

ORIGINAL ARTICLE

Antidiabetic Activity of Methanolic Leaf Extract and Different Fractions of *Zephyranthes Candida* in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Since long back, herbal medicines have been the highly-esteemed source of medicine; therefore, they have become a growing part of modern, high-tech medicine. *Zephyranthes candida* (ZC) has been mentioned in the Indian System of Traditional Medicine for the treatment of diabetes mellitus. The objective of this study was to evaluate the anti-diabetic activity of methanolic leaf extract of *Zephyranthes candida* (MLZ) and its different fractions in healthy and Streptozotocin (STZ)-induced diabetic rats. Healthy wistar and STZ-induced diabetic rats were treated orally with MLZ (100, 200 and 400 mg/kg) and glipizide (5 mg/kg) for 21 consecutive days. Blood samples were collected from the retro orbital plexus on 1st, 8th, 15th and 21st day. In another study, STZ-induced diabetic rats were treated with glipizide (5 mg/kg), fraction I [hexane: ethyl acetate (1:1)], fraction II [chloroform: methanol (1:1)] and fraction III [chloroform: methanol (2:8)]. Blood glucose levels and lipid profiles were determined using ERBA, semiautoanalyzer. The methanolic extract was further analyzed for phytochemical analysis. MLZ (100, 200 and 400 mg/kg) showed a significant reduction in blood glucose levels which were comparable to that of the standard anti-diabetic drug, glipizide. The total cholesterol, triglycerides and low density lipoproteins levels were significantly reduced by MLZ in diabetic rats. All the three fractions are also reduced the blood glucose levels after single oral administration ($p < 0.01$). In phytochemical analysis, MLZ showed the presence of flavonoids, glycosides and alkaloids. The present study results indicated that *Zephyranthes Candida* possess anti-diabetic and lipid lowering effects may be due to the antioxidant activity of flavonoids or alkaloids. Further studies are needed to elucidate the structures and to evaluate the exact mechanism of anti-diabetic action of the active components.

Keywords: *Zephyranthes Candida*, Diabetes Mellitus, Hypoglycemic, Antihyperlipidemic, Antihyperglycemic

Diabetes mellitus (DM) is a chronic metabolic disorder, characterized by increased blood glucose level as a result of an absolute or relative lack of insulin and failure of insulin to act on its targets tissue. It has also been associated with an increased risk for developing atherosclerosis due to alteration in the blood lipid profile and raising the risk of cardiovascular diseases [12]. Atherogenic dyslipidemia is characterized by

abnormal circulating lipid profile including low levels of high-density lipoprotein (HDL), elevated levels of low-density lipoprotein (LDL), and triglycerides (TG) often found in patients who are obese and have type 2 diabetes [3]. According to recent estimates, approximately 285 million people worldwide (6.6%) in the 20-79 year age group will have diabetes in 2010 and by 2030, 438 million people (7.8%) of the adult

population, is expected to have diabetes [4]. World Health Organization (WHO) estimated that the total number of people with diabetes in 2010 to be around 50.8 million in India, rising to 87.0 million by 2030 [5].

The currently-used anti-diabetic drugs show a loss of efficacy over time, a poor tolerability and low compliance due to numerous adverse effects, including severe hypoglycaemia, weight gain, oedema, nausea and gastrointestinal derangements. Lipid-lowering drugs are used to treat diabetic hyperlipidemia. Some of are associated with serious adverse side effects. Thus, new strategies were needed that allow a sustained glycaemic control and avoid hypoglycaemia and other side effects [6]. Most of the plants have been reported to have antihyperglycemic effects with less adverse effects and low toxicity as compared to synthetic compounds [7-8]. According to WHO, almost 70% of the diabetic patients use plants as a primary source of antidiabetic agents in order to satisfy their principal health care.

Zephyranthes candida belongs to the family Amaryllidaceae, is a perennial herb, used as folk medicine in many countries because of their pharmacological activities. The decoction of *Zephyranthes candida* leaves has been used by the herbal healers and tribes of Andhra Pradesh in India for the management of diabetes mellitus [7, 9]. The leaves contain alkaloid haemanthidine, nerinine, zephyranthin and tezettine. However, there are no reports on the antidiabetic and antihyperlipidemic properties of *Zephyranthes candida* till date. Therefore, the present study was planned to investigate the antidiabetic and antihyperlipidemic activity of *Zephyranthes candida* in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Drugs and chemicals

Glipizide was obtained from Mylan Laboratories Ltd, Hyderabad, Andhra Pradesh, India as gift sample. Streptozotocin (STZ) was purchased from Sigma-Aldrich, St. Louis, USA. Commercial diagnostic kits were obtained from Erba Mannheim (Transasia Bio-Medicals Ltd, Baddi Dist, Solan (HP), India for determination of blood glucose, total cholesterol (TC), triglycerides (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and very low density lipoprotein-cholesterol (VLDL-C). All other used chemicals were of analytical reagent grade.

Experimental Animals

Male wistar rats (9 weeks old; 180-200 g) were procured from National Institute of Nutrition, Hyderabad, Andhra Pradesh, India. The animals were housed in individual polypropylene cages under

standard laboratory conditions of light, temperature ($22 \pm 1^\circ\text{C}$) and relative humidity for at least two weeks before the beginning of experiment, to adjust to the new environment and to overcome stress possibly incurred during transit with a 12 h light/dark cycle. Animals were given standard rat pellets and drinking water *ad libitum*. The animals were fasted 12 h before the conduct of experiment and during the experiment they were withdrawn from water. The experiments were planned after the approval of Institutional Animal Ethical Committee of KVSRR Siddhartha College of Pharmaceutical Sciences, Vijayawada (993/PO/E/S/06/CPCSEA).

Collection of plant material

The plants of *Zephyranthes candida* were procured from Sri Veera Hanuman Nursery Rajahmundry, Andhra Pradesh, India and were authenticated by Dr. K. Madhava Chetty, Asst. Professor, Department of Botany, Sri Venkateswara University, Tirupati. A specimen was preserved in the laboratory for future reference. The leaves were air dried and grounded into a powdery fine texture and stored at room temperature in an air tight polythene bag prior to use.

Extraction

A hundred gram (100 g) ground sample was extracted with 500 mL of 70% methanol (40-60°C) in a soxhlet extractor for 12 h (plant material to solvent ratio was 1:5 w/v). The extract was concentrated to a semisolid mass using a rotary evaporator (at 45°C). For antidiabetic activity, the extract and its fractions were formulated as suspensions in normal saline with 1% sodium carboxy methyl cellulose (SCMC) as the suspending agent.

Purification of anti diabetic compounds using Column chromatography

Silica gel (60-120 mesh) was dried in an oven for 1 h at 100°C. About 20 g of this was packed on to a glass column (50 × 1 cm) fitted with cotton in hexane with flow rate of 1 mL/min. The crude extract was loaded on to silicagel chromatography and eluted successively with solvents and their combinations (Hexane: Ethyl acetate (9:1), Hexane: Ethyl acetate (1:1), Chloroform: Methanol (9:1), Chloroform: Methanol (1:1), Chloroform: Methanol (2:8), chloroform: Methanol (1:9), Methanol) based on polarity. Two bed volumes were taken as a fraction. Each fraction was analysed by qualitative TLC. Qualitative TLC plates were prepared by making slurry of 2 g of silica gel-G with 5 ml of water and spread over the plate mutually on 5×20 cm glass plate followed by air drying. The plates were then activated in oven for 1 h at 100°C. After activation the TLC plates spotted with crude extract and different fractions.

Preliminary phytochemical tests

The crude methanolic extract of *Zephyranthes candida* was subjected to qualitative tests for identification of different constituents like flavonoids, terpenoids, glycosides, saponins, alkaloids, tannins and aminoacids by using standard qualitative methods described by Trease & Evans, and Tona [10-11].

Experimental Design

Acute toxicity study of MLZ

The acute toxicity of the crude methanolic leaf extract of *Zephyranthes candida* (MLZ) was determined by using wistar rats (150-200 g), according to the method described by Karim et al., 2012 [12]. The animals were divided into four groups (n=6) and received the following single dose treatment orally.

Group I: Served as a control, received 0.5% SCMC suspension orally.

Group II: Treated with 1000 mg/kg of MLZ

Group III: Treated with 2000 mg/kg of MLZ

Group IV: Treated with 4000 mg/kg of MLZ

All the doses of the extracts were prepared by dissolving the extract in 0.5% SCMC suspension prior to administration. The animals were observed at 0, 30, and 60 min (for behavioral effects); 24, 48, and 72h (for physical effects); 1 week for any kind of pharmacological toxic effects, body weight changes, food and water consumption.

Evaluation of MLZ for hypoglycemic activity on Healthy Rats

At the end of the fasting period of 24 h, blood was withdrawn from the tail vein for initial blood glucose taken as zero time (0 h). Then animals were randomly divided into five groups of six animals each and given following oral treatment for 21 consecutive days, once daily.

Group I: Served as control, received 0.5% SCMC.

Groups II: Treated with glipizide (5 mg/kg).

Group III: Treated with MLZ (100 mg/kg).

Group IV: Treated with MLZ (200 mg/kg).

Group V: Treated with MLZ (400 mg/kg).

Blood samples were withdrawn from the tail vein on 1st, 7th, 15th and 21st day following treatment. The plasma was separated by centrifugation (Remi, R- 4C Compact model, Mumbai, India) at 6000 rpm for 6 min and stored at -20 °C until analysis. Blood glucose levels were determined as described by Trinder et al. [13].

Evaluation of MLZ for antihyperglycemic activity on STZ - induced diabetic rats

Induction of diabetes

The animals were fasted for 24 h and diabetes was induced by a single intraperitoneal injection of a freshly prepared STZ (40 mg/kg) diluted in 0.1 M sodium citrate buffer (pH 4.5) solution. STZ-treated animals were allowed to drink 5% glucose solution overnight to

overcome the drug induced hypoglycemia. About 72 h after the STZ administration, the animals get induced and their fasting blood glucose levels were measured. Rats with plasma glucose ranging from of 250 mg/dL were used for this experiment. The diabetic rats were randomized into five groups comprising of six animals in each groups as given following oral treatment for 21 consecutive days, once daily.

Group I: Served as control and received 0.5% SCMC.

Groups II: Treated with glipizide (5 mg/kg).

Group III: Treated with MLZ (100 mg/kg).

Group IV: Treated with MLZ (200 mg/kg).

Group V: Treated with MLZ (400 mg/kg).

Blood samples were withdrawn from the tail vein on 1st, 7th, 15th and 21st day following treatment. The plasma was separated by centrifugation (Remi, R- 4C Compact model, Mumbai, India) at 6000 rpm for 6 min and stored at -20 °C until analysis. Blood glucose levels were determined as described by Trinder et al. [13].

Evaluation of different fractions for antihyperglycemic activity on STZ-induced diabetic rats

Three fractions were selected based on the TLC for this study. Fraction I is hexane: ethyl acetate (1:1); Fraction II is chloroform: methanol (1:1) and Fraction III is chloroform: methanol (2:8). STZ- induced diabetic rats were divided into five groups comprising of six animals in each groups and given single oral treatment as follows.

Group I: Served as control and received 0.5% sodium CMC.

Groups II: Treated with glipizide (5 mg/kg).

Group III: Treated with fraction I (100 mg/kg).

Group IV: Treated with fraction II (100 mg/kg).

Group V: Treated with fraction III (100 mg/kg).

Blood glucose levels were determined before and after 1, 2 and 3 h of the treatment by GOD-POD method.

Biochemical parameters

At the end of the experimental period, all the animals were fasted overnight, anesthetized using ketamine (15 mg/kg) and midazolam (20 mg/kg) intra muscular injection), and sacrificed by cervical decapitation. Blood samples were collected in eppendorfs tubes the estimation of various parameters. The serum levels of TC [14], TG [15], HDL [16], LDL and VLDL [17] were estimated using semi autoanalyser (ERBA Chem 5 Plus, Mumbai, India).

Statistical analysis

Statistics for significance were calculated using Graph Pad Prism 5.0 software (San Diego, CA, USA). All the values of blood glucose and biochemical parameters were represented as mean \pm SD (standard deviation). When two groups were compared, Student's

Table 1. Effect of methanolic leaf extract of *Zephyranthes Candida* on the blood glucose levels of healthy rats (n=6).

Treatment	1 st Day	7 th Day	15 th Day	21 st Day
0.5% SMC	81.74 ± 9.89	79.06 ± 3.75 ^{NS}	74.48 ± 4.09 ^{NS}	77.17 ± 9.25 ^{NS}
Glipizide (5 mg/kg)	68.60 ± 8.38	56.53 ± 6.92 ^{**}	55.36 ± 6.09 ^{***}	48.89 ± 4.95 ^{***}
MLZ (100 mg/kg)	70.17 ± 7.54	70.03 ± 3.50 ^{NS}	66.36 ± 3.89 ^{NS}	60.55 ± 4.28 [*]
MLZ (200 mg/kg)	72.30 ± 4.04	64.36 ± 5.17 ^{NS}	62.64 ± 5.66 [*]	59.27 ± 5.23 ^{***}
MLZ (400 mg/kg)	71.26 ± 5.67	57.28 ± 5.62 ^{***}	50.83 ± 3.43 ^{***}	46.11 ± 2.41 ^{***}

All values are expressed as Mean ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ^{NS} $p > 0.05$ when compared to control group; (one-way ANOVA followed by Dunnett's post hoc test). MLZ, Methanolic leaf extract of *Zephyranthes Candida*.

Table 2. Effect of methanolic leaf extract of *Zephyranthes candida* (MLZ) on the blood glucose levels in streptozotocin (STZ)-induced diabetic rats (n=6).

Treatment	1 st Day	7 th Day	15 th Day	21 st Day
0.5% SMC	332.97 ± 23.91	331.53 ± 17.77 ^{NS}	316.93 ± 17.18 ^{NS}	335.22 ± 8.71 ^{NS}
Glipizide (5 mg/kg)	375.68 ± 7.04	226.33 ± 3.20 ^{***}	196.27 ± 2.49 ^{***}	191.70 ± 6.87 ^{***}
MLZ (100 mg/kg)	368.11 ± 6.37	332.75 ± 7.21 ^{***}	287.51 ± 12.81 ^{***}	252.37 ± 8.50 ^{***}
MLZ (200 mg/kg)	345.17 ± 12.8	283.04 ± 12.67 ^{***}	258.30 ± 11.06 ^{***}	216.79 ± 14.72 ^{***}
MLZ (400 mg/kg)	383.45 ± 6.62	284.22 ± 12.69 ^{***}	235.74 ± 12.56 ^{***}	212.90 ± 14.94 ^{***}

All values are expressed as Mean ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ^{NS} $p > 0.05$ when compared to control group; (one-way ANOVA followed by Dunnett's post hoc test). MLZ, Methanolic leaf extract of *Zephyranthes Candida*.

t-test was used. One-way ANOVA followed by Dunnett's post hoc multiple comparison tests, when more than two groups were compared. Differences between groups were considered significant at $p < 0.05$.

RESULTS

Phytochemical tests

Preliminary phytochemical analysis of the crude methanolic extract of *Zephyranthes candida* showed the presence of, flavonoids, glycosides, terpenoids, saponins, alkaloids and tannins. Amino acids were absent in the extract.

Identification and elucidation of the phytochemicals

In order to identify the responsible compounds for the antidiabetic activity, dry leaf methanolic extracts were partitioned with different solvents and their combinations based on polarity. The active fraction was eluted in Ethyl acetate: Methanol (7:3) as a green eluent which up on concentrated by air drying. The compound moved as a single spot on silicagel F₂₅₄ with different solvent systems like Hexane: Ethyl acetate (9:1), Hexane: Ethyl acetate (1:1), Chloroform: Methanol (9:1), Chloroform: Methanol (1:1), Chloroform: Methanol (2:8), chloroform: Methanol (1:9), Methanol.

Out which Chloroform: Methanol (2:8) have shown an R_f Value 0.6. This has to be further purity must be checked on High performance liquid chromatography (HPLC) and gas chromatography (GC) to confirm purity.

Acute oral toxicity study of MLZ

In acute oral toxicity study, ZC (1000, 2000 and 4000 mg/kg) did not produce abnormal behaviour and mortality was not recorded during 8 days after treatment with ZC. The body weight, food and water intakes of ZC administered rats were also normal in comparison to vehicle treated rats. Hence, doses of 100, 200 and 400 mg/kg were chosen for further experiments according to OECD guidelines for screening of the anti-diabetic activity [18].

Effect of MLZ on blood glucose levels of healthy rats

The effects of MLZ (100, 200 and 400 mg/kg) on the blood glucose levels of healthy rats in the 1st, 7th, 15th and 21st day are shown in Table 1. MLZ caused significant reduction in blood glucose levels from 70.17 ± 7.54 to 60.55 ± 4.28 mg/dL (with 100 mg/kg) and 72.30 ± 4.04 to 59.27 ± 5.23 mg/dL (with 200 mg/kg) and 71.26 ± 5.67 to 46.11 ± 2.41 mg/dL (with 400 mg/kg) in the 21st day against the control. The results of the present study clearly indicated that the MLZ exhibited significant hypoglycemic activity normal

Table 3. Effect of methanolic leaf extract of *Zephyranthes candida* on the lipid profiles of STZ-induced diabetic rats.

Parameter	0.5% SMC	GPZ (5 mg/kg)	MLZ (100 mg/kg)	MLZ (200 mg/kg)	MLZ (400 mg/kg)
TC	146.88 ± 9.58	75.36 ± 4.36*	121.11 ± 8.14	105.92 ± 8.22*	81.20 ± 5.02*
TG	163.24 ± 9.15	61.50 ± 5.78*	144.83 ± 9.47	98.01 ± 6.75*	62.55 ± 2.47*
HDL-C	18.53 ± 1.54	30.33 ± 1.55*	20.62 ± 2.14	24.33 ± 1.28*	29.00 ± 1.63*
LDL-C	122.47 ± 8.33	34.25 ± 2.45*	91.37 ± 3.88*	60.80 ± 4.32*	27.51 ± 2.46*
VLDL-C	42.05 ± 5.71	13.72 ± 1.84*	35.18 ± 2.36	22.62 ± 1.47*	12.45 ± 1.41*
TC/HDL-C	8.23 ± 1.47	2.83 ± 0.65*	6.25 ± 1.87	4.73 ± 0.82*	2.89 ± 0.69*
LDL/HDL-C	6.97 ± 1.36	1.51 ± 0.11*	4.74 ± 1.10	2.80 ± 0.56*	0.97 ± 0.24*

GPZ, Glipizide; TC: Total cholesterol; TG: Triglycerides; HDL-C: High density lipoprotein-cholesterol; LDL-C: Low density lipoprotein-cholesterol; VLDL-C: very low density lipoprotein-cholesterol. All values are expressed as Mean ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ^{NS} $p > 0.05$ when compared to control group; (two-way ANOVA followed by Bonferroni post hoc test).

Table 4. Effect of a single oral administration of various fractions of *Zephyranthes candida* on the blood glucose levels of streptozotocin (STZ)-induced diabetic rats (n=6)

Time (h)	Control	Glipizide	Fraction I	Fraction II	Fraction III
0	500 ± 35.25	454.33 ± 21.22 ^{NS}	380.0 ± 16.64 ^{NS}	414.33 ± 14.01 ^{NS}	292.33 ± 4.04**
1	443 ± 45.02	271.33 ± 15.94**	309.33 ± 81.81 ^{NS}	304 ± 45.31*	368.0 ± 47.12 ^{NS}
2	443 ± 40.52	270.66 ± 138.44**	260.33 ± 114.54**	221 ± 21.52***	395.33 ± 85.27 ^{NS}
3	443 ± 25.31	287.0 ± 67.13*	296.33 ± 133.61*	155 ± 17.24***	265.33 ± 12.01*

All values are expressed as Mean ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ^{NS} $p > 0.05$ when compared to control group; (one-way ANOVA followed by Dunnett's post hoc test). Fraction I, hexane: ethyl acetate (1:1); Fraction II, chloroform: methanol (1:1) and Fraction III, chloroform: methanol (2:8)

healthy rats, comparable to the effect exhibited by standard drug, glipizide.

Effect of MLZ and their fractions on blood glucose levels of STZ-induced diabetic rats

Table 2 shows the reduction of blood glucose levels in STZ-diabetic rats after treatment with glipizide and MLZ (100, 200 and 400 mg/kg). MLZ produced significant reductions in blood glucose levels after 7 days at all the selected doses in diabetic rats ($p < 0.001$). Blood glucose levels were reduced from 368.11 ± 6.37 to 252.37 ± 8.50 mg/dL (MLZ, 100mg/kg) and 345.17 ± 12.8 to 216.79 ± 14.72 mg/dL (MLZ, 200mg/kg) and 383.45 ± 6.62 to 212.90 ± 14.94 mg/dL (MLZ, 400mg/kg). Fractions also exhibited glucose reduction property after single dose administration to diabetic rats (Table 3).

Effect of MLZ on Lipid profile

Serum TC, TG, LDL, VLDL and HDL levels of the experimental groups of animals are shown in Table 4. Serum TC, TG, LDL and VLDL were significantly higher ($p < 0.05$) in STZ-induced diabetic rats (group II) than those in normal controls (group I). Decreased levels of HDL were observed in STZ induced diabetic rats compared to normal untreated mice. Treatment with MLZ and glipizide resulted in a significant decrease

($p < 0.05$) in TC, TG, LDL and VLDL levels compared to those in STZ-induced diabetic rats. Serum HDL levels were significantly increased ($p < 0.05$) in the diabetic treated group.

DISCUSSION

DM is a common health problem worldwide. It is not only kills, but is a major cause of retinopathy, nephropathy, gangrene, neuropathy, heart attacks and strokes [19]. Over recent years, there has been rapid expansion of different classes of antihyperglycaemic drugs. These drugs have diverse toxicological profiles because each possesses a unique pharmacological mechanism of action [20]. Indeed the cost of diabetes-related health care was estimated to be \$232 billion in 2007 [21]. The enormous costs of modern treatment indicate that alternate strategies for the prevention and treatment of diabetes must be developed. Since almost 90% of the people in rural areas of developing countries still rely on traditional medicines for their primary health care. More than 1200 species of organisms have been used ethnopharmacologically or experimentally to treat symptoms of DM.

Himalaya Diabecon was the renowned Himalaya herbals brand endorsed by over 250,000 doctors worldwide and used by customers in over 60 countries. It was an ayurvedic blend of herbal (*Commiphora*

wightii, *Gymnema sylvestre*, *Pterocarpus marsupium*, *Glycyrrhiza glabra*, *Casearia esculenta*, *Eugenia jambolana*, *Asparagus racemosus*, *Boerhaavia diffusa*, *Sphaeranthus indicus*, *Tinospora cordifolia*, *Swertia chirata*, *Tribulus terrestris*, *Phyllanthus amarus*, *Gmelina arborea*, *Gossypium herbaceum*, *Berberis aristata*, *Aloe vera*, *Triphala*) extracts, herbal (*Momordica charantia*, *Piper nigrum*, *Ocimum sanctum*, *Abutilon indicum*, *Curcuma longa*, *Rumex maritimus*, *Ficus racemosa*, *Acacia arabica*, *Areca catechu*, *Withania somnifera*, *Valeriana wallichii*, *Oroxylum indicum*, *Santalum album*, *Zingiber officinale*, *Eugenia jambolana*) powders and some minerals [22-24]. All the herbs are previously reported to have potential anti-diabetic activity in animal models with identified various mechanisms [25-28]. *Zephyranthes candida* has been mentioned in Indian system of traditional medicine to treat DM. In the present study, *Zephyranthes candida* exhibited good hypoglycemic and antidiabetic activity in STZ-diabetic rats. *Zephyranthes candida* also showed excellent antihyperlipidemic activity in diabetic rats, hence it can be used as one of the herb in herbal formulation or in the treatment of DM and dyslipidemia.

CONCLUSION

The present study results revealed that the methanolic extract of *Zephyranthes candida* showed significant hypoglycemic, antihyperglycemic and antihyperlipidemic activities due to alkaloidal content of the extract. Further investigations are needed to identify the lead molecule and to elucidate exact mechanism of action for antidiabetic effect.

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CONFLICT OF INTEREST

The authors declare that this research does not have any conflict of interest with anyone or any institute.

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