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Efficacy of methanolic extract of Piper longum on endothelial function in vitro and ex vivo

Vinay Kumar Kanakpura

Sumanta Kumar Goswami

Shekhar Dethe

Mohammed Naserrudin Inamadar
inamdarn@gmail.com

Original article

Efficacy of methanolic extract of *Piper longum* on endothelial function *in vitro* and *ex vivo*

Vinay Kumar Kanakpura, Sumanta Kumar Goswami, Shekhar Dethe, Mohammed Naserrudin Inamadar*

Abstract

Abstract: Piper longum has been used in several formulations in Ayurvedic system of medicine. The effect of herbal extract on diabetes induced endothelial dysfunction has not been studied. Therefore, we studied the effect of methanolic extract of Piper longum fruit (MEPL) on diabetes induced endothelial dysfunction using rat model. In addition, we studied the effect of MEPL on phenylephrine pre-contracted isolated rat thoracic aorta. MEPL dose dependently relaxed isolated rat aorta in vitro. Type 2 diabetes was induced in rats by administration of streptozotocin and nicotinamide. Diabetic rats were fed with MEPL at 22.5,45, and 90 mg/kg bw dose for 28 days and four weeks of induction of diabetes. A significant decrease in relaxant effect of acetylcholine (Ach) was observed on aorta isolated from diabetic rats when compared with effect of Ach on isolated aorta of normal rats. Though MEPL significantly relaxed isolated aorta of normal rats in vitro, the treatment of extract to diabetic rats did not increase responsiveness of Ach on aorta isolated from diabetic rats. Further studies need to be carried out to evaluate the effect of MEPL on diabetes induced endothelial dysfunction. MEPL might be effective in decreasing blood pressure in hypertension.

Keywords: Diabetes, endothelial dysfunction, Piper longum, aorta

Introduction

Diabetes mellitus is a metabolic disorder that affects more than 100 million people around the world. India is home to the highest number of diabetics in the world. Diabetic neuropathy, nephropathy, cardiopathy are the major complications of diabetes. Increase in blood pressure and decrease in erectile function results from endothelial dysfunction. Increase in the level of sugar, *advanced glycation end products* (AGEs), oxidative stress etc. are a few factors that cause endothelial dysfunction. Controlling level of sugar, AGEs and oxidative stress helps in the management of endothelial dysfunction[1-7]. Though, anti-diabetic medicines are helpful, herbal products might potentiate activity of anti-diabetic medicines[8]. Herbal product is also preferred in diabetes by some patients owing to low cost, least side effect of these preparations[9].

Piper longum is an important medicinal plant with multiple pharmacological activities including anti-inflammatory, antibacterial, antiamoebic and hepatoprotective activity[10]. Administration of ethanol extract of *Piper longum* by intravenous route was reported to decrease mean arterial pressure in rat[11]. Oil of *Piper longum* and aqueous extract of *Piper longum* have been reported to possess anti-diabetic activity[12, 13]. We evaluated the vasorelaxant effect of methanolic extract of *Piper longum* (MEPL) on isolated blood vessel from rat. In addition, we studied the effect of MEPL on endothelial function

Mohammed Naserrudin Inamadar
Department of Pharmacology, Al-Ameen College of Pharmacy, Bangalore, Karnataka, India. Email: inamdarn@gmail.com

Professor, Department of Pharmacology, College of Pharmacy, Jazan University, PO Box 114, Jazan, 82822, Kingdom of Saudi Arabia

* Corresponding Author

Vinay Kumar Kanakpura and Sumanta Kumar Goswami
Department of Pharmacology, Al-Ameen College of Pharmacy, Bangalore, Karnataka, India

Shekhar Dethe
Natural Remedies Private Limited, Electronic City, Bangalore, Karnataka, India

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ex vivo by evaluating the effect of Ach on isolated aorta from type 2 diabetic rats fed with MEPL and comparing with the response of Ach on aorta of non-diabetic rats.

Materials and Methods

Materials

Streptozotocin (Himedia), nicotinamide (Himedia), phenylephrine (Sigma-Aldrich Co), Ketamine hydrochloride (Neon Laboratory Ltd), xylazinehydrochloride (Indian Immunologicals Ltd), acetylcholine chloride (Sigma-Aldrich Co), d-Glucose anhydrous (ReaChem Laboratory Chemicals Pvt. Ltd.), Glucose assay kit (Span Diagnostics) were purchased. Other reagents used were of analytical grade.

Animals

Wistar albino rats bred at Al-Ameen College of Pharmacy, Bangalore, India and weighing 180-250 g were used for the study. Mice weighing 20-30 g were used for toxicity study. Permission for animal study was obtained from animal ethics committee of the institute. The animals were maintained at temperature 25-27 °C, and had free access to rat chow and drinking water.

For *in vitro* study, animals were divided in to two groups having eight rats each. For *ex vivo* study, animals were divided into four groups having six rats in each group.

Preparation of herbal extract

Dried fruits were purchased from Amruth Keshari Depot, Chickpet, Bangalore, Karnataka 560053. One kg of fruit was boiled with three liters of methanol under reflux, on a water bath at 60°C for 90 minutes and filtered. The above step was followed two times with the marc (residue) obtained from the first step. The filtrates were added together, and concentrated by boiling successively under vacuum[14].

Effect of *Piper longum* on isolated aorta of normal rat

Krebs Henseleit (K-H) salt solution [composition (mM): 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.5 $CaCl_2$, 25 $NaHCO_3$, 11 glucose] was prepared. The K-H solution was saturated with carbogen gas (95% oxygen and 5% carbon dioxide) to avoid K-H buffer acidosis, which occurs after prolonged gassing with carbogen. The pH of K-H solution was 7.4.

Animals were anesthetized with ketamine (65 mg/kg bw, i.p.) and xylazine (8 mg/kg bw, i.p.). Thoracic cavity was opened and thoracic aorta was located below heart. The aorta was freed from attached fats and a 2-3 cm aorta was cut. Then the tissue was washed in K-H solution aerated with carbogen gas till the tissues were mounted in four channel organ bath (AD Instruments, Australia). The aorta ring (3-4mm wide) was mounted between two steel hooks in an organ bath, filled with K-H solution, and maintained at 37°C. The steel hooks were stretched in opposite directions to exert 2 g tension on tissue. The aorta was washed after every 15 minutes and after 1 h, it was treated with Ach to ensure that endothelium was intact. After washing the tissues several times, different concentrations of MEPL were treated to evaluate possible contractile effect.

To study the relaxant effect of MEPL, the aorta was contracted by incremental doses of phenylephrine. The sub-maximal and ceiling doses of phenylephrine were observed. In the next set of experiment, the aorta was contracted by sub-maximal dose of phenylephrine and the maximum contraction achieved with this dose was considered as 100% effect. Different concentrations of MEPL were added to organ bath to study the relaxant effect of the extract in phenylephrine pre-contracted aorta.[15]

Acute toxicity study

For this study, mice were divided into two groups each containing six mice. One group was treated with vehicle (water with 1% Tween-80) and another group was treated with 2 g/kg bw dose of MEPL. The toxicity study was performed as per OECD guideline 425: Acute Oral Toxicity: Up-and-Down Procedure[16].

Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first four hours) and thereafter daily for a total period of 14 days for any adverse event (time of onset, length of recovery period). Mice were on

fast for four hours before dosing and were allowed to take food after two hours.

In case of any toxicity signs, animals were observed for additional toxicity reactions such as changes in skin and fur, eye, mucus membrane, respiratory system, circulatory system, autonomic system, central nervous system, somato-motor activity, behaviour pattern, number and duration of tremor, number and duration of convulsions, duration of salivation, number and duration of diarrhea, duration of lethargy, duration of sleep and duration of coma[16].

Induction of diabetes

Rats were divided into five groups. Four groups of rats were induced diabetes chemically. Type 2 diabetes was induced by administration of streptozotocin (65 mg/kg bw, i.p.; dissolved in ice-cold citrate buffer having pH of 4.5) and nicotinamide (90 mg/kg bw, i.p.) as described earlier[17].

Oral blood glucose tolerance test

To confirm induction of diabetes, oral blood glucose tolerance test was performed after 72 h of administration of streptozotocin and nicotinamide.

Blood glucose was estimated by using Glucose assay kit (Span diagnostics Pvt.). Oral glucose tolerance test (OGTT) was carried out to confirm the induction of type 2 diabetes. After 12 h of fasting rats were orally given a glucose challenge of 2 gm/kg bw. Blood glucose was determined by the above-mentioned method at 0, 30, 60, 90 and 120 mins after glucose challenge. A plot of blood glucose level obtained versus time was analyzed for impairment.

Effect of *Piper longum* on aorta of diabetic rats *ex vivo*

After induction of diabetes in rats, it was allowed for four weeks for development of endothelial dysfunction following which the treatment of MEPL (22.5, 45, and 90 mg/kg bw) was continued for 28 days. Low doses of MEPL were selected for administration because the higher doses might cause gastric irritation. After the end of treatment, the animals were anesthetized as aorta was removed as discussed earlier. Aorta was contracted by 10 μM phenylephrine and relaxed by different concentrations of Ach. The effect of Ach on aorta from diabetic rats treated vehicle and MEPL was recorded, and compared with that of a healthy rat.

Statistical analysis

Values are expressed as mean ± standard error of mean of 6-9 observations. Two way ANOVA followed by Bonferroni's post-hoc test was used to evaluate statistical significance.

Results

Preparation of herbal extract

The yield of the methanolic extract of *Piper longum* was approximately 10%. This value was similar to the reported value for percent yield of ethanol extract18. A greenish-black extract was obtained which was better soluble in dimethylsulfoxide (DMSO). For dosing the animals, a suspension of MEPL was prepared with 1% Tween-80.

Effect of *Piper longum* on isolated aorta of normal rat

Methanol extract of *Piper longum* did not contract isolated aorta of normal rat at a concentration upto 100 μg/mL.

Contractile effect of phenylephrine on isolated aorta of normal rat was observed at concentrations upto 30 μM. Ceiling effect was observed at 30 μM and 10 μM was selected as sub-maximal dose (Figure 1).

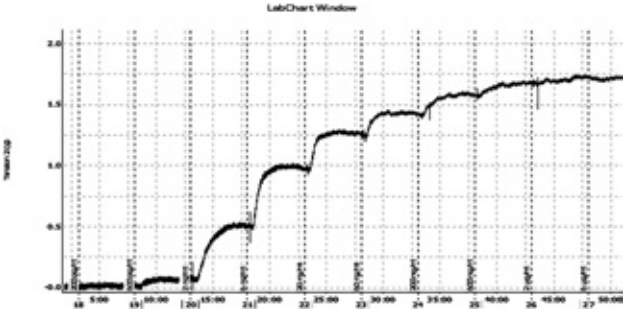


Figure 1. Contractile effect of phenylephrine on aorta of rat *in vitro*

MEPL dose dependently relaxed isolated aorta of normal rats. The significant relaxation was observed at 100 μg/mL (Figure 2). This data suggests that administration of this extract may be helpful in the management of hypertension.

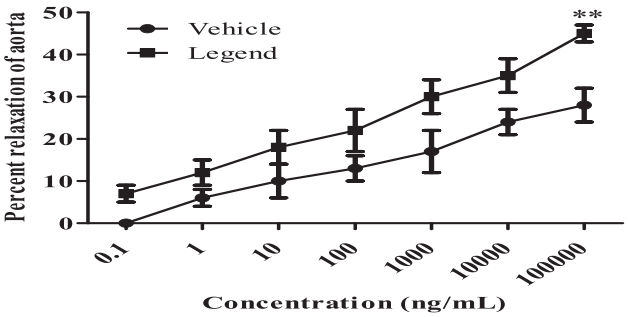


Figure 2. Effect of methanol extract of *Piper longum* on relaxation of isolated aorta.

The value is presented as mean ± standard error of mean (n=9). At 100 µg/mL, the extract significantly relaxes isolates aorta of normal rats in comparison to vehicle (Tween20 in water). ** p< 0.01.

Acute toxicity study

The extract was found to be safe at upto 2 g/kg bw. No death was observed. Adverse events such as writhing, change in body colour were not noticed. The animals treated with MEPL behaved in a similar way as those treated with vehicle. Further, lower doses of extracts such as 22.5, 45, and 90 mg/kg bw were selected for administration. Because *piper longum* is used as spice, we preferred to use lower doses for administration.

Oral blood glucose tolerance test

Blood glucose was found to be elevated in diabetic rats in comparison to the normal rats. While the fasting glucose levels were 108.44 ± 1.68mg/dL in control rats, the glucose levels increased to a maximum of 170.45 ± 1.17 mg/dL at 60 minutes after oral glucose challenge, which was brought back to values close to fasting level within 120 minutes. However, the diabetic rats showed very high fasting glucose values (322.79 ± 5.92 mg/dL) which increased further after the oral glucose challenge (Figure 3).

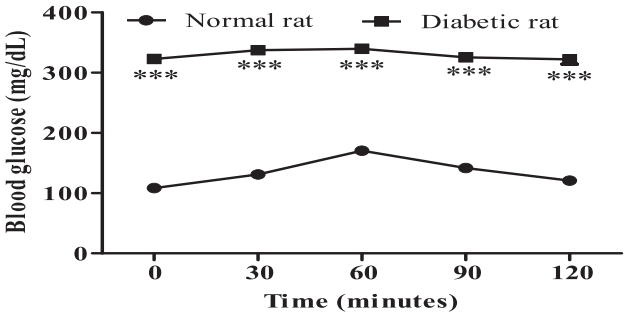


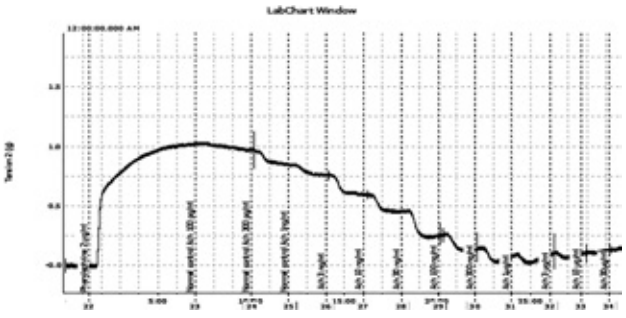
Figure 3. Oral glucose tolerance test.

The blood glucose level of normal rats increased significantly 1 h after administration of glucose when compared with 0 min time point and normalized after 2 h, whereas the blood glucose level of diabetic rat was higher before administration of glucose and was not affected much after glucose administration. Values are presented as mean ± standard error of mean. Two way ANOVA followed by Bonferroni's post-hoc test was used for statistical calculation. For normal rat, n=3 and for diabetic rats n=18. *** p< 0.001.

These changes could also be observed in form of difference in the AUC $_{0\text{-}120}$ min of normal (11.74 ± 5.04 g.min/dL) and diabetic (39.74 ± 0.29 g.min/dL) rats, which is found to be highly significant statistically (*** $p < 0.001$).

Effect of *Piper longum* on aorta of diabetic rats *ex vivo*

Ach-induced relaxation of blood vessel is known to be endothelium dependent. A dose dependent relaxation is seen with Ach and approximately 90% relaxation of phenylephrine pre-contracted aorta from normal rat was observed at 2 µM. However, Ach at 2µM could relax aorta from diabetic rat only upto 50%. Treatment of MEPL to diabetic rats at 22.5, 45, and 90 mg/kg bw dose did not increase responsiveness of Ach to relax aorta (Figure 4). This data suggests that MEPL might not be helpful in the management of endothelial dysfunction. Further studies are required to ascertain prophylactic treatment of *Piper longum* on diabetes induced endothelial dysfunction.



Effect of Ach on aorta of normal rat

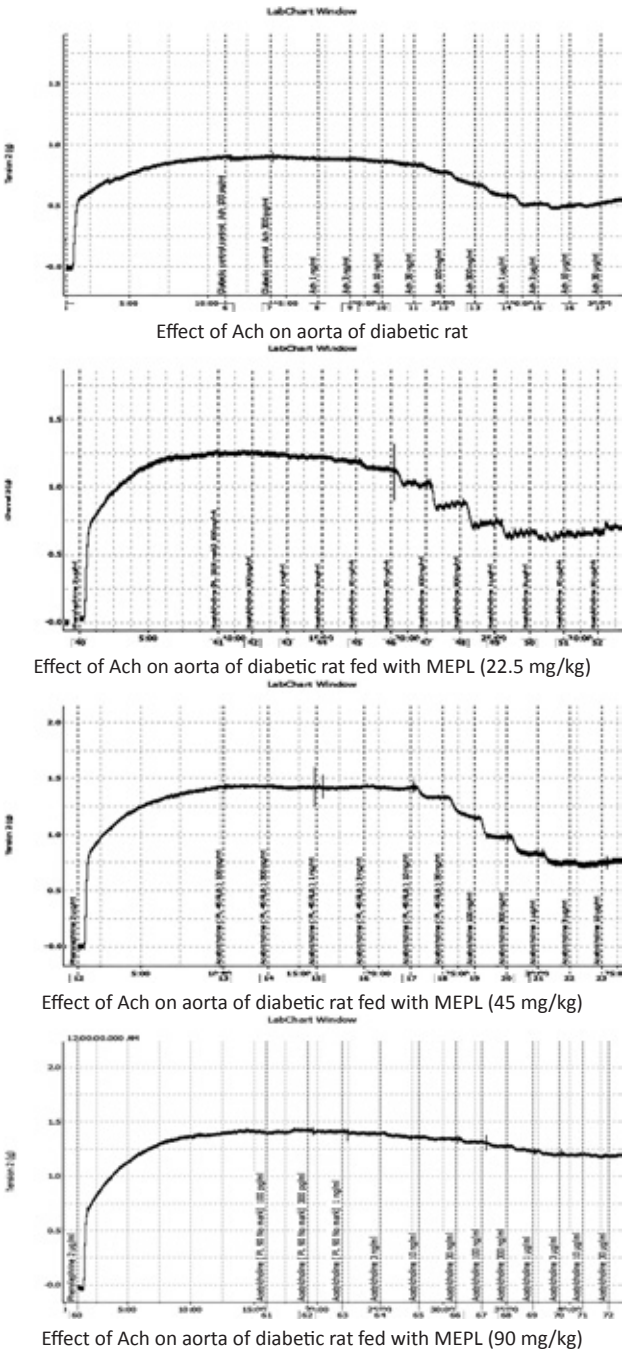


Figure 4. Effect of treatment of methanol extract of *Piper longum* on diabetes-induced endothelial dysfunction.

Though Ach could relax phenylephrine pre-contracted normal aorta, the relaxant effect of Ach was decreased by 50% after 56 days of induction of diabetes. Treatment of MEPL did not improve relaxant effect of Ach on aorta of diabetic rat.

Discussion

Piper longum is one of the most popular medicinal plants. The extract of dose dependent relaxed isolated aorta from rat suggest possible use in the management of high blood pressure. Shoji et al., reported that dehydropipernonaline, an amide isolated from *Piper longum* dilates blood vessel[19]. Though, we did not estimate level of dehydropipernonaline in MEPL, role of dehydropipernonaline in dilating isolated rat aorta in this study cannot be refuted. Treatment of this extract at 22.5, 45, and 90 mg/kg bw doses for 28 days after four weeks of induction of diabetes did not potentiate relaxant effect of Ach on isolated aorta of diabetic rat. This suggests that these doses and duration of treatment may not be suitable to treat diabetes-induced endothelial dysfunction and related vascular complications.

Many physiological processes are known to be altered in diabetes, which leads to development of endothelial dysfunction. Endothelium has limited capability for self-repair. Therefore, endothelial repair is achieved through the contribution of circulating endothelial progenitor cells (EPCs). In diabetes, alteration in function of EPC including reduced proliferation, adhesion, migration, and incorporation into tubular structures is observed. Diabetic EPCs also display functional impairment, such as reduced proliferation, adhesion, migration, and incorporation into tubular structures. It is reported that in case of endothelial dysfunction, Ach may contract endothelium by its action on M3 muscarinic receptor. Hyperglycemia also initiates apoptosis of endothelial cells, which involve activation of p38 mitogen–activated protein kinase (MAPK) and c-Jun Nterminal protein kinase (JNK). The apoptotic endothelial cells or degradation product including microparticles (endothelial microparticles) triggers the atherosclerotic process. Similarly, the contraction of blood vessel resulting from endothelial dysfunction can aggravate cardiac complications.[5]

An alteration in levels of enzymes, which regulate tone of endothelial smooth muscle is also reported in diabetes. Nitric oxide/ cyclic guanosine monophosphate path way is responsible for relaxation of endothelial smooth muscle. In nerves and endothelium, nitric oxide synthase metabolize arginine to nitric oxide that diffuses into smooth muscle to stimulate soluble guanylyl cyclase for

synthesizing cyclic guanosine monophosphate. Protein kinase G and ion channels modulated by cyclic guanosine monophosphate relax endothelial smooth muscle. Uncoupling of nitric oxide synthase in diabetes generates superoxide that converts nitric oxide into peroxynitrite, a reactive oxidant. The oxidative stress due to increase in the level of peroxynitrite damage endothelium, which is one of the factors in the development of endothelial dysfunction.[20, 21]

Contraction of endothelium is regulated by Rho-kinase. Angiotensin-II and endothelin-1 binds with their respective G-protein coupled receptor AT1R and ETAR respectively to activate Rho, a G-protein. Rho stimulates Rho kinase that inhibits myosin light chain by phosphorylating it. This favours increase in level of phoshorylated myosin light chain and decrease in level of myosin light chain that results in contraction of endothelial smooth muscle. The level and activity of Rho kinase is reported to be increased in diabetes. In addition, Rho kinase inhibits nitric oxide synthase. This process favours contraction of endothelial smooth muscle and results in dysfunction of endothelium.[4, 21-23]

Conclusion

Methanolic extract of *Piper longum* relaxes endothelial smooth muscle of aorta from normal and healthy rat. But, the administration of this extract did not increase sensitivity of Ach in the aorta of diabetic rats. However, we did not evaluate the effect of diabetes-induced endothelial dysfunction on blood pressure and effect of MEPL on blood pressure. *Piper longum* extract standardized with the level of dehydropipernonaline might be a good research tool to evaluate its effect on endothelial dysfunction. Further studies need to be carried out to evaluate preventive use of methanol extract of *Piper longum* on endothelial dysfunction. Future studies emphasizing the use of standardized extract, use of other models of endothelia dysfunction and additional study design including preventative use of extract can satisfactorily evaluate the usefulness of this extract in endothelial dysfunction.

Acknowledgements

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