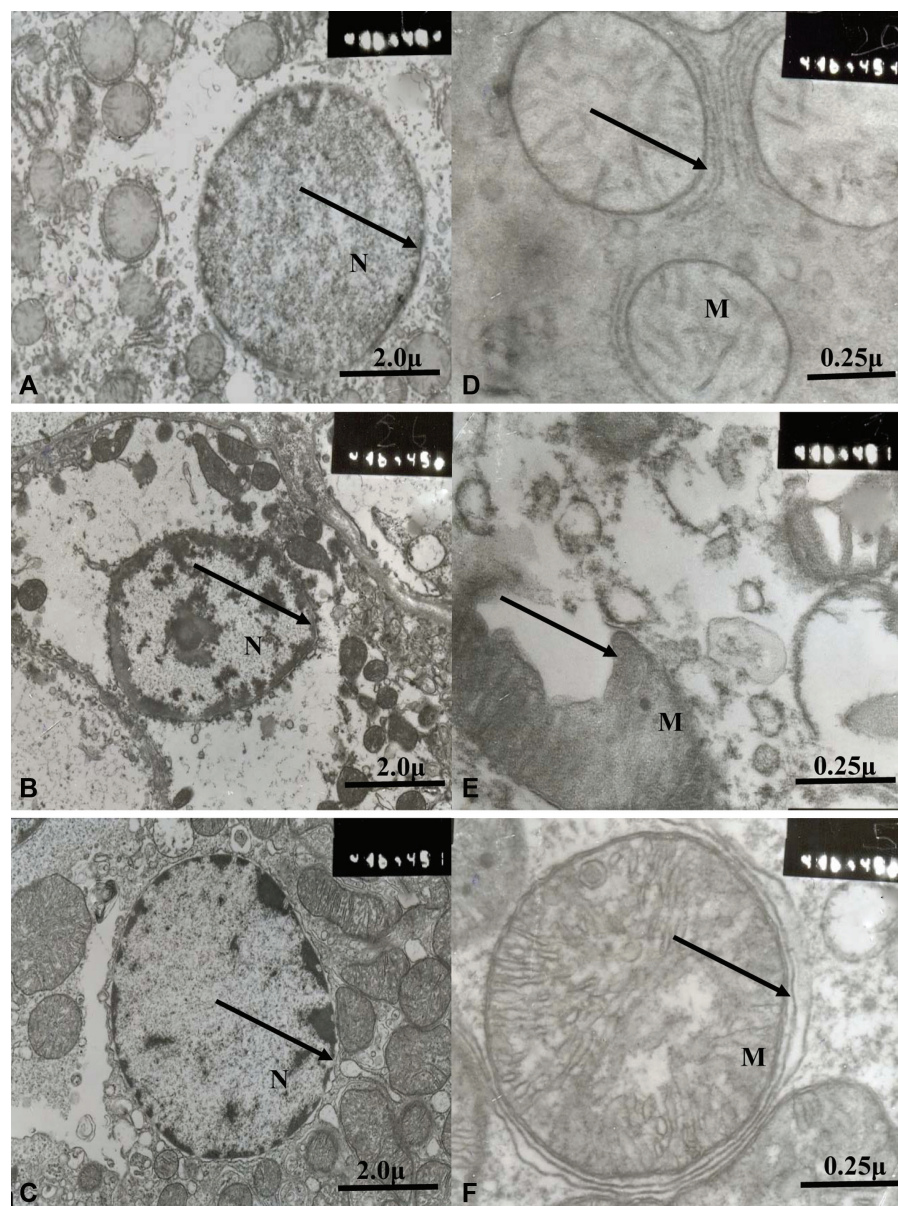


Fig. 1 Transmission electron micrograph showing the nuclei (N) in the liver of normal mice (A), diabetic mice (B), and *Potentilla fulgens* extract-treated diabetic mice at magnification $\times 5,000$ (C). Transmission electron micrograph showing the mitochondria (M) in the liver of normal mice (D), diabetic mice (E), and *P. fulgens* extract-treated diabetic mice (F) at magnification $\times 40,000$.

enzymes. However, the highest percentage of inhibition was shown against α -glucosidase. This suggests that the *P. fulgens* extract may contain several inhibitory substances, thereby resulting in the inhibitions of these enzymes to different extents under the assay conditions used.

In vivo Studies

Marked differences were observed in the ultrastructural features of the nucleus and of the mitochondria in the liver and in the kidneys of diabetic mice compared with the normoglycemic mice, as shown in ►Figs. 1–2. The nucleus is one of the most prominent cellular organelles, and its shape and size play an important role in cellular function.³⁸ The normal liver and kidney cells revealed normal features of euchromatic nucleus (that is, with normal chromatin distribution), with fewer nuclear heterochromatic contents. The contours of the nuclei were round, with the nuclear membrane showing normal structures, such as regular outline

and the absence of any prominent membrane protrusion or invagination (►Figs. 1A & 2A). The mitochondria were well-maintained in the form of typical oval and elliptic shapes, with smooth surfaces and clear outlines. The outer and inner mitochondrial membranes were intact, without breakage. The cristae were arranged in the form of a concentric ring or a vertical line, congested and clear. No abnormalities, such as membrane distortion or vacuolization, were observed in the mitochondrial matrix (►Figs. 1D & 2D). The electron microscopic observations of the diabetic liver and kidney sections showed evagination and invagination of the nuclear envelope, as well as apoptotic cell nuclei with peripheral heterochromatin condensation and margination (►Figs. 1B & 2B). This provides enough evidence that alloxan-induced diabetes is likely to cause apoptosis in liver and kidney cells, as previously reported.^{39,40} Hyperglycemia-mediated apoptosis has also been well-documented in several previous studies.^{41–43} The mitochondria are considered to be pivotal

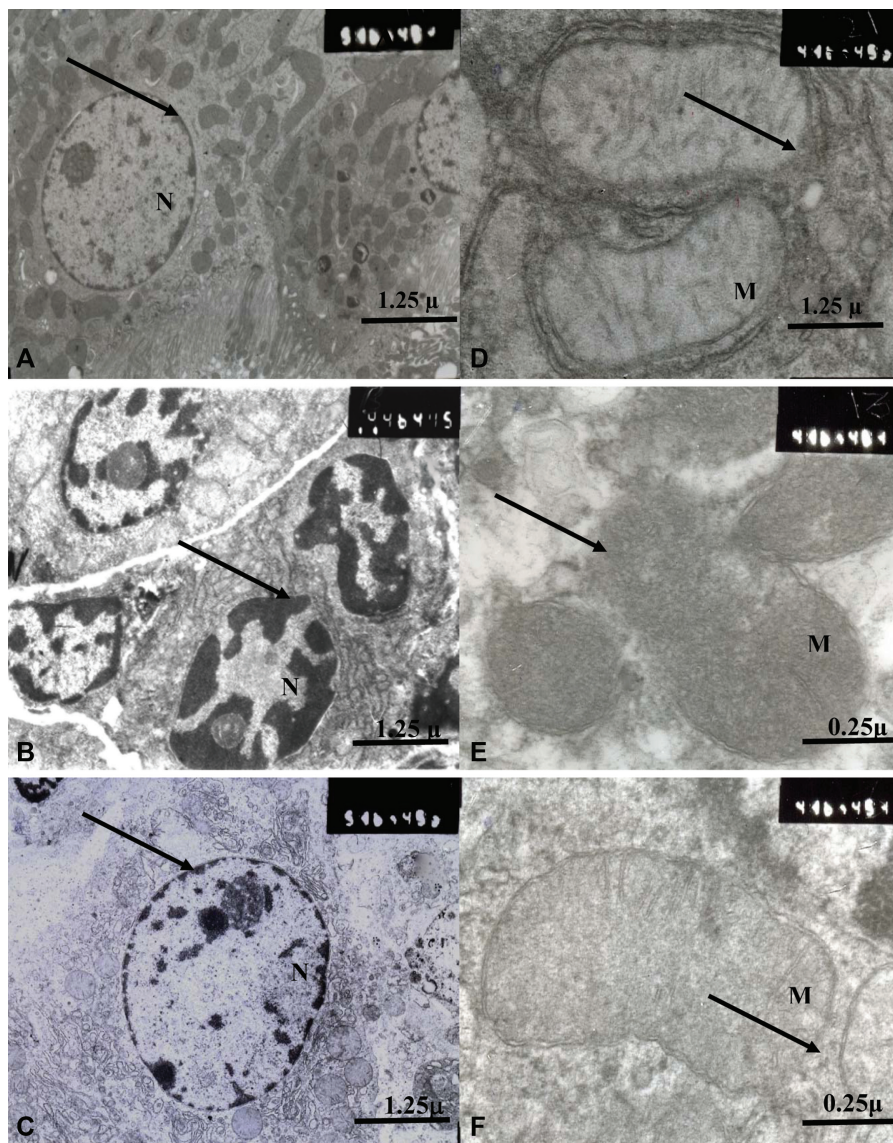


Fig. 2 Transmission electron micrograph showing the nuclei (N) in the kidneys of normoglycemic mice (A), diabetic mice (B), and *Potentilla fulgens* extract-treated diabetic mice (C) at magnification x 8,000. Transmission electron micrograph showing the mitochondria (M) in the kidneys of normal mice (D), diabetic mice (E), and *P. fulgens* extract-treated diabetic mice (F) at magnification x 40,000.

organelles in determining cell destiny, and act as an 'on-off' switch, modulating autophagy and apoptosis in the process of cell death.^{44–46} In the present study, some mitochondria exhibited breakage of the outer membrane at places, while some showed distortion of the inner cristae in the liver and in the kidneys of diabetic mice (►Figs. 1E & 2E), and these features have been reported in the pancreas of alloxan-induced diabetic mice.⁴⁷ Apoptosis is widely assumed to involve the mitochondrial permeability transition pore complex (MPTPC), which opens a small inner-membrane pore that leads to the influx of water, ions, and small molecules, causing the mitochondrial matrix to swell and the outer membrane to rupture.^{48,49} Vacuolization is also observed in the liver and in the kidneys of diabetic mice, which indicates an autophagic cell death, morphologically characterized by an accumulation of vacuoles.⁵⁰ Previous reports demonstrated that autophagy normally removes the aggregated or misfolded proteins induced by diabetes to defend against diabetes-induced mitochondria damage.⁵¹ The effect of the *P. fulgens* extract treatment was clearly visible, with an improvement in the ultrastructural features of the liver and of the kidney sections in diabetic mice, with a pronounced normalized appearance of the nuclei, as seen in the normal mice (►Figs. 1C & 2C). The ultrastructural abnormalities observed in the mitochondria were improved in the treatment with the *P. fulgens* extract (►Figs. 1F & 2F). In previous reports, the *P. fulgens* extract has shown to normalize the lipid profile⁸ and the level of enzymes such as hexokinase,⁸ aldose reductase,^{19–21} sorbitol dehydrogenase,²² and the antioxidant enzymes⁹ in diabetic mice. This suggests the possible reason for its beneficial effect on the ultrastructural changes of the studied tissues observed in diabetic mice.

The lens of the eye is comprised of highly ordered fiber cells that are covered anteriorly by a monolayer of epithelial cells. Fiber cells are hexagonal in cross-section, and are arranged in a honeycomb-like pattern, forming an array of regularly aligned, concentric rings that comprise the bulk of the lens.⁵² The fiber lens are developed all around the lens equator, eventually meeting and forming end-to-end associations with corresponding fibers from other segments of the lens. Any disruption to this organization impairs light transmission and lens function, because a loose but intimate and regular bonding of lens fibers is essential for the normal functioning of the lens (►Fig. 3).

Mature lens fibers lack cellular organelles and contain a highly concentrated protein solution to enable light refraction.⁵³ The transmission electron micrograph of the cross sections of the lens revealed disorganized fiber patterns in diabetic mice, in which the arrangement of the fibers was distorted compared with that observed in the normal lens fibers. This is in line with previous reports that hyperglycemic states adversely affect the lens fiber and morphology patterns, resulting in structural alterations.⁵⁴ In diabetic mice treated with the *P. fulgens* extract, a regular alignment of the lens fibers was observed. Although the lens segments were not as compact as observed in normal lenses, the honeycomb-like pattern with a regularly aligned array was

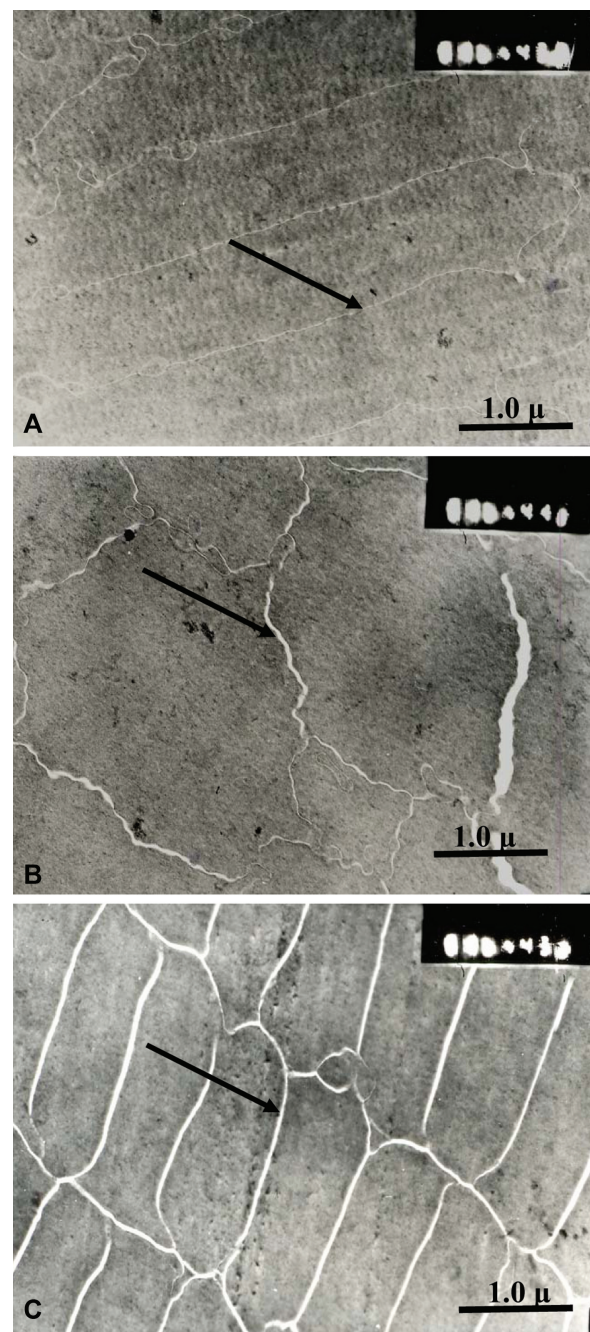


Fig. 3 Transmission electron micrograph showing the eye lens fibers of normoglycemic mice (A), diabetic mice (B), and *Potentilla fulgens* extract-treated diabetic mice (C) at magnification $\times 10,000$.

seen to be regular, and the alterations in the fiber pattern were minimized in *P. fulgens* extract-treated diabetic mice. Many other plants are known for their ability to restore the alloxan-induced morphological damage in different organs.^{47,55}

Conclusion

In conclusion, the experiment performed in the present study revealed that the *P. fulgens* root extract could inhibit enzymes such as amylase, α -glucosidase, β -glucosidase, and

lipase under in vitro conditions, and exhibit protective effects against the ultrastructural changes observed in the liver, in the kidney and in the eye lens of diabetic mice. Studying these effects has added new elements to the understanding of the antidiabetic property of this plant extract.

Acknowledgments

We would like to thank the DBT (Department of Biotechnology, Ministry of Science & Technology, New Delhi) DST-FIST (Department of Science & Technology- Fund for Improvement of S&T Infrastructure, Ministry of Science & Technology, New Delhi) Department of Biochemistry and the Department of Biotechnology & Bioinformatics, NEHU, Shillong, India, for providing the basic necessary facilities and financial assistance. We would also like to thank the staff of the TEM instrument, SAIF, NEHU, Shillong, India, for their assistance in sample preparation and viewing.

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Erratum: The category of DOI: 10.1055/s-0038-1669934 has been changed from Review Article to Original Article.