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Original Article

ENHANCEMENT OF ANTI-DIABETIC ACTIVITY OF 4-HYDROXYISOLEUCINE IN COMBINATION WITH NATURAL BIOAVAILABILITY ENHANCERS

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ABSTRACT

Objective: This study investigates for the first time the antidiabetic activity of 4-hydroxy isoleucine (4-OH Ile) in combination with natural bioavailability enhancers, piperine and ginger oleoresin.

Methods: Alloxan induced diabetic rat model was used for studying the antidiabetic activity of 4-OH Ile in combination with natural bioavailability enhancers to study its effect on body weight, fasting blood glucose level and oral glucose tolerance test.

Results: 4-OH Ile present in *Trigonella foenum-graceum* seeds is an amino acid that shows a significant activity in type II diabetes. Due to its glucose dependent effect and devoid of side effects, 4-OH Ile is used for management of type II diabetes. The antidiabetic activity of 4-OH Ile was found to be significantly increased in combination with piperine and ginger oleo resin. However, piperine has demonstrated better effect as compared to ginger oleoresin. Histopathological studies of pancreas were also done to further verify the effect of natural bioenhancers.

Conclusion: The results of this study places piperine and ginger oleo resin as a mode of increasing activity of 4-OH Ile and thereby reducing their cost of the treatment.

Keywords: 4-Hydroxyisoleucine, Bioavailabilty enhancers, Type II diabetes.

INTRODUCTION

Fenugreek (*Trigonella foenum-graceum* L.) is an annual herbaceous aromatic leguminous, widely cultivated in Mediterranean countries and Asia, as it is a popular food (home remedies) consumed in various ways. The pods contain about 10–20 yellowish seeds rich in proteins (30% dry matter) and with a pleasing appetizing aroma [1]. Besides, the seeds contain sapogenins used for medicinal steroids synthesis, steroidal saponins which are responsible for the hypocholesterolemic activity of fenugreek, as well as the free amino acid 4-hydroxyisoleucine (near 80% of free amino acids present in fenugreek seeds) that is responsible for the hypoglycemic activity of fenugreek [2-4]. Its precursors in the seeds are suspected to be 4-hydroxyisoleucine, which is the major amino acid present [5;6].

Most important property of *T. foenum graecum* is its antidibetic action which has been explored in many research articles. In India, the seeds of fenugreek (*T. foenum graecum*) have been used traditionally as a treatment for diabetes. Beneficial effects of the seeds have been evidenced in experimental diabetic animals and in both type 1 and type 2 diabetic subjects [7;8]. It has been reported that alcoholic extract of fenugreek seeds has an antidiabetic efficacy in streptozotocin-induced diabetic rats. It is observed that a fraction rich in testa and endosperm of fenugreek seeds decreased the hyperglycemia and glycosuria accompanied by a reduction in the high levels of plasma glucagon and somatostatin in diabetic dogs [9]. The gum fraction isolated from the seeds was shown to be effective in reducing plasma glucose [10].

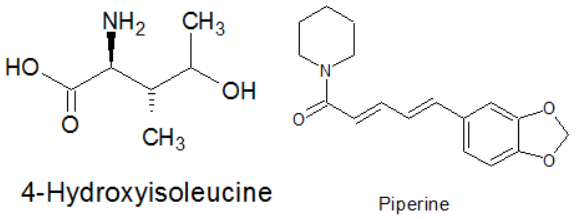
The magical component responsible for its extraordinary hypoglycemic effect was found to be 4-hydroxyisoleucine. 4-Hydroxyisoleucine (4-OH Ile) is a natural nonproteinogenic amino acid present in *T. foenum graecum* seeds possessing insulinotropic biological activity [11-13] 4-OH Ile increases glucose-induced release of insulin. In contrast to several types of pharmacological drugs that have been used for the treatment of type II diabetes (e. g. sulfonylureas), the insulin response mediated by 4-OH Ile is strictly dependent on the glucose concentration. This unique property of 4-OH Ile allows us to avoid undesirable side effects such as hypoglycemia in the therapy of type II diabetes. Thus, 4-OH Ile seems a promising dietary supplement in the treatment and prevention of this chronic disease [14].

This study aims at increasing the anti-diabetic activity of 4-OH Ile by combining it with natural bio enhancers. Taking the cost of 4-OH Ile into consideration this combination will reduce the expense of regimen for type II diabetes.

Hydroxyisoleucine (28%)

Fenugreek plant and its seeds both shows antidiabetic activities. The amino acids 4-hydroxyisoleucine, alkaloid trigonelline, flavonoid glycosides and gallactomannen have been reported to be responsible for the antidiabetic activity of fenugreek. It is difficult to isolate 4-hydroxyisoleucine in pure form therefore 4-hydroxyisoleucine enriched fraction of the fenugreek extract is being used as an antidiabetic agent [15]. This product has been patented as a synergistic composition to be used for the treatment of diabetes mellitus under the trade name Sugaheal®. In the present study this 4-hydroxyisoleucine (28%) and trigonelline enriched fraction of fenugreek extract has been used for studying the antioxidant efficacy.

Piperine, an amide alkaloid obtained from the mature fruits of *Piper nigrum* and *P. longum,* is the first and till date the most potent bioenhancer to be discovered [1]. The bioenhancing activity of piperine and the concept of BA enhancement using piperine both were discovered and scientifically validated in 1979 in India [16]. It was studied successfully to reduce the dose of the drug and cost of the treatment. Collaborative studies conducted by Cadila Labs Ltd. at RRL, Jammu, led to successful launching of the well known anti-TB drug Rifampin (200 mg) along with bioenhancer piperine (10mg) under the trade name 'Risorine', in 2009. In the above case, Rifampicin's conventional dose of 450 mg has been reduced to 200 mg with the same bioavailability [17]. Piperine brings about its bioenhancing activity by inhibition of drug efflux pump (PGP_2) and by inhibiting enzymes such as $CYP1A_1$, $CYP1B_1$, $CYP1B_2$, $CYP1E_1$ and $CYP3A_4$. All the drugs metabolized by these enzymes are influenced by bioenhancer piperine [18]. All categories of drugs like cardiovascular, respiratory, CNS, GIT, anticancer, immunomodulatory drugs, antibiotics, several other classes of drugs and nutraceuticals are greatly influenced by piperine. It is interesting to note that piperine brings about its bioenhancing effect in a dose of 10 mg in all formulations irrespective of the dose of combination drug.



4-Hydroxyisoleucine Piperine

The drug consist of rhizome of the plant *Zingiber officinalis* Roscoe, family Zingiberaceae, commonly known as ginger. Many drugs are found to be more active when used in combination with bioenhancer products developed from ginger. The effective range for ginger as a bienhancer is 10-150 mg. Class of drugs which has shown enhanced activity are antiretrovirals, CVS drugs, CNS drugs, anti-inflammatory, antiarthritic, antitubercular, antileprotic, antiulcer and many other therapeutic agents [19, 20].

MATERIALS AND METHODS

Plant material, Chemicals and reagents

All crude drugs namely Black pepper, *Piper nigrum* and Ginger, *Zingiber officinalis,* were procured from reliable sources. Fenugreek seeds, Black pepper and Ginger rhizomes were procured from local market of Bilaspur. Alloxan, a standard diabetic inducer was purchased from Lobal Chemie.

4-hydroxy isoleucine (28%)

Dried mature seeds of Fenugreek, *Trigonella foenum graecum,* family Leguminosae were first subjected to screening for the presence of total amino acids and trigonelline using thin layer chromatography on pre-coated silica gel TLC plates using n-butanol: acetic acid: water in a ratio of 12:8:2 and initial scanning using UV at 254 nm for the presence of trigonelline. Ninhydrin reagent was used for color development of total amino acids [21].

Dried mature Fenugreek seeds in a quantity of 1 Kg were flaked in a flaker to expose the inner core, resulting in flakes of average 15 mm in size. The flakes were then subjected to hydro-alcohol extraction using 6 liters of isopropyl alcohol: water mixture in a ratio of 50:50 at 35^{0}C for 12 hours. The resultant liquid (about 5500 ml) was concentrated to a final volume of 150 ml under vacuum at 45-50^{0}C. This liquid was extracted with 3x50 ml of n-hexane to remove fats and lipids. The defatted concentrate was diluted with demineralized water to a final volume of 500 ml. This liquid was subjected to fine filtration through 200-mesh size to remove insolubles.

The filtered liquid was then passed through a glass column of 500 mm length x 25 mm diameter containing strong acid cation exchange resin in H^{+}form freshly regenerated with 600 ml of 3% HCl in water, followed by washing to neutral pH. After passing the liquid, the column was washed with de-mineralized water to neutral pH. The loaded amino acid and trigonelline were eluted with 200 ml of 0.5 N ammonia solution. The ammonia liquid was circulated in the column until it attained a stable P^{H} of 8.0.

The resultant solution was then passed through a glass column of 800 mm length x 25 mm diameter containing 200 ml of freshly regenerated weak acid cation resin in gel form. The eluent from this column was a colourless, neutral liquid having only compounds such as amino acids and trigonelline present in the ratio as in the mother seed. The product was spray dried with the conditions of co-current air flow, inlet temperature, 165^{0}C, outlet temperature 85^{0}C with automizer revolutions of 30, 000 rpm. The resultant granules from the spray drying process was found to be free flowing and suitable for formulation.

The resultant powder reffered to as Sugaheal® was further screened by HPLC for amino acids by derivatization using working standard of (99%) of 4-hydroxyisoleucine as a dinitrofluorobenzene derivative (347 nm), and trigonelline using UV. HPLC analysis of Sugaheal®on JASCO HPLC/lC 2000 with UV-2075 detector and reverse phase C-18 column L1 as defined in USP30/NF25 with 5µ particle size (diameter 250 mm X 4.6 mm). Results of the HPLC studies has indicated the content of Sugaheal® as 4-hydroxy isoleucine (28.44%), trigonelline (31.91%) and remainder as Galactomannen [22].

Piperine

Dried ripe fruits of black pepper, *Piper nigrum* were defatted with petroleum ether (60-80^{0}) in soxhlet extractor for 24 hrs. The extract was dried and further extracted with ethyl alcohol (95%) for 48 hrs. The total ethyl alcohol extract was cooled and filtered to remove fine particles if necessary, and concentrated under reduced pressure to yield total alcohol extract in 2.5% yield. The concentrated solution was kept in an ice bath, and water was added drop wise (about 30 ml will be required) to precipitate piperine. Piperine was collected on a sintered glass funnel. It was further recrystallized from acetone: hexanes (3:2) to afford pale yellow crystals of piperine (7%). Thin layer chromatographic study of isolated piperine along with the authentic primary standard has shown a single spot of R_f 0.52, in solvent system toluene-ethyl acetate (7:3) when spread with Dragendorff's reagent [16;17].

Ginger oleo resin

About 250g of completely dried ginger was powdered in a mechanical grinder, finely shifted and subjected to continuous hot percolation process using soxhlet apparatus, by using solvents petroleum ether and then with ethanol for 24 and 48 hrs respectively. The extracts thus obtained were concentrated to a thick brownish yellow semi-solid mass using Rotary vacuum evaporator or water bath. The thick pasty mass was added to water to precipitate oleo-resin. The practical yield was found to be 2.9 % [23]. The oleo-resin extract was subjected to thin layer chromatography using silica gel G and petroleum ether: ethyl acetate (7:3) as a solvent system. The Rf values of the oleo-resin constituents were found as per the reported values in the literature.

Antidiabetic activity

Animals

The Sprague-Dawley rats of either sex weighing between 150-300 g were employed in this investigation. They were housed under standard conditions of temperature 22^{o} C (±3^{o}C) humidity 35% to 60%, and light (12:12 hr light/dark cycle) in polypropylene rat cage. The experimental protocol was submitted and approved by Institutional Ethical Committee, Guru Ghasidas Vishwavidyala, Bilaspur.

Experimental design

In experiment total 30 rats (24 diabetic survival rats and 6 normal rats) were used. The rats were divided into 5 groups each group comprising 6 rats (n=6). All rats were marked and randomly divided into eleven groups, each group comprising of six animals. Weight of individual rat was taken on electrical signal pan balance and numbering was done to each rat. Group I: Normal untreated rats given vehicle orally for 10 days. Group II: Diabetic rats (Alloxan induced) given vehicle orally for 10 days. Group III: Diabetic rats given *T. foenum-graecum* extract containing 28 % 4-OH Ile orally for 10 days. Group IV: Diabetic rats given *T. foenum-graecum* extract containing 28 % 4-OH Ile orally along with piperine for 10 days. Group V: Diabetic rats given *T. foenum-graecum* extract containing 28 % 4-OH Ile orally along with ginger oleo resin for 10 days.

Dose was calculated according to the weight of individual rat and was administered orally by suspending it in 2% w/v Tragacanth suspension. The dose for *T. foenum graecum* extract with 28% 4-OH Ile, piperine and Ginger oleo resin was calculated as 400 mg/kg [22-24], 15-30 mg/kg [25] and 30 mg/kg respectively.

Induction of diabetes in rats

Alloxan (170 mg/kg body weight) [26] was dissolved in ice cold saline, pH maintained 4.5 by citric acid. The animals were fasted overnight and DM was induced by single i. p. injection of freshly prepared Alloxan (170 mg/kg body weight) in ice cold saline, pH maintained at 4.5 by citric acid. The animals were given 3% glucose solution orally just after Alloxan injection to avoid hypoglycaemia.

They were also allowed to drink 5% glucose solution for 24 hrs to overcome the drug induced hypoglycaemia. The animals were considered as diabetic, if their blood glucose values were above 200-250 mg/dl after 72 hrs i.e. 3rddday of injection. The treatment was started on 4thday and this was considered as the first day of treatment and was continued for 10 days.

RESULTS

Effect on body weight

Body weight and fasting blood glucose were monitored on 0 day, 5th day and 10th day respectively. Results of the effect of 4-OH Ile and bioenhancers on body weight is given in table-1 and fig-1.

Table 1: Effect of 4-OH Ile and bioenhancers on body weight of animals

S. No.	Groups	Body weight		
		0 Day	5 Day	10 Day
1	Normal	168.00±4.76	169.16±5.30	169.66±5.03
2	Diabetic	180.66±4.92	161.50±5.09**	154.66±3.89**
3	4-OH Ile	192.06±4.93	198.83±4.35**	211.50±2.12**
4	4-OH Ile+Piperine	194.33±3.73	188.50±3.84**	202.50±4.25**
5	4-OH Ile+GOR	195.66±4.47	198.83±6.04**	191.33±4.36**

GOR-Ginger oleo-gum resin

Stastistical method: One way ANOVA followed by Dunnett's multiple comparison tests (N=6); values are expressed as mean±SEM; *P<0.05 as compared to diabetic control; **P<0.01 as compared to diabetic control.

Effect on fasting blood glucose level

On treatment with fenugreek seed powder containing 28 % 4-OH Ile (400 mg/kg body weight) alone and along with piperine and (GOR) the fasting mean blood glucose levels on 0 day (after being diabetic) i.e. 246.83±2.72 mg/dl, 260.66±1.66 mg/dl and 246.00±0.71 mg/dl reduced to 124.33±1.49 mg/dl, 118.50±3.37 mg/dl and 131.83±3.14 mg/dl respectively on 10th day. Blood glucose was estimated using a commercial diagnostic kit. (Accu check active glucometer). Results of the effect on fasting blood glucose level is given in table 2 and fig. 2.

GOR-Ginger Oleo-gum Resin

Stastistical method: One way ANOVA followed by Dunnett's multiple comparison tests (N=6); values expressed as mean±S. E. M.; ***P<0.001 values are expressed as mean±S. E. M.; ***P<0.001as compared to diabetic control group. P<0.001 as compared to control group.

Oral glucose tolerance test

On the last day of treatment an oral glucose tolerance test (OGTT) was performed after overnight fasting. Blood was sampled from the tail vein of rat at a time of 0 min (baseline), 30, 60 and 120 min after an oral glucose load of 3.0 g/kg of bodyweight. Food but not water was withheld from cages during the course of fasting. Results of the effect of 4-OH Ile and bioenhancers on oral glucose tolerance test is given in table 3 and fig. 3.

Table 2: Effect of 4-OH Ile and bioenhancers on fasting blood glucose level

S. No.	Groups	Fasting blood sugar level		
		0 Day	5 Day	10 Day
1	Normal	102.50±5.70*	106.00±4.69*	107.83±6.41*
2	Diabetic	248.16±4.16	253. 50±2.94**	263.16±3.09**
3	4-OH Ile	246. 83±2.72	189.5±2.60**	124.33±1.49**
4	4-OH Ile+Piperine	260.66±1.66	132.16±2.49***	118.50±3.37***
5	4-OH Ile+GOR	246.00±0.71	146.00±2.99***	131.83±3.14***

Table 3: Effect of 4-OH Ile and bioenhancers in oral Glucose tolerance test

S. No.	Groups	Oral glucose tolerance test			
		0 min	30 min	60 min	120 min
1	Normal	107.83±6.41	159±5.56*	137.16±4.02*	123.5±3.38*
2	Diabetic	263.16±3.09	314±2.55**	359.16±7.07**	292.5±3.28**
3	4-OH Ile	124.33±1.49	144.16±2.34**	144.66±2.98**	128.83±2.27**
4	4-OH Ile+Piperine	118.50±3.37	162±7.51**	147±30.11**	139.66±16.98**
5	4-OH Ile+GOR	131.83±3.14	148.16±2.58**	174.83±2.62**	145.83±4.04**

GOR-Ginger oleo-gum resin

Stastistical method: One way ANOVA followed by Dunnett's multiple comparison tests (N=6); values are expressed as mean±S. E. M.; *P<0.001 as compared to control group. **P<0.001as compared to diabetic control group.

Histopathological results

After a rest period of one week followed by treatment, one animal from each group was sacrificed. Pancreas was dissected out and immediately fixed in 10% neutral buffer formalin. Stored pancreases were sent to Medical College, Raipur for histopathology. The sections were cut and stained with haematoxylin and eosin using standard protocol for the preparation of slide. Prepared slides were collected and histomorphologic observation was done under photomicroscope (Primostar microscope with digital camera-ZEISS) [27].

All pancreatic cells of the normal rats were present in their normal proportions. The acinar cells which stained strongly are arranged in lobules with prominent nuclei. The islet cells are seen embedded within the acinar cells and surrounded by a fine capsule. Pancreatic cells of the Alloxan-diabetic rats (control) showing a progressive distortion in the histoarchitecture of the pancreatic parenchyma and stroma compared to normal group.

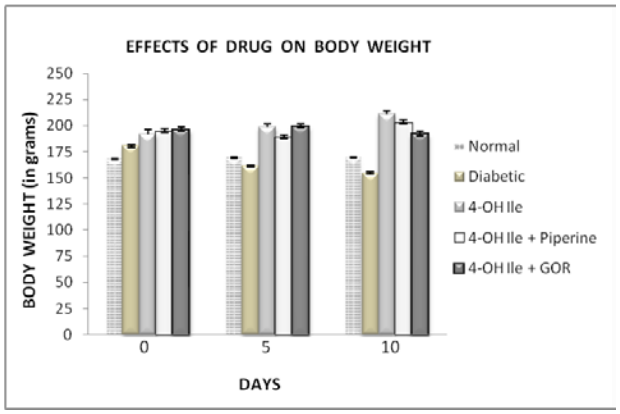
DISCUSSION

In present study the experimental groups alloxan induced diabetic rats were treated with fenugreek seed powder containing 28 % 4-

OH Ile alone and along with bioenhancers i.e. piperine and ginger oleogum resin (GOR). The experiment was conducted in accordance with parallel design i.e. each group received single formulation, single time. After completion of the study protocol, it was found that the blood glucose level and body weight improved significantly (P<0.001) as compared to diabetic control.

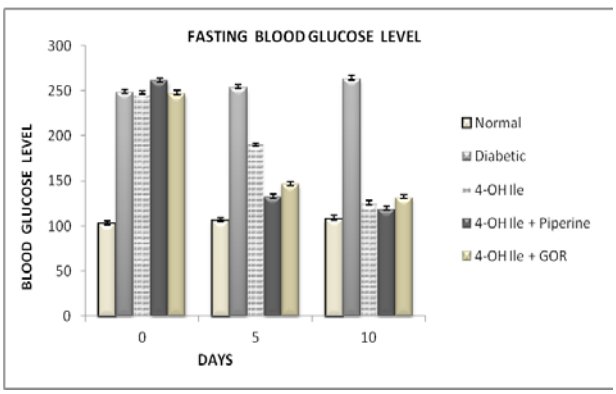
Alloxan induced diabetic rats exhibited decreased body weight, polyphagia, polydipsia associated with decrease in endogenous insulin and hyperglycaemia. Treatment with extracts to diabetic rats increases body weight and also decrease in elevated blood sugar level. These effects may be attributed to either inhibition of increase in insulin output or inhibition of intestinal absorption of glucose or increase in glucose metabolism or combination of all.

4-OH Ile displays an *in vitro* insulinotropic activity, which is of great interest, and that its stimulating effect is related to the immolation of glucose concentration in the medium as shown in isolated pancreatic beta cells. Administration of 28% 4-OH Ile alone and along with piperine and ginger oleo-gum resin increases body weight in Alloxan diabetic rats (table-1, Graph-1). The ability to protect body weight loss seems to be as a result of its ability to reduce hyperglycaemia.



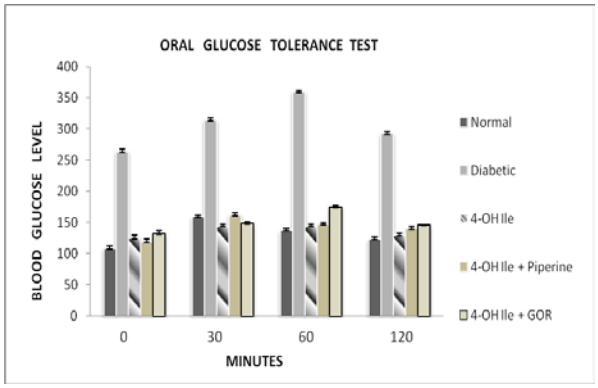
Graph 1: Effect of 4-OH Ile (28%) and bioenhancers on body weight of diabetic rats

Results of 4-OH Ile alone and along with bio enhancers has indicated significant decreases in fasting blood glucose level in diabetic rats (table 2, Graph 2). Fasting blood glucose level of 4-OH Ile with piperine (118.50±3.37) has been the most significant as compared to 4-OH Ile (124.33±1.49), and 4-OH Ile with GOR (131.83±3.14) when correlated with the values of normal groups (107.83±6.41). This prominent activity of OH Ile in combination with piperine may be due to bioavailability enhancement potential of piperine.



Graph 2: Effect of 4-OH Ile and bioenhancers on fasting blood glucose level of diabetic rats

Results of blood glucose level of diabetic rats subjected to oral glucose tolerance test in comparison with values obtained in normal rats (Table-3, Graph-3) indicated significant decrease of the blood glucose level almost to the normal level. In case of 4-OH Ile (128.83±2.27), 4-OH Ile with piperine (139.66±16.98) and 4-OH Ile with GOR (145.83±4.04) as compared to the values of normal groups (123.5±3.38). Significant antidiabetic activity of 4-OH Ile has been reported to be due to its ability to decrease insulin resistance, inhibiting some of the absorption of sugars through the intestine into the blood stream [28] and by enhancing glycogen synthesis in muscle cells following exercise by up-regulating the activity of insulin receptors in muscles [29]. 4-Hydroxyisoleucine is reported to be metabolized 4-hydroxy-3-methyl-2-keto pentanoate aldolase (HMKP aldolase) producing bacteria as they use it as a sole carbon source. Bioenhancers may be playing a protective role in this metabolic process to enhance the antidiabetic activity of 4-hydroxyisoleucine.



Graph 3: Effect of 4-OH Ile (28%) and bioenhancers on oral glucose tolerance test in diabetic rats

Histopathological study of diabetic untreated rats revealed degeneration of pancreatic islet cells, which was due to alloxan used in this experiment. Signs of regeneration of β-cells have been reported following consumption of some plant extracts and isolated compound alone and with bioenhancer. However it is difficult to predict the exact percentage of regeneration that has taken place after treatment. Transmission electron microscopy (TEM) needs to be done for better understanding of the results.

CONCLUSION

The present data demonstrated that bioenhancers i.e. piperine and ginger oleo-resin increased the anti-diabetic activity of 4-OH Ile probably by preventing its metabolism and making it readily available in blood plasma. These in vivo studies of 28 % 4-OH Ile with natural bioenhancers may be helpful for the development and application of 28 % 4-OH Ile as a promising antidiabetic agent. The formulation if prepared can be considered as safe supplementary therapy for a long term and effective management of diabetic patients. However, the drug–drug interaction potential should be confirmed by further *in vivo* studies. Further systematic studies in humans in vitro and *in vivo* are also needed to identify the effect and mechanism of piperine as a bioenhancing agent.

ACKNOWLEDGEMENT

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CONFLICT OF INTERESTS

Declared None

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