**Research article**

*Hydrolea zeylanica* improves cognitive impairment in high-fat diet fed-streptozotocin-induced diabetic encephalopathy in rats via regulating oxidative stress, neuroinflammation, and neurotransmission in brain

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**ABSTRACT**

Type2 diabetes mellitus is a progressive metabolic disorder characterized by β-cell dysfunction with the increase in hepatic glucose synthesis and insulin resistance which leads to microvascular complications like diabetic encephalopathy that impairs cognitive dysfunctions, and dementia. The green and leafy vegetables of *Hydrolea zeylanica* are used in diet as rich source of nutrition, dietary fibers in reducing malnutrition and keeps in control the blood sugar level to treat diabetes related vascular complications. This study investigated the effect of hydroalcohol extracted fraction of leaves of *H. zeylanica* (HHZ) on high-fat diet fed-streptozotocin (HFD/STZ)-induced diabetes encephalopathy in experimental rats, and quantified the flavonoids, nutrients contents by HPLC analysis. HHZ demonstrated potential cellular antioxidant protection in ORAC, CAP-e tests. HHZ showed mixed competitive inhibition towards acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) activities, and exhibited dose dependent inhibition to both neurotransmitter activities. After 4 weeks administration of HHZ (oral, 300, and 400 mg/kg b.w.) in HFD/STZ-induced diabetic rats, HHZ-400 significantly (*p* < 0.001) improved the learning and memory impairment with the reduction in serum glucose and elevation in insulin level in encephalopathy rats. It also significantly (*p* < 0.001) improved oxidative (MDA, SOD, CAT, and GSH), and proinflammatory markers (TNF-α, IL-6, and hs-CRP) with the reduction in cholinesterase (AChE, BChE) and β-secretase (BACE1, BACE2) activities as evidenced by histological architecture of cortex in diabetic encephalopathy rats. Diet rich source of flavonoids e.g., quercetin, caffeic acid, rutin, gallic acid, and ferulic acid, nutrients, and vitamins in *H. zeylanica* enhanced the cellular antioxidant protection by reducing oxidative stress, neuroinflammation and neurotransmission in the brain of diabetic encephalopathy rats.

1. Introduction

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized by abnormalities in insulin synthesis, and sensitivity in pancreatic β-cells resulted in excessive rise in blood glucose level. Hyperglycemia is directly, or indirectly associated with abnormal carbohydrate, lipid, and protein metabolism (WHO, 2019). Type2 diabetes mellitus (T2DM) is characterized by β-cell dysfunction, increased hepatic glucose synthesis, and insulin resistance (IR) leads to various macrovascular (coronary artery disease, peripheral arterial disease, and stroke), and microvascular complications e.g., diabetic nephropathy, neuropathy, retinopathy and encephalopathy. Insulin resistance, hyperinsulinemia, hyperglycemia with β-cell dysfunction, increased hepatic glucose synthesis, and insulin resistance (IR) leads to various macrovascular (coronary artery disease, peripheral arterial disease, and stroke), and microvascular complications e.g., diabetic nephropathy, neuropathy, retinopathy and encephalopathy. The brain is a relevant site in diabetic encephalopathy, oxidative stress and inflammation are the key factors involved in initiating the cleavage of amyloid precursor protein (APP) at β-site, or within β-amyloid (Aβ) peptide affect neurotransmission in brain causes memory loss and dementia (Rezai-Zadeh et al., 2005; Sima, 2010). Several antioxidant and anti-inflammatory therapeutics such as proliferator-activated receptor (PPAR-γ) agonists (thiazolidinediones,
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rosiglitazone, troglitazone), C-peptide, and glucagon-like-peptide1 (GLP-1) analogues have been developed to prevent the progression of diabetic encephalopathy (Pipatpiboon et al., 2012). However, these drugs are contraindicated in patients with renal insufficiency, risk of heart failure with antihypertensive agents and also, the patient reported side effects like nausea, vomiting, anorexia, reported, and for these reasons, there is a problem with drug of choice in diabetic encephalopathy patients (Bhutada et al., 2010).

Hydroela zeylanica (L.) Vahl (Hydroelaeeae) is an aquatic tufted perennial creeper native to India and other Southeast Asian countries. In India, green and leafy vegetables of H. zeylanica are used in diet which are rich source of nutrition, dietary fibers in reducing malnutrition, and keeps in control the blood sugar level (Kanthasamy Kalaichelvi, 2016; Pareek and Kumar, 2014). Since ancient times, the Chorei tribal community of Assam, India uses young leaves of H. zeylanica to treat diabetes related vascular complications like retinopathy, neuropathy, renal diseases, and encephalopathy (Banik et al., 2010). The authors reported R-limonene, perillartine, N-formyl-L-lysine, limonen-6-ol, pivalate, lidocaine, and galactomelanin in H. zeylanica (HHZ) were responsible in improving glycemic control in T2DM rats (Swain et al., 2020). This study investigated the effect of HHZ on high-fat diet fed-streptozotocin (HFD/STZ)-induced diabetes encephalopathy by regulating oxidative stress, neuro-inflammation and neurotransmitters in the brain of experimental rats, and quantified the flavonoids, nutrients contents by HPLC analysis. STZ-induced diabetic encephalopathy has been associated with oxidative stress implicated in pathogenesis of cholinergic dysfunction, and depression leads to AD. The present study investigated cellular protection of HHZ in diabetic encephalopathy rats.

2. Material and methods

2.1. Chemicals and reagents

Fluorescein sodium salt (FluChE), Butyrylcholinesterase (BChE), S-butrylylthiocholine chloride (SBTC), Acetylthiocholine iodide (ATCI), Streptozotocin (STZ), glibenclamide (GLB), dithiobis-2-nitrobenzoic acid (DTNB), GSH, phenazinum methosulfate (PMS), thiobarbituric acid (TBA), ethylendiamine tetraacetic acid (EDTA), nitro blue tetrazolium (NBT), thio-barbituric acid (TBA), trichloroacetic acid (TCA), hydrogen peroxide (H₂O₂), triton-X-100, Ferric chloride, hydroxylamine hydrochloride, and bovine serum albumin (BSA) were purchased from Sigma Aldrich, USA and were used in all experiments. All HPLC solvents were procured from SRL Pvt. Ltd., Mumbai, India.

2.2. Nutritional analysis of H. zeylanica

The dried leaf sample of H. zeylanica was taken for amino acids, fat soluble vitamins and flavonoid analysis by HPLC (Nexera UHPLC; Shimadzu) analysis methods following by standard HPLC conditions (Samse and Vorarat, 2007; Wang et al., 2016; Zhao et al., 2016) were carried out at National Institute of Food Technology, Entrepreneurship and Management- Thanjavur (NIFTEM-T).

2.3. Preparation of bioactive fraction of H. zeylanica (HHZ)

Hydroela zeylanica (L.) Vahl (Hydroelaeeae) leaves were collected from Pipili, Puri District of Odisha, India (latitude: 20°12'23.62"N; longitude: 85°53'40.72"E; altitude: 51'). The taxonomical identification was done by Dr. P. C Panda, Principal Scientist, Regional Plant Resource Centre, Bhubaneswar, and the voucher specimen deposited at the herbarium of our Centre (7727/RPRC). The shade dried leaves were finely ground (600 g) hot macerated with 70% alcohol (1:4) at 70–80 °C for 72 h on a continuous process by collecting extract each 4h interval. The extracted fraction (HHZ) dried under decreased pressure by rotary-vacuum evaporator (R-100, Buchi, Switzerland) and kept at 4 °C for further use (Swain et al., 2020).

2.4. Cellular antioxidant capacity assay of HHZ

2.4.1. Oxygen radical absorbance capacity (ORAC) assay of HHZ

ORAC assay of HHZ was carried out (Kanhar et al., 2018; Thaipong et al., 2006). 2,2’-azobis (2-amidinopropane) hydrochloride (AAPH) radical (150 mM) was prepared freshly in PBS (75 mM, pH 7.4). To initiate the reaction, the stock solution of trolox (20 μM) and sodium fluorescein (FL, 150 μM) was prepared in PBS (75 mM, pH 7.4). The mixture was made with total volume of 200 μL in PBS (75 mM, pH 7.4) which contained diluted antioxidant/HHZ (25 μL) and FL solution (150 μL). The mixture was pre-incubated for 20 min at 37 °C then AAPH solution was added rapidly, and the plate was immediately placed in the ELISA microplate reader (Synergy H1MF, BioTek, USA) and the fluorescence was recorded every min at 37 °C for 80 min with excitation and emission filter 485 and 520 nm, respectively. A blank was prepared using PBS instead of antioxidant solution. Data were exported to excel sheet for calculation. By subtracting the area under curve (AUC) of Trolox from the AUC of blank, the AUC was determined. t = 0/fluorescence sample, t = 0 from the normalized curves. Net AUC and AUC were determined by the following formula.

\[ \text{AUC} = 1 + \sum f_i/f_0 \]

Where \( f_0 \) was the initial fluorescence reading at 0 min and \( f_i \) is the fluorescence reading at (i) time.

2.4.2. Cell based antioxidant protection in erythrocytes (CAP-e) assay of HHZ

CAP-e assay of HHZ was performed (George et al., 2014; Kanhar et al., 2018). HHZ was prepared (1 mg/mL) with normal saline (0.9%) at physiological pH mixed and incubated for 20 min. Fresh RBCs (from Wistar rats) were centrifuged at 2400 rpm for 10 min and the supernatant was collected for further use. RBCs treated with various diluted HHZ and kept in dark in rockinig condition for 2 h. RBCs with untreated HHZ was taken as negative control, and RBCs with oxidizing agent but not with antioxidant test taken as positive control. Antioxidants unable to enter into cells were removed by centrifugation, and the supernatant were collected for the oxidation reaction. Oxidative damage was done by addition of AAPH in presence of dichlorofluorescein diacetate (DCF-DA), and the degree of antioxidant damage was recorded by measuring fluorescence intensity of HHZ in ELISA microplate reader (Synergy H1MF, BioTek, USA). Inhibition of oxidative damage was calculated as the reduced fluorescence intensity of the test. Gallic acid was taken as reference drug, and IC₅₀ was calculated.

2.5. Cholinesterase inhibition assays of HHZ

Acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) inhibition activities of HHZ were estimated by using Ellman’s reagent
Cholinesterase enzymes hydrolyse the substrates and produce thiocholine, which reacts with DTNB forms yellow colour 5-thio-2-nitrobenzoate anions at 405 nm. In one set of reaction, 96-well microplate was taken with 120 μL sodium phosphate buffer (50 mM; pH 8), various concentration of HHZ (1 mg/mL), 20 μL of AChE (0.22 U/mL), and 10 μL of DTNB (3 mM in 50 mM sodium phosphate buffer; pH 8). For BChE inhibition assay, another set of reaction was prepared with same recipes in place of AChE, 20 μL of BChE (0.4 U/mL) was taken. Reaction mixtures were incubated at 37 °C for 25 min, then 10 μL of substrate acetylcholine iodide (ATCI; 15 mM) and S-butyrylthiocholine chloride (SBTC; 15 mM) were added in AChE, and BChE reaction mixture, respectively. Hydrolysis of AChE and BChE were observed by the formation of yellow colour 5-thio-2-nitrobenzoate anion with 15 min, which were detected at 405 nm by microplate ELISA reader (Synergy H1MF, BioTek, USA). Galantamine was considered as reference drug. IC50 of HHZ were determined.

2.6. Mode of cholinesterase inhibition assay of HHZ

Mode of inhibition of HHZ on cholinesterase (AChE, and BChE) was performed (de la Torre et al., 2012). Two reaction mixture were set for AChE and BChE, in one set it was carried out with 20 μL of AChE (0.22 U/mL), 10 μL of DTNB (3 mM) and various concentrations of acetylcholine iodide (ATCI) (0.05–3 mM) in sodium phosphate buffer (50 mM; pH 8) with final volume makeup with sodium phosphate buffer (50 mM; pH 8) for AChE inhibitory activity. Mode of inhibition on BChE was carried out by following the above protocol, however S-butyrylthiocholine chloride (SBTC) was added to BChE instead of ATCI. Both the reaction mixtures were incubated at 37 °C for 25 min. A double reciprocal Lineweaver-Burk (LB) plot was plotted between 1/[v] and 1/[s]. Kinetic values were applied by transforming data to LB plot and graphs were plotted.

2.7. Experimental animals

The Institutional Animal Ethical Committee (IAEC) of the Regional Plant Resource Centre, Bhubaneswar (1807/G0/R/S/15/CPCSEA) approved the protocol to conduct experiments on animals by following the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA). Wistar albino rats of either sex (150–180 g) was acquired from Imagenex India Pvt. Ltd., Bhubaneswar, India, and housed in adequately ventilated polypolyethylene cages at 25–30 °C with 12 h light/dark cycles. Rats had access to standard pallets and water ad libitum. Before the experiment began, rats were given a week to adapt laboratory environment.

2.7.1. Induction of diabetes

After complete acclimatization to laboratory conditions, rats were divided in two dietary regiments. Rats of 10 no. were in normal control (NC) group fed with normal pallet diet (NPD), while 30 no. of rats in other group were fed with high fat diet (HFD; 58% fat, 25% protein and 17% carbohydrate) for first two weeks. After 2 weeks of dietary manipulation, the overnight fasted HFD rats were received a single dose of STZ (40 mg/kg, i.p.) in 0.1 M citrate buffer (pH 4.5) while, NPD-fed rats received equivalent volume of normal saline. After 7 days of STZ-induction, diabetic status of each rat was confirmed by testing fasting blood glucose level ≥300 mg/dL from tail tip (glucometer, Accu-check, India) and was randomly divided into 4 different groups (Swain et al., 2020).

2.7.2. Animal grouping and treatment

Rats were divided into 5 different group (n = 6 rats). Group-I was normal control group received normal saline (0.9%, 1 mL/kg) with NPD diet, whereas HFD/STZ-induced rats randomly divided into 4 different groups. As, Group-II was HFD/STZ-induced negative control group received 0.9% normal saline, Group-III was positive control group received glibenclamide (5 mg/kg, p.o.), Group-IV was HHZ-300 group received HHZ (300 mg/kg, p.o.), and Group-V was HHZ-400 group received HHZ (400 mg/kg/p.o.). The treatment was continued for 30 days beginning from the day of conformation of diabetes in HFD/STZ-induced rats.

2.8. Behavioural analysis

On the 27th day of experimental period, the behavioural study was conducted to understand the learning and memory functions of experimental rats by performing novel object recognition test (NOR).

2.8.1. Novel object recognition test (NOR)

The novel objective recognition (NOR) test was conducted in an open wooden box (80 cm long X 50 cm high X 60 cm wide) to understand and recognize the familiar objects (F1 & F2) and novel object (N) as non-spatial memory condition in experimental rats (Malik et al., 2013). The experiment was conducted in 3-sections (habituation, training, and test section). On 27th day, rats were taken for habituation and placed in the open box to explore the empty box without any objects for 6 min. After 24 h of habituation session, 2nd phase training was conducted to explore two identical rectangular shape familiar objects as object-1 (F1) and object-2 (F2). The objects were fixed in two adjacent corners (~9 cm from the wall), each rat was freely allowed to explore F1 and F2 for 6 min and the exploration time with F1 and F2 were recorded (sec). Exploration was defined as pointing the nose at an object from a distance of ≤2 cm and/or touching it with the nose. After this session, all animals were returned to their home cage. The 3rd phase of experiment was conducted after 24 h of this session with the same animals, but one of the familiar objects, F2 was replaced with a cylindrical heavy novel object (N), and then rats were allowed to explore the objects F1, and N for 6 min. The exploration time with F1, and N were recorded, and the discrimination index (DI) was calculated. Throughout the session, objects and open filed box were cleaned with 70% ethanol to avoid confounding error encouraged by the influence of odour. The discrimination index (DI) for NOR was calculated by following DI = (TfTf)/(Tf + Tn); where Tf is the exploration time of the novel object (N) and Tn is the exploration time of the familiar objects (F1 & F2).

2.9. Collection of blood and preparation of tissue homogenate

After completion of the treatment period, rats were sacrificed after an overnight fasting. Blood sample of each rat were collected, processed, and kept at -80 °C until further examination. The whole brain of rats was promptly removed, washed with normal cold saline. A section of the brain was homogenized in 10X PBS, and centrifuged at 4000 rpm for 10 min (5424 R, Eppendorf, Germany) to eliminate cell debris to get clear supernatant. The supernatant was kept at -80 °C in a deep freezer (U410-86, Eppendorf, Germany), rest of the brain was fixed in 10% formalin for histological investigation.

2.10. Effects of HHZ on serum glucose and insulin levels in T2DM rats

Serum glucose (SG) of all experimental rats were estimated (Corin Clinical System, India). Serum insulin (SI) levels were estimated by using the ELISA kit (Monobind Inc., USA). Protocols were followed as provided by the manufacturer, and absorbances were recorded by multimode microplate reader (Synergy H1MF, BioTek, USA).

2.11. Effects of HHZ on brain antioxidant markers

The MDA content (nM/mg protein) in brain tissues of experimental rats was determined (Wright et al., 1981). In superoxide dismutase (SOD), the inhibition of cytochrome-C reduction in xanthine-xanthine oxidase system was quantified, and the rate of rise in absorbance per min was calculated, and the results were expressed as U/mg of protein.
Catalase activity (CAT) was recorded by measuring the reduction in absorbance during the decomposition of H₂O₂ at 240 nm at 1 min intervals (U/mg proteins). The reduced glutathione (GSH) concentration was determined, and the results were expressed as mM/mg protein (Swain et al., 2020).

2.12. Effects of HHZ on brain inflammatory markers

Various inflammatory markers in brain tissues e.g., tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) were measured using the standard ELISA model by rat-specific ELISA kit (Fine Biological Technology Co. Ltd., Wuhan, China). Absorbance was measured at specified wavelength as instructed in the kit using ELISA microplate reader (Synergy H1MF, BioTek, USA), and results were expressed as pg/mL, pg/mL, and ng/mL, respectively.

2.13. Effects of HHZ on AChE and BChE level in brain

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) level in brain tissues of experimental rats were evaluated by colorimetric method. A reaction mixture of 100 μL of tissue homogenate supernatant, 75 μL DTNB (3 mM), and Tris-HCl (50 mM; pH 8) were taken and the reaction was initiated by adding 25 μL of ATCI/SBTC to the reaction mixture for the estimation of AChE/BChE. A multimode ELISA microplate reader (Synergy H1MF, BioTek, USA) was used to record the change in absorbance at 405 nm at 1 min interval up to 10 min, and the results were expressed as nM/min/g protein. The reactions were performed in triplicates to calculate the rate of enzyme activities (Ellman et al., 1961).

\[
R = 5.74 \times 10^{-4} \times A/CO; \text{ where, } R = \text{ rate of moles of substrate hydrolysed/min/g tissue; } \]
\[
A = \text{ change in absorbance/min; and } CO = \text{ actual concentration of the tissue (mg/mL).}
\]

2.14. Effects of HHZ on β-secretase (BACE1 and BACE2) in brain

A rat ELISA kit based on the principle of standard sandwich ELISA model with their respective antibodies (Fine Biological Technology Co. Ltd., Wuhan, China) was used to determine the level of BACE1, and BACE2 in brain tissues. Absorbance of final reaction mixture was measured at 450 nm using a multimode ELISA reader (Synergy H1MF, BioTek, USA), and results were presented in ng/mL.

2.15. Histopathological examination

The brain tissues of all experimental rats were dissected, and fixed in 10% formalin (neutral buffered) for 24 h before washed in water. Tissues were dehydrated with increasing concentration of absolute alcohol (50, 70, 95, and 100%), and then rinsed with distilled water. The dehydrated tissues were fixed in paraffin blocks, and cut into 5 μM thick slices using a microtome (MRM-ST, Medimeas, India). Then the slices were stained with haematoxylin and eosin (H&E), and the cellular architecture of brain tissues were observed under inverted microscope (LYNX NIB100, Lawrence & Mayo, India) at X40.

2.16. Data analysis

Origin 2019b software was used to examine the data. The standard error of the mean (±SEM) was used to represent the descriptive data. To compare the mean, one-way ANOVA was conducted using Tukey’s post hoc analysis with the significance level at p < 0.05.
from AAPH produces peroxyl free radical upon thermal decomposition), and allows the fluorescent signal to persist. The results in Figure 2A showed that the net AUC of HHZ was 14.73 ± 0.98 which was found comparable to reference drug trolox (22.10 ± 0.78). The change in fluorescence intensity reflects the increase in fluorescence decay and HHZ as an antioxidant inhibited the fluorescence decay by neutralising peroxyl free radicals (Cao et al., 1996).

3.4.2. Effects of HHZ on cell-based antioxidant protection in erythrocytes (CAP-e) assay

CAP-e assay was performed to evaluate the cellular antioxidant potential of HHZ in living cells (RBCs obtained from Wistar rats). The cellular protection abilities of HHZ were analysed by the reduction in fluorescent intensity of DCF-DA (fluorescent probe) in presence of peroxyl radical generator AAPH. Results in Figure 2B showed that the IC50 of HHZ was recorded 30.97 ± 0.76 μg/mL which was found comparable to gallic acid (22.82 ± 0.59 μg/mL). The results obtained in ORAC and CAP-e studies go beyond previous reports showed that the presence of flavonoids, nutrients, and vitamins in HHZ were responsible in protecting cellular membranes, and lipoproteins from intracellular ROS produced by peroxyl radicals (Swain et al., 2020).

3.5. Effects of HHZ on cholinesterase activities

In vitro acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition potential of HHZ were studied. It was found that the IC50 of HHZ was 43.16 ± 0.18 and 48.50 ± 0.75 μg/mL for AChE, and BChE inhibition activities, respectively. The results were found comparable to galantamine inhibition with IC50 of 25.19 ± 0.43, and 24.25 ± 0.82 μg/mL in AChE, and BChE activities, respectively (Fig. 2C and D). These findings supported the notion that flavonoids, nutrients, and vitamins in HHZ were effective antioxidants to resist neurotoxic oxidants and inflammatory mediators in preventing neurodegeneration in AD (Cho et al., 2012; Orhan et al., 2013).

3.6. Mode of cholinesterase inhibition study of HHZ

The Lineweaver-Burk (LB) plot was used to investigate the kinetic mode of inhibition of AChE, and BChE by HHZ. Enzyme inhibition kinetics was estimated by the construction of LB plot plotted from velocity of reaction vs substrate concentration (1/v 1/s; where ‘s’ was substrate concentration and ‘v’ was reaction velocity. The type of AChE and BChE inhibition triggered by HHZ demonstrated mixed competitive inhibition as intersection at Y-axis with Vmax 0.5 and 0.8, respectively (Fig. 2E and F). The bioactive components in HHZ competed at active sites of AChE, and BChE by preventing the substrates (ATCI and SBTC) (de la Torre et al., 2012).

3.7. Effects of HHZ on NOR test in STZ-induced diabetic encephalopathy rats

The NOR test was carried out to evaluate the effect of HHZ on cognitive functions in diabetic encephalopathy rats (Table 1). During the training session, there was significant (p < 0.001) decrease in the mean exploration time for familiar objects F1 (6.83 ± 0.47 s), and F2 (6.50 ± 0.42 s) in STZ-induced diabetic group as compared to normal. However, HHZ-400 significantly (p < 0.001) increased the mean exploration time for F1 (15.83 ± 0.33 s), and F2 (16.16 ± 0.38 s) and found comparable to GLB-treated positive control. The comparison of time exploration in training session persists similar in both F1 and F2 objects for all the experimental groups (Table 1). Whereas in the experimental session, rats were spent more exploration time in novel object (N) than the familiar object (F1). It was found that STZ-induced diabetic encephalopathy rats spent significantly (p < 0.001) more exploration time in object F1 (12.00 ± 0.25 s), and N (21.36 ± 0.42 s), and found comparable to GLB-treated positive control. The discrimination index (DI) was calculated based on exploration time in both F1 and N objects for all the experimental groups (Table 1). Whereas in the experimental session, rats were spent more exploration time in novel object (N) than the familiar object (F1). It was found that STZ-induced diabetic encephalopathy rats spent significantly (p < 0.001) more exploration time in object F1 (12.00 ± 0.25 s), and N (21.36 ± 0.42 s), and found comparable to GLB-treated positive control. The discrimination index (DI) was calculated based on exploration time in both F1 and N of all experimental rats. The DI score in STZ-induced group (0.03 ± 0.01) significantly (p < 0.001) reduced as...
compared to normal. But, the DI score significantly (p < 0.001) elevated in HHZ-400 (0.27 ± 0.01) and found close to GLB-treated positive control (0.30 ± 0.01) (Table 1). In the test session, all treated groups exploring more time with N over F1 indicated different objects retention ability and discrimination skills, including remembering capacity from the previous incident. However, STZ-induced diabetic encephalopathy rats showed less exploration time with both objects due to the lack of retention and discrimination skills. The cognitive functions in treated groups were increased by various metabolic changes in the brain due to the presence of flavonoids, nutrients, and vitamins in HHZ (Ennaceur, 2010).

3.8. Effects of HHZ on serum glucose and insulin in STZ-induced diabetic encephalopathy rats

STZ-induced diabetic encephalopathy rats showed significant (p < 0.001) elevation in serum glucose (SG) level (391.31 ± 11.03 mg/dL) as compared to normal. Whereas, HHZ-400 treated group significantly (p < 0.001) reduced the SG level (167.89 ± 1.03 mg/dL) and found comparable to the GLB-treated positive control (148.29 ± 2.89 mg/dL) (Table 2). Elevation of SG level in STZ-induced diabetic rats indicated the pancreatic β-cell destruction and insulin resistance. But the SG level significantly improved in HHZ-treated group may be correlated to the presence of flavonoids, nutrients, and vitamins which directly impact on restoring the β-cell destruction and insulin sensitivity in STZ-induced diabetic encephalopathy rats. As shown in Table 2, STZ-induced diabetic encephalopathy rats showed significant (p < 0.001) reduction in insulin (SI) level (10.22 ± 0.17 μIU/mL) as compared to normal. But, oral dosage of HHZ-400 (22.10 ± 0.78 μIU/mL) and found close to the GLB-treated positive control (17.10 ± 0.13 μIU/mL) due to the presence of flavonoids (quercetin, caffeic acid, rutin, gallic acid, ferulic acid) and nutrients, vitamins (retinol acetate, vitamin K, α-tocopherol and cholecalciferol). This result ties well with previous findings wherein the bioactive components in HHZ were responsible in improving glycaemic control by proliferating

Figure 2. Cellular antioxidant, anticholinesterase activities and mode of inhibition of hydroalcohol extracted fraction of Hydrolea zeylanica (HHZ). A. depicts the area under curve (AUC) in oxygen radical absorbance capacity (ORAC) assay of HHZ with net AUC 14.73 ± 0.98 as compared to the reference drug trolox (22.10 ± 0.78). B. depicts % inhibition of cell-based antioxidant protection in erythrocytes (CAP-e) assays of HHZ with IC_{50} 3.97 ± 0.76 μg/mL as compared to the reference drug gallic acid (22.82 ± 0.59 μg/mL). C. depicts Acetylcholinesterase (AChE) inhibition of HHZ with IC_{50} 43.16 ± 0.18 μg/mL as compared to the reference drug galanthamine (25.19 ± 0.43 μg/mL). D. depicts Butyrylcholinesterase (BChE) inhibition of HHZ with IC_{50} 48.50 ± 0.75 μg/mL as compared to galanthamine (24.25 ± 0.82 μg/mL). E&F. represent the mode of inhibition of AChE and BChE activities by plotting Lineweaver-Burk (LB) plot with Vmax 0.5 (approx.), and 0.8 (approx.), respectively in a different substrate concentration (0.05-3 mM/lit) which signifies competitive inhibition (reversible) in the presence of HHZ. All the experiments were performed in triplicate and results were expressed as mean ± SEM.
Significant pancreatic β-cells, regulating glucose homeostasis and reducing insulin resistance in encephalopathy rats (Babu et al., 2013; Swain et al., 2020).

### Effects of HHZ on brain oxidative markers in STZ-induced diabetic encephalopathy rats

In hyperglycaemia, lipid peroxidative MDA level is relatively high in brain because it contains high amount of fatty acid which are susceptible towards oxidation. We observed in Table 2 that the MDA level significantly (p < 0.001) elevated (14.37 ± 1.52 nM/mg protein) in STZ-induced group as compared to normal. While, HHZ-400 significantly (p < 0.001) reduced the MDA level (1.79 ± 0.07 nM/mg protein), and found comparable to GLB-treated positive control (1.30 ± 0.10 nM/mg protein). SOD is the 1st line antioxidant defence catalyses superoxide anion to O2 and H2O2. The activity of SOD significantly (p < 0.001) in STZ-induced group (5.64 ± 0.51 U/mg protein) as compared to normal. But, HHZ-400 significantly (p < 0.001) restored the SOD activity (10.77 ± 0.10 U/mg protein), and found comparable to GLB-treated positive control (12.64 ± 0.30 U/mg protein) (Table 2). HHZ administration normalized the level of antioxidant enzymes MDA, SOD, CAT and GSH in brain tissues of STZ-induced diabetic encephalopathy rats. In support to our previous report, these findings provide additional information about flavonoids (quercetin, caffeic acid, rutin, gallic acid, ferulic acid) and nutrients, and vitamins (retinol acetate, vitamin K, α-tocopherol and cholecalciferol) were responsible in preventing lipid peroxidation and neutralising the generation of ROS in brain and restores antioxidant defence in encephalopathy subjects (Calis et al, 2020; Gomaa et al., 2019; Mukherjee et al., 1994).

### Effects of HHZ on neuroinflammatory markers in STZ-induced diabetic encephalopathy rats

The pathophysiology of diabetic encephalopathy has been associated with the inflammation cytokines e.g., TNF-α, IL-6, and hs-CRP in brain.

### Table 1. Effects of HHZ on Novel object recognition (NOR) test in STZ-induced diabetic encephalopathy rats.

| Groups       | Normal object recognition (NOR) | Discrimination index (DI) |
|--------------|---------------------------------|---------------------------|
|              | Exploratory time in sec          |                           |
|              | F1 object                        | F2 object                  |
| Normal Control | 21.21 ± 0.57                    | 21.00 ± 0.44              |
| Negative Control | 6.83 ± 0.47***                  | 6.50 ± 0.42***            |
| Positive Control | 17.00 ± 0.44***                  | 17.50 ± 0.42***           |
| HHZ-300       | 11.33 ± 0.33***                  | 11.35 ± 0.32***           |
| HHZ-400       | 15.83 ± 0.30***                  | 16.16 ± 0.31***           |

Novel object recognition (NOR) test was carried out, and found that HFD/STZ-induction caused significant (***p < 0.001) cognitive impairment in negative control encephalopathy rats as compared to normal. However, oral administration of GLB and HHZ significantly (**p < 0.001) improved cognitive function in contrast to the negative control. Standard drug-glibenclamide (GLB 0.5 mg/kg b.w., i.p.), HHZ-300, HHZ-400: hydroalcohol extracted fraction of leaves of *H. zeylanica* at 300 and 400 mg/kg b.w., respectively. Statistical analysis was carried out by one-way ANOVA followed by Tukey’s post hoc analysis. Values were represented as mean ± SEM (n = 6).

### Table 2. Effects of HHZ on Glycaemic, Oxidative and Inflammatory markers in serum, and brain tissues of STZ-induced diabetic encephalopathy rats.

| Biochemical parameters | Normal Control | Negative Control | Positive Control | HHZ-300 | HHZ-400 |
|------------------------|---------------|------------------|-----------------|---------|---------|
|                         | Estimation of serum glucose (SG) and serum insulin (SI) |                 |                 |         |         |
| SG (mg/dL)             | 87.64 ± 0.78  | 391.31 ± 11.03***| 148.29 ± 2.89***| 238.69 ± 3.84***| 167.89 ± 3.84***|
| SI (μg/mL)             | 23.93 ± 0.50  | 10.22 ± 0.17###  | 17.10 ± 0.13###  | 12.26 ± 0.16###  | 15.96 ± 0.12###  |
| Estimation of brain oxidative markers |                 |                 |                 |         |         |
| MDA (nM/mg protein)    | 1.16 ± 0.05   | 14.37 ± 1.52###  | 1.30 ± 0.10###  | 2.64 ± 0.30###  | 1.79 ± 0.07###  |
| SOD (U/mg protein)     | 14.22 ± 0.09  | 5.64 ± 0.51###   | 12.64 ± 0.30###  | 8.62 ± 0.51###  | 10.77 ± 0.10###  |
| CAT (U/mg protein/min) | 18.82 ± 0.46  | 6.33 ± 0.70###   | 14.87 ± 0.24###  | 10.51 ± 0.27###  | 13.39 ± 0.28###  |
| GSH (mM/mg protein)    | 29.06 ± 0.52  | 8.21 ± 0.38###   | 27.49 ± 0.44###  | 19.01 ± 1.20###  | 26.06 ± 0.60###  |
| Estimation of neuro-inflammatory markers |                 |                 |                 |         |         |
| TNF-α (pg/mL)          | 63.55 ± 2.63  | 139.91 ± 11.49***| 72.27 ± 0.26***  | 90.22 ± 1.20***  | 77.54 ± 1.12***  |
| IL-6 (pg/mL)           | 247.03 ± 3.36 | 844.88 ± 27.90### | 341.53 ± 15.25### | 528.20 ± 20.40### | 444.83 ± 12.76### |
| hs-CRP (ng/mL)         | 110.61 ± 2.53 | 310.85 ± 3.53### | 127.37 ± 1.37### | 200.45 ± 2.08### | 148.89 ± 2.81### |

Statistical analysis was carried out by one-way ANOVA followed by Tukey’s post hoc analysis. Values were represented as mean ± SEM (n = 6). The glycaemic markers (SG and SI), oxidative markers (MDA, SOD, CAT and GSH) and neuro inflammatory markers (TNF-α, IL-6 and hs-CRP) in serum and brain significantly (**p < 0.001) affected in STZ-induced negative control group (40 mg/kg b.w., i.p.) as compared to normal. However, the rest of the experimental group (GLB, HHZ-300, HHZ-400) significantly (**p < 0.001) improved serum glycaemic markers, brain oxidative, and inflammatory markers in STZ-induced diabetic encephalopathy rats. Standard drug-glibenclamide (0.5 mg/kg b.w., i.p.), HHZ-300, HHZ-400: hydroalcohol extracted fraction of leaves of *H. zeylanica* at 300 and 400 mg/kg b.w. respectively. TNF-α-tumor necrosis factor α, IL-6- Interleukin 6 and hs-CRP- high-sensitivity C-reactive protein.
tissues of diabetic subjects (Liu et al., 2012). In the current study, STZ-induced group significantly (p < 0.001) elevated the level of TNF-α (139.91 ± 11.49 pg/mL), IL-6 (844.00 ± 27.90 pg/mL), and hs-CRP (310.85 ± 3.53 ng/mL) in brain hippocampus as compared to normal. While, pre-treatment of HHZ-400 significantly (p < 0.001) restored the level of TNF-α (77.44 ± 1.12 pg/mL), IL-6 (444.83 ± 12.76 pg/mL), and hs-CRP (148.81 ± 2.81 ng/mL), and were found comparable to GLB-treated positive control (AChE; 32.84 ± 0.43 nM/min/gm protein) and found comparable to GLP-treated positive control (AChE; 25.71 ± 0.62 nM/min/gm protein) and found comparable to GLP-treated positive control (AChE; 25.71 ± 0.62 nM/min/gm protein) and found comparable to GLP-treated positive control (AChE; 25.71 ± 0.62 nM/min/gm protein) and found comparable to GLP-treated positive control (AChE; 25.71 ± 0.62 nM/min/gm protein). The elevated activities of AChE and BChE in STZ-induced group resulted in reduced cholinergic action and diminution of acetylcholine (Kumar and Singh, 2017). But, HHZ reduced the cholinesterase activities by managing STZ-induced oxidative stress which regulates glucose metabolism, insulin signalling in diabetic encephalopathy rats (Calis et al., 2020; Gomaa et al., 2019).

3.11. Effects of HHZ on cholinesterase activity in STZ-induced diabetic encephalopathy rats

Cholinergic neurotransmitters e.g., acetylcholine (ACh) and butyrylcholine (BCh) are highly expressed in the hippocampus and cerebral cortex of brain and responsible for cognitive functions which are catalysed by the hydrolysis of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The increase in cholinesterase activities cause neuronal function impairment as found mostly in chronic diabetes patients (Mesulam et al., 2002). In hyperglycaemia, reduced insulin transmission in brain lowers ACh synthesis and alters the insulin signalling pathway leads to cognitive impairment (Dubey et al., 2020). As shown in Fig. 3A and B, the cholinesterase (AChE: 54.63 ± 0.36 and BChE: 50.06 ± 0.64 nM/min/gm protein) activities significantly (p < 0.001) elevated in STZ-induced rats as compared to normal. While, pre-treatment of HHZ-400 significantly (p < 0.001) reduced the level of AChE and BChE, and found comparable to GLB-treated positive control (AChE: 36.42 ± 0.43 and BChE: 31.15 ± 0.44 nM/min/gm protein) and found comparable to GLP-treated positive control (AChE: 26.51 ± 0.44 nM/min/gm protein). The enhanced levels of AChE and BChE in STZ-induced group resulted in reduced cholinergic action and diminution of acetylcholine (Kumar and Singh, 2017). But, HHZ reduced the cholinesterase activities by managing STZ-induced oxidative stress which regulates glucose metabolism, insulin signalling in diabetic encephalopathy rats. The rich source of flavonoids (quercetin, caffeic acid, rutin, gallic acid, ferulic acid) and nutrients, and vitamins in HHZ may act on the inflammatory pathways, and suppress neuro-inflammation by managing STZ-induced oxidative stress in encephalopathy rats (Calis et al., 2022; Gomaa et al., 2019).

3.12. Effects of HHZ on β-secretase level in STZ-induced diabetic encephalopathy rats

β-secretase e.g., BACE1 and BACE2 prominently expressed in brain, and pancreas are involved in initiating Abeta-generation by the cleavage of amyloid-precursor protein (APP) at β-site, or within β-amyloid (Aβ)-peptide (Basi et al., 2003). So, BACE is a prime target site for the therapeutic intervention to inhibit Abeta-generation in AD. In the current research, STZ-induced diabetic encephalopathy rats showed significant (p < 0.001) increase in the level of BACE1 242.86 ± 1.50, and BACE2 24.51 ± 0.41 ng/mL as compared to normal. But, pre-treatment with HHZ-400 significantly (p < 0.001) reduced the level of BACE1 108.01 ± 1.69, and BACE2 15.07 ± 0.27 ng/mL, which were comparable to
normal control, the glial cells (GC) were arranged in intact and regular form of GC and E cells (Figure 4E). Similar recovered protective activities. However, HHZ-400 administered group showed an adequate, and effective improvement in cellular architecture of the cortex with an intact and regular form of GC and E cells (Figure 4E). Similar recovered protective activities.

**4. Conclusions**

Potential of *Hydrolea zeylanica* leaves (HHZ) for the prevention and cure of STZ-induced diabetic encephalopathy was supported by various experimental studies. HHZ demonstrated promising cellular antioxidant protection in ORAC and CAP-e studies. *In vitro* test results showed HHZ has significant inhibition potential against cholinesterase inhibition (*AChEI, and BChEI*) activities with mixed competitive inhibition towards *AChE, and BChE*. HHZ-400 significantly (*p < 0.001*) reduced the serum glucose, and elevated the serum insulin level in blood with the improvement in oxidative (MDA, SOD, CAT, and GSH), and inflammatory markers (TNF-α, IL-6, and hs-CRP) in brain tissues. It also significant (*p < 0.001*) reduced cholinesterase (*AChE, and BChE*) and β-secretase (*BACE1, and BACE2*) activities as evidenced by histological architecture of brain cortex in diabetic encephalopathy rats. The rich source of flavonoids as quercetin, caffeic acid, rutin, gallic acid, ferulic acid, and nutrients and vitamins (retinol acetate, vitamin K, α-tocopherol and cholecalciferol) in the leaves of *H. zeylanica* enhanced the cellular antioxidant protection by reducing oxidative stress, neuroinflammation, and cholinergic transmission in the brain of diabetic encephalopathy rats. The detail mechanism on genetic expression studies is required to substantiate further to understand the therapeutic action of *H. zeylanica* in the pathogenesis of diabetic encephalopathy.

**3.13. Histological observation**

We explored the histological variations in the brain cortex of all experimental group rats by haematoxylin and eosin (H&E) staining. In normal control, the glial cells (GC) were arranged firmly and appeared in regular round, or oval shape with a larger cell structure along with healthy eosinophil cells (E) in an intact form (Figure 4A). In contrast, STZ-induced diabetic encephalopathy rats had deformed cortex with abate, shrunken GC, and E cells with several necrotic cells (N) in the cortex region due to the neurotoxic effects of HFD/STZ-induced oxidative stress (Figure 4B). The oral administration of HHZ-300 showed moderate improvement in the cortex region (Figure 4D). However, HHZ-400 administered group showed an adequate, and effective improvement in cellular architecture of the cortex with an intact and regular form of GC and E cells (Figure 4E). Similar recovered cortex region with all normal cellular architecture were found in oral administration of GLB group (Figure 4C). The histological observation reflected that HHZ-400 significant restored the cellular structural integrity in the brain cortex of STZ-induced diabetic encephalopathy rats which supported the current experimental finding on its neuroprotective activities.

**Figure 4.** Histopathological observations of brain cortex (T.S.) in STZ-induced diabetic encephalopathy rats. A. depicts brain tissue of normal control with normal histological architecture with healthy and well-arranged cortical appearance with glial cells (GC), neural cells (NC), eosinophil (E) with no observation of cellular necrosis. B. shows brain histology of STZ-induced rat with severely damaged cellular architecture, irregular arrangements of glial cells (GC), necrotic cells (N) with neuronal degeneration. C. shows brain histology of GLB-treated positive control with improved cellular architecture and well-arranged of glial cells (GC), neural cells (N), eosinophil (E). D & E. shows brain histology of HHZ-300 and HHZ-400 treated brain of STZ-induced rats with recovery from neuronal degeneration with improved glial cells (GC), neural cells (NC), eosinophil (E). HHZ-300: hydroalcohol extracted fraction of leaves *H. zeylanica* 300 mg/kg b.w.; HHZ-400: hydroalcohol extracted fraction of leaves *H. zeylanica* 400 mg/kg b.w.

**Declarations**

**Author contribution statement**

Sandeep Kumar Swain; Atish Kumar Sahoo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Umesh Chandra Dash: Performed the experiments; Analyzed and interpreted the data; analysis tools or data; Wrote the paper.

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**Data availability statement**

Data will be made available on request.
