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HYPOGLYCEMIC AND NEUROPROTECTIVE PROPERTIES OF *CYPERUS ROTUNDUS* ON TYPE 2 DIABETIC RATS AND SHSY5Y NEURONAL CELLS

Latha P[1*], Chandra Sekhar K.B[2], and Sujatha D[3]

[1] Research Scholar, Jawaharlal Nehru Technological University, Anantapuramu, A.P, India.
[2]Director, OTRI, Jawaharlal Nehru Technological University, Anantapuramu, A.P, India
[3]Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati- - 517502, A.P, India.
***Corresponding author E-mail:** lathaudayan94@gmail.com

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ABSTRACT

The tubers of *Cyperus rotundus* was widely used in traditional folklore medicine for diabetes mellitus. In the present study, the ethanolic extract of *Cyperus rotundus* (EECR) was investigated for its hypoglycemic action in Streptozotocin (STZ) induced diabetic rats and its neuroprotective effect on MTT assay, and hyperglycemia - induced Reactive Oxygen Species (ROS) on SHSY5Y neuronal cells. The total phenolic and flavonoid content of the extract and *in vitro* alpha - amylase and alpha - glucosidase inhibitory activity were studied. Type 2 diabetes mellitus was induced in male wistar rats by administration of STZ (40 mg/kg/i.p). The rats were divided into six groups (n=6), group-I normal control, group II STZ induced diabetic control, group III Glibenclamide-5mg/kg/p.o, groups IV, V, &VI were treated with ethanolic extract of *Cyperus rotundus* at doses 100, 200, & 400 mg/kg/p.o respectively. The protective effect of EECR on SHSY5Y neuronal cells was studied in MTT assay and hyperglycemia induced ROS by using dye carboxy-H_2DCFDA. The results showed that the EECR reversed the STZ induced biochemical changes like increased blood glucose, SGPT, SGOT, lipid profile (TC, TG, LDL, VLDL), and improved HDL levels. The EECR restored the changes in histopathology of pancreas, body weight and significantly inhibited the *in vitro* alpha amylase and alpha glucosidase action. STZ induced cytotoxicity in MTT assay and hyperglycemia - induced ROS in SHSY5Y neuronal cells was significantly ameliorated by EECR. The findings of our study suggest that the EECR has antidiabetic and neuroprotective activity.

INTRODUCTION:

Type 2 diabetes mellitus is the endocrine disorder characterized by hyperglycemia with metabolic abnormalities. The prevalence of diabetes in the world is drastically increasing and it is will rise to 473 million by 2045 and the major part in this increase number will be contributed by the urban people in developing countries. It is expected that India will lead China in the population with diabetic people. The complications of type 2 diabetes mellitus were the major cause of morbidity and mortality in the world affecting vital organs[1].

The conventional drugs have its own limitations for its use in diabetes mellitus which gained the attention of natural medicines in the treatment of diabetes mellitus. The glycemic changes induced by insulin resistance in diabetic people contributes to the neurological changes in the brain[2]. Hyperglycemia in brain induces the generation of reactive oxygen species (ROS) that is responsible for the neurodegeneration in CNS[3]. Animal studies reveal that insulin plays a significant role in hippocampal synaptic plasticity and regulates learning, memory, and cognitive function[4]. The essential oil of Cyperus *rotundus* was found to contain β-sitosterol, cyperene, cyperol, flavonoids, sesquiterpenoids, ascorbic acid, and polyphenols[5]. The tubers were widely used as diuretic, diaphoretic, astringent, stomach and bowel complaints, fever, thirst, fainting, hypertension, vomiting, cough, dyspnea, diarrhea, intermittent fever, worm infestations and for its diabetes property in traditional folklore medicine[6]. In the present study, an attempt is made to investigate the hypoglycemic and neuroprotective activity of ethanolic extract of *Cyperus rotundus* was investigated in streptozotocin - induced diabetic rats and SHSY5Y neuronal cells.

MATERIALS AND METHODS:

Plant material collection:

The tubers of *Cyperus rotundus* (Family- Cyperaceae) used in the study was obtained from nearby areas of Chittoor district, Andhra Pradesh and authenticated by Dr. K. Madhava Chetty, Sri Venkateswara University, Tirupati.

Extract preparation:

The tubers are shade dried, powdered coarsely, soaked in petroleum ether for 72 hrs and extracted with ethanol by a simple soxhlation process. The extract obtained was stored in a desiccator for experimental studies.

Preliminary Qualitative analysis: The phytoconstituents present in the plant extract was screened according to standard procedures [7].

Inhibition Assay on α- glucosidase and α- amylase: *In vitro* α- glucosidase and α- amylase inhibition assay was performed according to standard methods and the IC_{50} values of EECR were determined [8-11].

Estimation of total phenol and flavonoid content: The total phenolic and flavonoid content of the EECR was performed using gallic acid and quercetin as standard respectively[12].

Experimental Animals:

The study was approved by Institutional Animal Ethics Committee (IAEC) of Sree Vidyanikethan College of Pharmacy, and all the male adult wistar rats for the experimental study were kept at temperature 21 ± 3^0 C with relative humidity 40-70 %, and maintained light/dark cycle (12/12 h) with food and water ad libitum. All the experimental procedures were followed according to CPCSEA guidelines.

Experimental design:

Toxicity studies: The acute toxicity study was performed as per acute toxic class method - OECD 423 guidelines and was found to be safe upto dose 2000mg/kg[13].

Oral Glucose Tolerance Test (OGTT): OGTT was performed according to Tahara et al., 2011 and the blood samples were periodically collected at 0, 30, 60, 90, & 120 minutes intervals using one - touch glucometer[14].

Induction of diabetes: STZ (40mg/kg/i.p) in 0.1 M citrate buffer at pH 4.5 was administered to rats. After 4 days, the rats with fasting blood glucose levels ≥ 200 mg/kg were considered diabetic and included in the experimental study[15].

Experimental Protocol:

The rats were divided into six groups (n=6), Group I-Normal control (vehicle 0.5% CMC), Group II-Diabetic control (STZ 40 mg/kg i.p), Group III-Glibenclamide (5mg/kg/p.o), Group IV, V & VI received 100,200, & 400 mg/kg p.o of EECR respectively. The treatment is continued for 15 days for all the groups except diabetic control group.

Estimation of biochemical parameters:

The blood samples were collected from the retroorbital plexus in overnight fasted rats for estimation of blood glucose, liver enzyme levels (SGOT & SGPT), lipid profile (TC, HDL, TG) by using Erba Mannheim diagnostic kits. Friedewald formula was used for determination LDL and VLDL levels. Later the rats were sacrificed by cervical dislocation method, liver and pancreas were isolated for liver glycogen estimation and histology of pancreas respectively.

Liver glycogen estimation: The tissue liver glycogen content was estimated by using anthrone reagent method[16].

Histopathological studies: The pancreatic tissues were sectioned to 4µm using microtome and stained with eosin and hematoxylin for histopathological studies.

Cell Culture: The SH-SY5Y neuronal cells were cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, Sodium bicarbonate, Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml) and were kept at 37^0C in a humidified 5% CO_2 incubator.

Cytotoxicity assay:

The EECR at various concentrations (5µM, 50µM, & 100µM) were added to 96 well plate and incubated for 30 minutes. To this STZ (30mM) and 15 mg of MTT was added. The viability of cells was evaluated by recording the absorbance in microplate reader at a wavelength of 570 nm[17].

Hyperglycemia- induced ROS:

Hyperglycemia- induced ROS was measured spectrofluorimetrically by using dye carboxy-H2DCFDA and the fluorescence was detected by using excitation and emission wavelengths at 430 nm and 580 nm respectively by TECAN multimode reader (Infinite 200 pro)[18].

Statistical analysis:

All data are presented as mean ± standard error mean of 6 animals. Data were analyzed statistically by graph pad prism 5 using Dunnett's't' test, p values were considered significant at $p < 0.05$.

RESULTS:

Preliminary phytochemical analysis:

The phytochemical constituents present in the EECR were alkaloids, flavonoids, tannins, sterols, terpenoids, carbohydrates, and glycosides.

Acute Toxicity study:

The acute toxicity studies revealed that EECR was safe up to 2000 mg/kg in rats and did not show any mortality and behavioral changes.

Total flavonoid and phenol content:

The total flavonoid content present in the EECR was 1.88 ± 0.03 mg of quercetin equivalents /g dry material. The total phenolic content present in EECR was 0.33 ± 0.72 mg of gallic acid equivalents /g dry material (Table-1).

Effect of EECR on α-glucosidase and α-amylase Inhibition Assay:

The IC50 value of EECR on α-glucosidase and α-amylase were 8.61 ± 2.12, and 188.01 ± 9.24 respectively.

Similarly, the IC_{50} value of acarbose on α-glucosidase and α-amylase was 2.04 ± 0.02, and 89.71 ± 0.65 respectively. The EECR showed significant inhibitory effect on α-glucosidase and α-amylase compared to acarbose (Table-2).

Effect of EECR on OGTT:

The EECR significantly ($p < 0.05$) reduced the elevated levels of blood glucose in overnight fasted rats shown in Fig-1.

Effect on Blood Glucose:

Glibenclamide treated groups significantly ($p < 0.05$) lowered the elevated blood glucose levels. Diabetic rats treated with EECR at various doses reduced the blood glucose levels and significant ($p < 0.05$) effect was observed at dose 400 mg/kg compared to diabetic control rats (Table-3)..

Effect of EECR on liver enzymes, Liver glycogen content, and Body Weight:

The elevated levels of SGOT and SGPT were significantly decreased in rats treated with EECR at dose 400 mg/kg to that of glibenclamide treated group. The decreased liver glycogen content was restored to normal in glibenclamide and EECR treated groups. Diabetic rats showed decrease in body weight but rats treated with EECR significantly reversed the weight loss (Table-3).

Effect of EECR on lipid profile:

The increased levels of TC, TG, LDL, VLDL, and decreased levels of HDL cholesterol in diabetic rats were restored to normal levels in glibenclamide treated groups. The EECR significantly ($p < 0.05$) improved the levels of HDL and reversed the elevated levels of TC, TG, LDL, and VLDL. The EECR at dose 400mg/kg showed significant ($p < 0.05$) effect similar to glibenclamide group (Table-4).

Effect of EECR on pancreas:

Histological studies revealed that the EECR dose - dependently improved the number of beta islet cells and their size by restoring the pancreas to normal (Fig - 2).

Effect of EECR on SHSY5Y Neuronal Cells MTT assay and ROS:

STZ at dose 30mM concentration decreased the cell viability to 49.19 ± 0.14.The EECR at 100 µM significantly improved the cell viability in MTT assay, and also decreased the hyperglycemia induced ROS and showed protective effect on SHSY5Y neuronal cells (Table 5 & Fig 3).

DISCUSSION:

Type 2 diabetes mellitus is a chronic metabolic disorder that causes damage to multiple organs like kidney, heart, liver, eye, and neurons. It is mainly due to impairment in the insulin secretion, or insulin resistance[19]. Several studies have shown that the type 2 diabetic people are more prone neurodegenerative changes similar to Alzheimer's disease. Poor glycemic control and impaired insulin signaling mechanism in the brain was the major cause for abnormalities in the brain[2]. Streptozotocin produces diabetes in rats similar to type 2 diabetes mellitus in humans and it is widely used animal model for screening hypoglycemic agents[20]. In the current study, the protective effect of ethanolic extract of *Cyperus rotundus* was investigated for its hypoglycemic activity in STZ induced diabetic rats and protective effect on cytotoxicity induced by STZ in MTT assay and ROS generated by hyperglycemia in SHSY5Y neuronal cells. The EECR markedly reduced the blood glucose levels in oral glucose tolerance test and in diabetic rats. α-glucosidase and α-amylase plays an important role in the absorption of glucose form the gut.

Table 1: Effect of EECR on Total phenol and Flavonoid content

Concentration of extract	Phenolic content (mg of gallic acid equivalent/ g dry material)	Flavonoid content (mg of quercetin equivalent/ g dry material)
EECR(100µg/ml)	0.33 ± 0.72	1.88 ± 0.03

All the values were given as mean ± SEM of three observed readings.

Table 2: Effect of EECR on α-glucosidase and α-amylase Inhibition Assay:

	α-glucosidase inhibition (IC_{50} µg/ml)	α-amylase inhibition (IC_{50} µg/ml)
Acarbose	2.04 ± 0.03	89.71 ± 0 .65
EECR	8.61 ± 2.12	188.04 ± 9.21

All the values were given as mean ± SEM of three observed readings.

Table 3: Effect of EECR on Blood Glucose, Liver Enzymes, Liver Glycogen Content, Body Weight

Groups	Blood glucose levels (mg/dl)	SGOT(mg/dl)	SGPT (mg/dl)	Liver glycogen content (mg/100 mg wet wt.)	Body weight (gm)	
					Initial	Final
Control	96.17 ± 2.95	83.5 ± 3.34	30.33 ± 1.20	100.5 ± 1.67	190.2 ± 3.80	195.8 ± 3.1
Diabetic Control	281.3 ± 7.13*	176.3 ± 3.56*	88.82 ± 0.91*	54.20 ± 2.86*	188.5 ± 1.31	164.2 ± 3.8
Glibenclamide (5mg/kg)	142.8 ± 3.83#	102.4 ± 2.22#	43.51 ± 2.24#	96.18 ± 1.93#	190.3 ± 4.65	200.3± 1.33
EECR-100	206.2 ± 4.07#	152.12 ± 3.38#	79.10 ± 2.46#	68.67 ± 1.12#	187.5 ± 2.82	191.7 ± 2.4
EECR -200	196.8 ± 1.72#	135.7 ± 2.37#	70.67 ± 0.99#	78.17 ± 2.64#	190.5 ± 3.35	192.5 ± 2.1
EECR -400	161.2 ± 2.77#	112.8 ± 2.04#	51.67 ± 2.28#	82.01 ± 2.73#	191.7 ± 2.79	197.5 ± 1.1

All values are expressed as mean ± SEM for n=6 animals. Significance was determined by one way ANOVA followed by Dunnett's't' test. *$p < 0.05$ compared with control, # $p < 0.05$ compared with diabetic control.

Table 4: Effect of EECR on lipid profile

Groups	TC (mg/dl)	TG (mg/dl)	HDL(mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Control	64.12 ± 1.97	54.26 ± 1.58	45.13 ± 2.10	38.06 ± 2.2	17.4 ± 2.21
Diabetic Control	130.01 ± 2.61*	148.08 ± 2.42*	20.4 ± 0.91*	88.96 ± 2.98*	55.03 ± 2.78*
Glibenclamide (5mg/kg)	67.13 ± 1.66#	74.33 ± 1.53#	38.99 ± 2.46#	40.13 ± 2.81#	26.14 ± 2.04#
EECR -100	102.05 ± 3.67#	124.02 ± 1.89#	28.41 ± 1.52#	72.34 ± 2.01#	42.72 ± 2.39#
EECR -200	88.71 ± 3.99#	98.51 ± 2.92#	30.28 ± 1.78#	62.13 ± 3.46#	36.12 ± 2.81#
EECR -400	72.33 ± 0.71#	82.36 ± 0.99#	34.91 ± 1.37#	51.33 ± 1.89#	31.03 ± 1.96#

All values are expressed as mean ± SEM for n=6 animals. Significance was determined by one way ANOVA followed by Dunnett's't' test. *$p < 0.05$ compared with control, # $p < 0.05$ compared with diabetic control.

Table 5: Effect of EECR on STZ Induced Cytotoxicity in MTT Assay

S.No	Drugs	IC_{50} values
1.	STZ (30mM)	49
2.	STZ + EECR- 5µM	37
3.	STZ + EECR- 50µM	32
4.	STZ + EECR- 100µM	16

All the values were given as mean ± SEM of three observed readings.

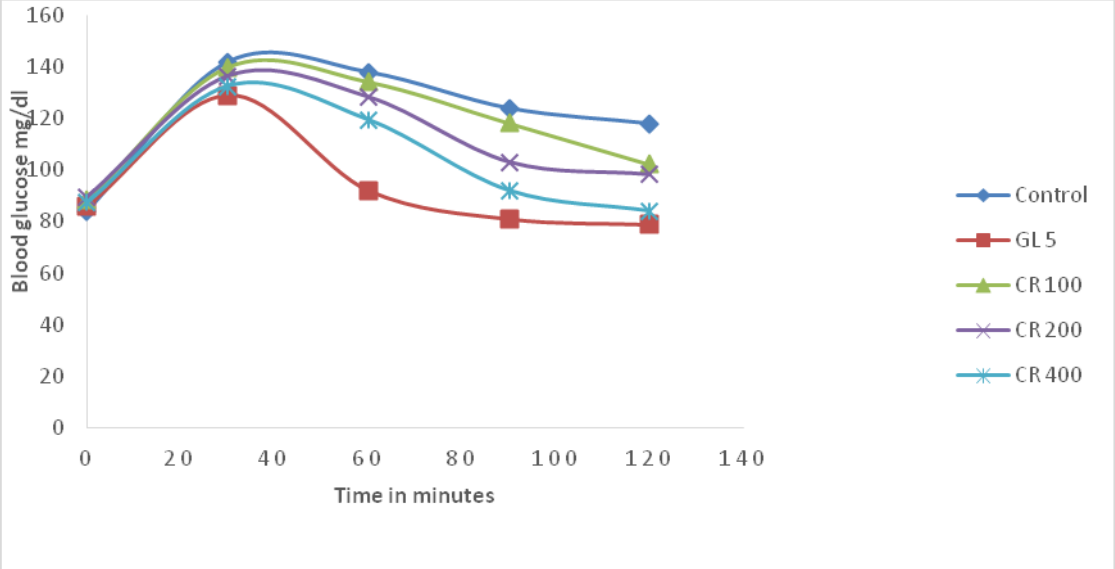


Figure 1: Effect of EECR Oral Glucose Tolerance Test

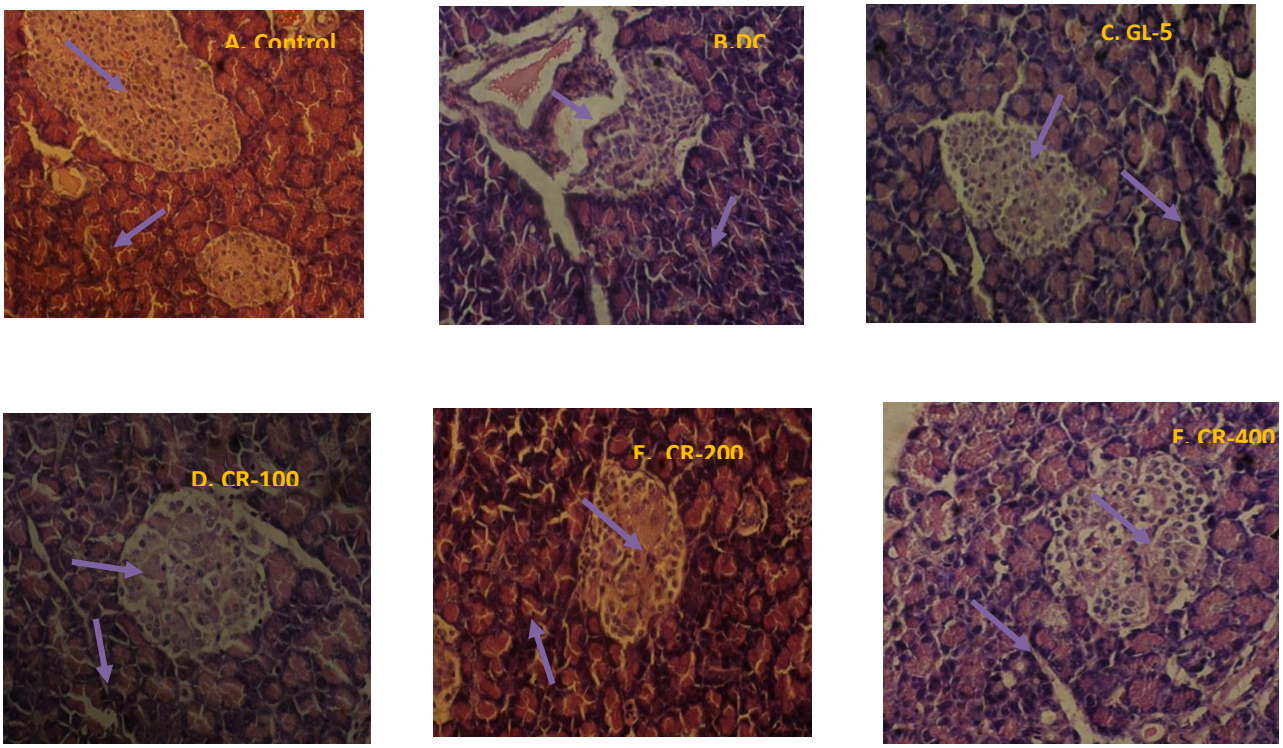
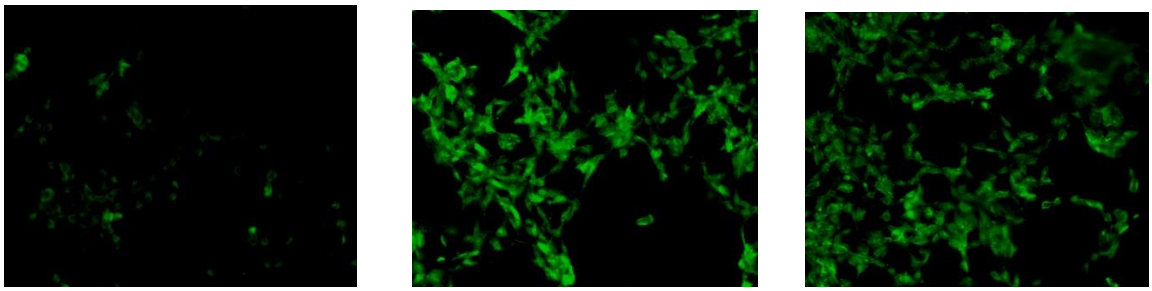


Figure 2: Histology of pancreas

Normal control (A), shows the intact beta islet cells and acinar cells. DC group (B), pancreatic cells were degenerated with decreased number of beta islets. Glibenclamide group (C), shows restored beta islet cells with increase in number. EECR 100 (D), EECR 200 (E), and EECR 400 (F) treated rats dose dependently regenerated the pancreas and increased the number beta islet cells.



Control **250mM Glucose** **EECR100µM**

Figure 3: ROS Staining with H_2DCFDAin SHSY5Y Neuronal Cells

Numerous studies have reported that inhibition of these enzymes significantly reduces the postprandial raise in blood glucose levels[21]. The EECR showed a potent inhibitory effect on α-glucosidase and α-amylase compared to standard acarabose. The liver is the major site for storage of glycogen, in diabetic rats due to altered metabolism glycogen content was depleted. Rats treated with EECR significantly improved the glycogen content. The raise levels of TG, TC, VLDL, LDL, and decreased levels of HDL are the risk factors for coronary heart disease[22]. Diabetic control rats showed a marked increase in lipid profile with decreased HDL levels. Supplementation of EECR in diabetic rats restored the alterations in the lipid profile which shows its lipid - lowering potential. The drastic change in the body weight of diabetic rats was due to impaired metabolism and rats treated with EECR at various doses significantly restored body weight to normal. Histological studies in pancreatic tissue sections showed degeneration and decreased β-cell mass in diabetic rats. In rats supplemented with EECR significantly alleviated these changes and improved the β-cell mass in the pancreas. The EECR significantly increased the cell viability and reverted the changes induced by STZ on SHSY5Y neuronal cells in MTT assay that signifies its cytoprotective action. Similarly, hyperglycemia - induced ROS on neuronal cells was restored to normal on treatment with EECR at dose 100µM suggesting its protective role in neuronal cells. Several studies have reported that the polyphenols and flavonoids present in the plants induce insulin secretion and improve the lipid abnormalities. The findings of the study reveal that the above actions might be due to the presence of phenols and flavonoids abundantly in the EECR.

CONCLUSION:

The EECR showed marked inhibition on α-glucosidase and α-amylase enzymes, alleviated the abnormalities in STZ induced diabetic rats, restored the histological changes, improved the liver glycogen content, and body weight. It also significantly ameliorated the hyperglycemia - induced ROS and STZ induced cytotoxicity on SHSY5Y neuronal cells. The study suggest that the EECR has potent hypoglycemic and neuroprotective action and supports the traditional use of the ethanolic extract of *Cyperus rotundus* in diabetes. Further studies are needed to explore its molecular mechanism and its clinical use in humans.

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