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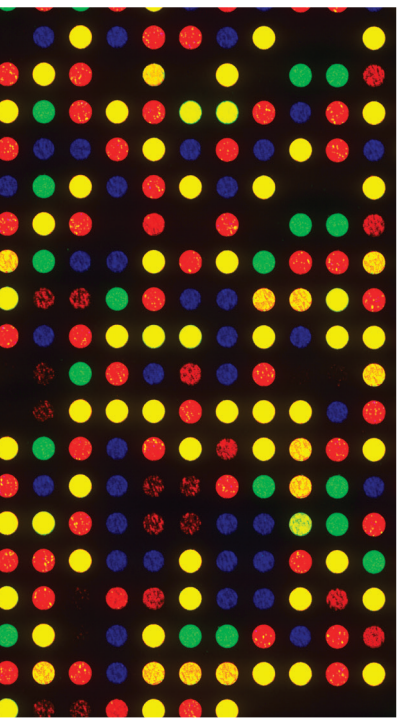
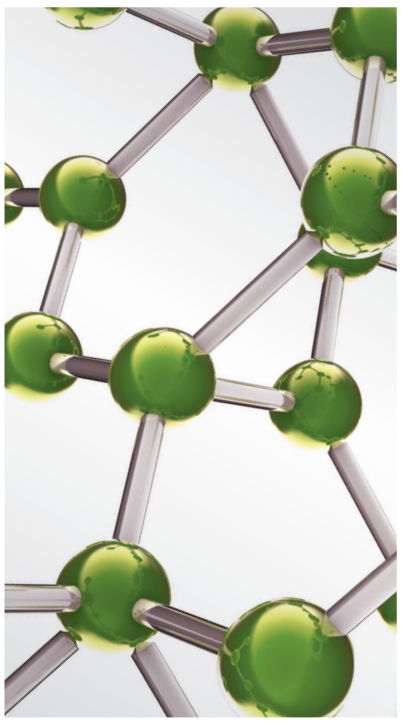
Medicinal Plants in Management of Type 2 Diabetes and Neurodegenerative Disorders

Guest Editors: Gan Siew Hua, Mohammad Amjad Kamal, Marcelo M. S. Lima, Mohamed Ibrahim Khalil, Visweswara Rao Pasupuleti, and Gjumrakch Aliev



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Editorial

Medicinal Plants in Management of Type 2 Diabetes and Neurodegenerative Disorders

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With advancement in modern medical sciences, a variety of major diseases such as type 2 diabetes and neurodegenerative disorders (ND) including Alzheimer's disease, Parkinson's disease, and the mood disorders still prevail. Medicinal plants have the potential to prevent, control, or cure some of these diseases. For example, some plants have constituents useful as antiaging potentials due to the presence of antioxidants. Many of the plants have high medicinal values to be further explored. However, determining specific components having specific pharmacological activity and their clinical effects still remains a challenge. This issue is a compilation of 10 intriguing papers focusing on medicinal plants with potential for management of diabetes and ND.

Berberine (Ber) is an isoquinoline derivative alkaloid isolated from several Chinese medicines such as *berberidis radix*, *phellodendri chinensis cortex*, *coptidis rhizoma,* and *mahoniae caulis*. In recent decades, much attention has been placed on its significant antidiabetic activities. Z. Wang et al. conducted a research to improve the problem posed by the plant's low solubility and poor membrane permeability by incorporating nanosuspension technology. Due to the enhanced absorption posed by the technology, they concluded that Ber has good potential as a novel potential antidiabetic agent for both functional food and pharmaceutical purposes.

The paper by N. Giribabu et al. focused on *Vitis vinifera* (Linn.) which belongs to Vitaceae family. Besides having antidiabetic activity, *V. vinifera* also has a wide range of pharmacological activities such as inhibition of platelet aggregation and low density lipoprotein oxidation, antioxidant, antimicrobial, and anticancer activities. In their research paper, the authors explored the potential of the seed extract to protect against diabetes-induced liver damage for the first time. The authors found that it has the ability to confer near normal activity levels for various key enzymes involved in liver carbohydrate metabolism in diabetes. In addition, liver histology findings following administration of *V. vinifera* seed ethanolic extract were also very promising. The authors hypothesized that these positive changes may be due to decrease in liver oxidative stress in diabetes and suggested that the seed extract of a Muscat variety of *V. vinifera* helps in liver protection in diabetes.

N. Giribabu et al. investigated *Centella asiatica*, an herb traditionally used to improve memory on prevention of hippocampus dysfunction in diabetic rats. They reported that administration of *C. asiatica* leaf aqueous extract to diabetic rats maintained near normal ATPases activity levels and prevented the increase in the levels of inflammatory and oxidative stress markers in the hippocampus. The authors suggested that *C. asiatica* leaf protects the hippocampus against diabetes-induced dysfunction which could help to preserve memory in diabetes.

P. niruri is another medicinal plant reported to possess antidiabetic and kidney protective effects. N. Giribabu et al. investigated the phytochemical constituents and *in vitro*

antioxidant activity of *P. niruri* leaf aqueous extract on oxidative stress and antioxidant enzymes levels in diabetic rat kidney. They found that administration of *P. niruri* leaf aqueous extract for 28 consecutive days prevented the increase in the amount of lipid peroxidation product, malondialdehyde, and the diminution of superoxide dismutase, catalase, and glutathione peroxidase activity levels in the kidney of diabetic rats. In addition, *P. niruri* leaf aqueous extract also exhibited *in vitro* antioxidant activity while phytochemical screening of the extract indicated the presence of polyphenols. The authors concluded that *P. niruri* leaf extract protects the kidney from oxidative stress induced by diabetes.

In another paper, I. Aziz et al. explored the potential of Spirulina platensis (a commonly used nutritional supplement due to its high protein and antioxidant content) as a neuroprotective agent in both experimental focal and global cerebral ischemia-reperfusion injuries in rats. They found that pretreatment with Spirulina significantly improved the rats' locomotor function and ameliorated the histological changes and neurological deficits thus suggesting that Spirulina possesses potential benefits in improving hind limb locomotor function and reducing morphological damage to the spinal cord which could offer alternatives to the limited management available for spinal cord injury.

S. N. Talukdar et al. reviewed the potential of *Momordica dioica* (a perennial, dioecious, cucurbitaceous climbing creeper), native to Asia for its phytochemical, ethnobotanical, phytotherapeutical, and pharmacological properties based on traditional view (including Ayurveda) along with recent scientific observations. Their effort can help many researchers justify the dynamic ethnobotanical and phytotherapeutical roles of several plants for future research as well as stimulating new ideas for therapeutic roles of *Momordica dioica* against diabetes, cancer, ND, and other life threatening disorders.

The *in vitro* and *in vivo* effects of *Cuscutae Semen* (CS) (a widely used traditional herbal medicine) on neurotoxicity were investigated by M. Ye et al. The *in vitro* assay indicated that CS attenuated 1-4-methyl-4-phenylpyridinium- (MPTP-) induced cell death, increased reactive oxygen species generation, and activated glutathione peroxidase. *In vivo*, immunohistochemistry assay for tyrosine hydroxylase revealed a significant loss of nigral dopamine neurons with parallel activation of the microglia and increased production of reactive oxygen species was also observed in the substantia nigra and striatum. The MPTP-induced loss of nigral dopamine neurons was, however, partly inhibited by CS suggesting that CS may be useful for the treatment of ND such as Parkinson's disease which warrants further investigation.

Another medicinal plant, *Codonopsis lanceolata* (*C. lanceolata*), has previously been employed for lung inflammatory diseases such as asthma, tonsillitis, and pharyngitis. J. B. Weon et al. evaluated the effects of fermented *C. lanceolata* on learning and memory impairment induced by scopolamine by using the Morris water maze and passive avoidance tests. To elucidate the possible mechanism of cognitive enhancing activity, the authors also measured acetylcholinesterase activity, brain-derived neurotrophic factor, and cyclic AMP response element-binding protein expression in the brain of mice. They found that the administration of fermented *C. lanceolata* reduced scopolamine-induced memory impairment in the Morris water maze and passive avoidance tests. Interestingly, it also inhibited acetylcholinesterase activity while the level of cyclic AMP response element-binding protein phosphorylation and neurotrophic factor expression in hippocampal tissue were significantly increased indicating that fermented *C. lanceolata* can ameliorate scopolamine-induced memory deficits in mouse and may be an alternative agent for the treatment of Alzheimer's disease in the future.

M. Rasool et al. reviewed the use of various phytochemicals in the treatment of ND. Many of the phytochemicals could be used as promising therapeutic agents because many have anti-inflammatory, antioxidative, and anticholinesterase activity. The review covers the various researches related to phytochemicals in management of ND.

Honey is the only insect-derived natural product with therapeutic, traditional, spiritual, nutritional, cosmetic, and industrial value. In addition to having excellent nutritional value, honey is a good source of physiologically active natural compounds, such as polyphenols. Unfortunately, there are very few current researches investigating the nootropic and neuropharmacological effects of honey, and these are still in their early stages. M. M. Rahman et al. reviewed the nootropic effects of honey, such as memory-enhancing effects, as well as neuropharmacological activities including anxiolytic, antinociceptive, anticonvulsant, and antidepressant activities. The review may help impact the use of honey for specific ND, apoptosis, necrosis, neuroinflammation, synaptic plasticity, and behavior-modulating neural circuitry in the future.

Overall, by compiling these papers, we hope to enrich both readers and researchers alike on the vast potential of natural products for the treatment of diabetes and ND for which treatment based on modern medicine is still lacking.

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Research Article

Berberine Nanosuspension Enhances Hypoglycemic Efficacy on Streptozotocin Induced Diabetic C57BL/6 Mice

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Berberine (Ber), an isoquinoline derivative alkaloid and active ingredient of Coptis, has been demonstrated to possess antidiabetic activities. However its low oral bioavailability restricts its clinical application. In this report, Ber nanosuspension (Ber-NS) composed of Ber and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) was prepared by high pressure homogenization technique. Antidiabetic effects of Ber-NS relative to efficacy of bulk Ber were evaluated in streptozotocin (STZ) induced diabetic C57BL/6 mice. The particle size and zeta potential of Ber-NS were 73.1 ± 3.7 nm and 6.99 ± 0.17 mV, respectively. Ber-NS (50 mg/kg) treatment via oral gavage for 8 weeks resulted in a superior hypoglycemic and total cholesterol (TC) and body weight reduction effects compared to an equivalent dose of bulk Ber and metformin (Met, 300 mg/kg). These data indicate that a low dosage Ber-NS decreases blood glucose and improves lipid metabolism in type 2 diabetic C57BL/6 mice. These results suggest that the delivery of Ber as a nanosuspension is a promising approach for treating type 2 diabetes.

1. Introduction

Type 2 diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia resulting from relative insulin deficiency and insulin resistance in various tissues. The worldwide prevalence of diabetes is estimated to rise to more than 200 million people and may subsequently reach 300 million by 2025 [1–3]. Several oral therapeutic agents are the first choice treatments of type 2 DM. The purpose of these oral hypoglycemic agents is to ameliorate the underlying metabolic disorder, associated with inadequate insulin secretion, insulin resistance, and increased hepatic gluconeogenesis. However, they have limited efficacy and occasionally produced severe side effects such as weight gain, hypoglycemia, gastrointestinal disturbances, liver injury, heart failure, and bloating [4–6].

Berberine (Ber), an isoquinoline derivative alkaloid isolated from several Chinese medicines, such as Berberidis Radix (Chinese name: Sankezhen), Phellodendri chinensis cortex (Chinese name: Huangbo), Coptidis Rhizoma (Chinese name: Huanglian), and Mahoniae Caulis (Chinese name: Gonglaomu), is commonly used as a quality control marker [7–9]. In recent decades, much focus has been put on its significant antidiabetic activities [10–12]. Previous studies showed that Ber exhibited hypoglycemic properties and exhibited insulin sensitization in type 2 diabetic rats by activation of AMPK [13, 14]. Moreover, Ber can ameliorate diabetic complications, such as cardiac dysfunction, endothelial dysfunction, and nephropathy on diabetic rats [15, 16]. Recent clinical investigations indicated that Ber was a potential and safe hypoglycemic drug to treat type 2 diabetic patients with dyslipidemia at an oral dose of 0.3–0.5 g three times daily

[17]. Despite the promising biological effects, Ber is poorly absorbed, resulting in low bioavailability after oral administration. It has been reported that the oral bioavailability of Ber in rats was 0.68% [18]. The low absorption and bioavailability of Ber is still not fully understood. Recently, several studies have proposed some interpretations; since Ber is a lipophobic compound, it is restrained from passing through the membranes of intestinal cells. Secondly, Ber acts as a substrate of several ATP-binding cassette transporters, such as P-glycoprotein (P-gp) and multidrug resistance-associated protein. To circumvent these pitfalls, several strategies, like absorption enhancer, self-microemulsion, and solid lipid nanoparticles, have been used to increase its bioavailability [14, 18–20].

Nanocrystal suspension, nanosuspension (NS) for short, is a carrier-free nanoparticles system containing only pure drug crystal and minimum surfactant and/or polymer for stabilization [21]. Reduction of particle size by nanocrystal technology to the nanoscale usually leads to a significant increase in drug solubility and dissolution rate with an obvious improvement in drug bioavailability. Liversidge and Cundy reported that [22], in the same dosage, danazol NS with the average particle size of 169 nm could obtain the C_{max} as high as 3.01 mg/ml and the bioavailability of 82% in beagle dogs, while the commercially available danazol suspension with the average particle size of 10 mm could only obtain the C_{max} of 0.20 mg/ml and the bioavailability of 5%. It could be found that NS significantly enhanced the oral absorption of danazol, a poorly water-soluble drug. A few techniques have been used to prepare drug loaded NS, including nanoprecipitation, pearl-milling, high speed homogenization, sonication, and high-pressure homogenization (HPH) [23]. Among these techniques, the HPH method with a high productivity and a lower level contamination which is favorable for implementation of industrial products has shown great superiority over other methods. In this study, we evaluate hypoglycemic activity of Ber-NS relative to efficacy of bulk Ber in streptozotocin induced diabetic C57/BL 6 mice.

2. Materials and Methods

2.1. Preparation of the Ber-NS. HPH technique was applied to prepare Ber-NS. Briefly, D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS, BASF, Germany) of 1.0% and hypromellose (HPMC, Dow Chemicals, Dartford, UK) of 0.5% were dissolved in distilled water. Ber (chloride form, purchased from Aladdin industrial corporation, Shanghai, China) powder of 0.5% was dispersed in the aqueous surfactant solution using high speed homogenization 5000 rpm for 10 min (IKA T18 basic ULTRA-TURRAX, Germany). Then the premix was passed through a Lab HPH (APV-2000, Germany); 5 cycles were performed at 500 bar and 15 cycles at 1500 bar.

2.2. Characterization of the Ber-NS. The particle size, polydispersity index, and Zeta potential measurements were performed on a Nano-ZS90 (Malvern Instruments Ltd., Malvern, UK) thermostated at 25°C. The sample was diluted 50 times with bidistilled water before the measurements. Each value reported is the average of three measurements. DSC was recorded on a DSC 204 F1 (NETZSCH, Germany). 5–10 mg of samples were sealed in aluminum pans and heated at 10°C/min from 30°C to 300°C and under N_2 flow (100 ml/min).

2.3. Animal Model and Treatment. Female C57BL/6 mice 7-8 weeks old, body weights 15–20 g, were purchased from the medical experimental animal center of Guangdong Province, China. The National Institute of Health guidelines for the care and use of laboratory animals were followed in all animal experimental procedures. The animals, housed individually in plastic cages, were allowed free access to food and water at all time and were maintained on a 12 h light/dark cycle in a controlled temperature (20 to 25°C) and humidity (50 ± 5%) environment for 1 wk before use. The normal chow diet consisted (as a percentage of total kcal) of 12% fat, 60% carbohydrate, and 28% protein and a high-fat diet (HFD) consisted of 41% fat, 41% carbohydrate, and 18% protein. C57BL/6 mice with normal diets and with HFD are generally used as nondiabetic controls and a diabetic model, respectively [24]. After 4 weeks of feeding, the mice were administered fast for 12 h and then received intraperitoneal injection with 120 mg/kg streptozotocin (STZ, Sigma, St. Louis, MO, USA) in citrate solution (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.5). After 3 weeks, diabetic mice (blood glucose concentrations ≥10.5 mmol/L) were administered Ber-NS, bulk Ber (50 mg/kg/d, BN group, Ber group, $n = 10$), and metformin (300 mg/kg/d, Met group, $n = 10$) via oral gavage daily for 8 weeks or treated with vehicle control (Con group, $n = 10$), respectively. Normal mice as control (Nor group, $n = 10$) were administered citrate buffer alone and treated with vehicle control for 8 weeks. Ber and Met were dissolved in saline. All the mice were allowed to continue to feed on their respective diets until the end of the study. Then, biochemical parameters were performed as described below.

2.4. Biochemical Assays. Fasting blood samples collected from the orbital cavity were added into precooled tubes containing EDTA (final concentration 4 mmol/L) and centrifuged at 2,500 ×g for 20 min at 4°C. Plasma glucose, cholesterol, and triglyceride levels were measured with commercial kits (Rongsheng-Biotech Company, Shanghai, China).

2.5. Statistical Analysis. Results were expressed as mean ± standard deviation (SD). Student's t-test was used to compare the mean differences between samples using the statistical software SPSS version 16.0 (SPSS, Chicago). In all cases $P < 0.05$ was considered statistically significant.

3. Results

3.1. Particle Size Analysis and Zeta Potential. The mean particle size and polydispersity index (PDI) were measured

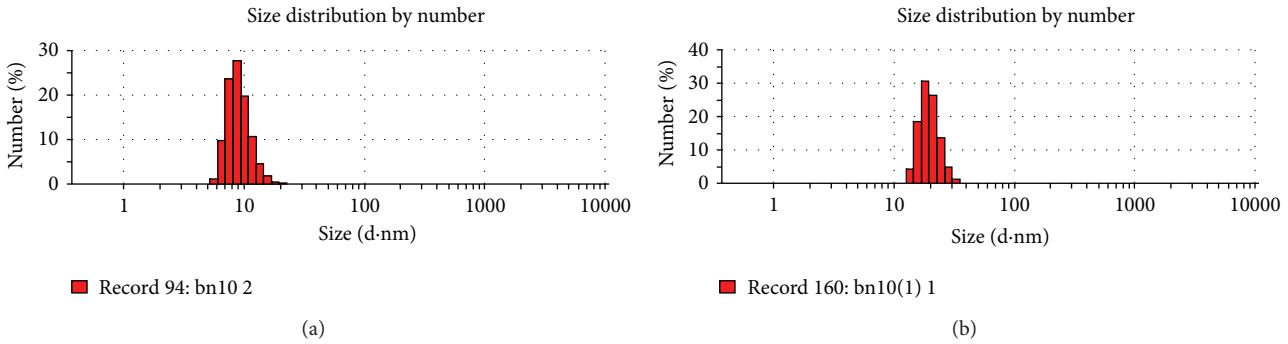

Figure 1: The particles size of Ber-NS ((a) day 0, (b) day 240).

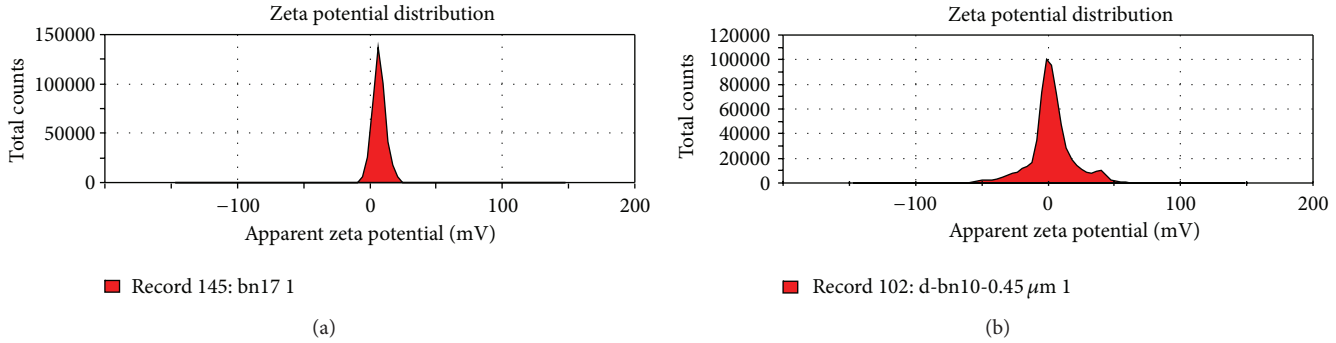

Figure 2: The zeta potential of Ber-NS ((a) day 0, (b) day 240).

immediately after the preparation of the NS. The mean particle size with PDI 0.302 was 72.4 nm. At eight weeks later, the particle size with a PDI of 0.462 was 294.6 nm (Figure 1). The PDI is a measure of particles size distribution. The values less than 0.3 indicate a high degree of homogeneity in particle size and vice versa. The zeta potential of Ber-NS at day 0 and day 240 were 6.95 and 2.5 mV, respectively (Figure 2).

3.2. DSC. DSC spectrograms are given in Figure 3. The thermogram of Ber showed a sharp endothermic peak at 189°C, corresponding to the melting point of the crystalline form of the drug. In the thermal curve of the physical mixture, the fusion endothermic peak of Ber was much lower than the crystalline substance and shifted to a lower temperature as a consequence of the interaction between Ber, HPMC, and TPGS. However, the thermal curve of the Ber-NS showed a complete disappearance of the Ber endothermic peaks, indicating the formation of an amorphous solid dispersion.

3.3. Pharmacodynamics of Ber-NS on Glucose and Lipid Metabolism. To evaluate the effect of Ber-NS on glucose and lipid metabolism, control and diabetic mice were treated with metformin (Met), Ber, Ber-NS, and plasma glucose, total cholesterol, and triglycerides analyzed. As shown in Table 1, diabetic mice exhibited higher fasting blood glucose (FBG), total cholesterol (TC), and triglyceride (TG) levels compared to those of control. Ber (50 mg/kg) treatment decreased FBG levels compared with Con groups. In contrast, BN (50 mg/kg) treatment resulted in a superior hypoglycemic and TC and body weight reduction effects compared to an equivalent dose of bulk Ber and Met (300 mg/kg). These data indicate that a low dosage Ber-NS decreases blood glucose and improves lipid metabolism in type 2 diabetic C57BL/6 mice.

4. Discussion

Ber is the major active ingredient of rhizome coptidis, a popular traditional Chinese herb used for the treatment of infection and inflammation. Many animal studies and clinical trials have proved that Ber has significant hypoglycemic effect, even comparable to metformin [14]. Although hypoglycemic effect of Ber is so enticing, it has not yet been used clinically as an antidiabetic drug, mainly because of its low bioavailability (<1%) [18]. As a result, Ber has to be administered repeatedly and at high doses (1500–2000 mg/d) when used in diabetic patients [24]. Although high dose of Ber decreases the blood glucose, it causes major gastrointestinal side-effects, which greatly limits its clinical application. So enhancing the bioavailability of Ber will not only increase its hypoglycemic effect, but also reduce its gastrointestinal side effects. Until recently, there is no multicenter, well controlled, long-term clinical trial to evaluate the efficacy of Ber in the treatment of diabetes, due to its low bioavailability. There were a few reports focusing on the development of new dosage forms of Ber to increase its bioavailability, such as using the intestinal absorption enhancer, self-microemulsion, and solid lipid nanoparticles [14, 18–20]. In this investigation, we first studied the effect of Ber-NS on glucose homeostasis

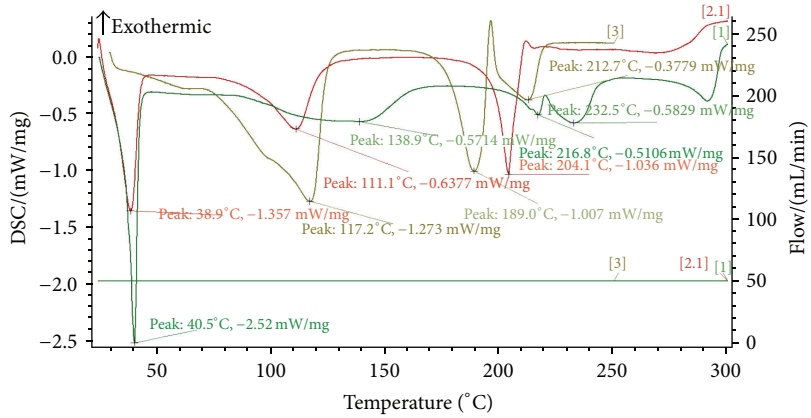

Figure 3: DSC thermograms of [1] Ber-HPMC-TPGS physical mixture, [2] Ber-NS and [3] Ber.

Table 1: Changes induced by Ber-NS in biochemical parameter on diabetic C57BL/6 mice.

Group	Dose (mg/kg)	Mice		Body weight (g)		FBG (mmol/L)	TC (mmol/L)	TG (mmol/L)
		Initial	Final	Initial	Final			
Nor	—	10	10	30.1 ± 1.0	31.9 ± 0.7	5.88 ± 0.53	2.39 ± 0.09	0.88 ± 0.10
Con	—	10	10	27.9 ± 1.2	30.7 ± 0.8	$8.08 \pm 0.85^{***}$	$3.34 \pm 0.21^{***}$	$1.10 \pm 0.12^{**}$
Met	300	10	10	28.6 ± 1.8	33.3 ± 3.6	$6.92 \pm 0.59^{\#}$	3.13 ± 0.36	0.99 ± 0.16
Ber	50	10	9	28.7 ± 1.6	29.3 ± 3.5	$6.81 \pm 0.63^{\#}$	2.97 ± 0.70	0.94 ± 0.18
BN	50	10	10	28.6 ± 1.7	32.1 ± 3.4	$6.05 \pm 0.45^{\#\#\#,\Delta}$	$2.72 \pm 0.64^{\#}$	0.97 ± 0.21

Eight weeks after Ber-NS treatment, biochemical parameter of the age-matched normal control group (Nor), STZ induced diabetic group (Con), Metformin treated diabetic group (Met), bulk Ber treated diabetic group (Ber), and Ber-NS treated diabetic group (BN). Data are presented as mean ± SD from 10 animals ($n = 10$) for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Nor; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Con; $^{\Delta}P < 0.05$ versus bulk Ber.

to assess if NS could enhance Ber antidiabetic effects. The present study showed that Ber reduced hyperlipidemia and hyperglycemia in diabetic mice, which were consistent with other studies [13, 14]. FBG was decreased by Ber treatment in type 2 diabetic mice induced by high-fat diet and low dose STZ. The therapeutic effects of Ber-NS were obviously improved when prepared with NS. It showed that the ability of NS to enhance the efficacy of Ber was most probably by improving its bioavailability and increasing its blood level.

More than 95% of the Ber-NS particles showed a small particle size (107.4 nm) (Figure 1). NS were prepared using a HPH method which results in small particles. Small particle size less than 200 nm is desired for being usually invisible to the reticuloendothelial system and for circulating over a prolonged period of time in vivo. Moreover, a zeta potential of $\geq \pm 25$ mV is recommended for achieving stable dispersions. This is attributed to the existence of repulsive forces between the particles, preventing them from contacting each other and agglomerating. Neutral particles obtained presently can thus be considered intrinsically stable as no interparticulate molecular interactions (both attractive and repulsive) are expected from these particles. In contrast to the neutral and negatively charged particles, the positively charged nanoparticles are taken up more rapidly by the cell membranes [20].

5. Conclusion

In the present study, the low solubility and poor membrane permeability of Ber [25] were enhanced by NS technology. This study demonstrated that Ber-NS possessed excellent antidiabetic activity in diabetic mice models. Moreover, Ber-NS produced a superior hypoglycemic and TC and body weight reduction and less adverse effects compared with bulk Ber and Met. Therefore, Ber-NS may be explored as a novel potential antidiabetic agent for the functional food and pharmaceutical purpose. This study also provides evidences to support the therapeutic effects of compound NS for treatment of diabetes in China. Despite of the promising results from our current investigation, there are still a plethora of practical issues which may be difficult to reconcile for the ultimate use of Ber-NS for the novel target-therapy in diabetes management.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Vitis vinifera (Muscat Variety) Seed Ethanolic Extract Preserves Activity Levels of Enzymes and Histology of the Liver in Adult Male Rats with Diabetes

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The effect of *V. vinifera* seeds on carbohydrate metabolizing enzymes and other enzymes of the liver in diabetes is currently unknown. We therefore investigated changes in the activity levels of these enzymes following *V. vinifera* seed extract administration to diabetic rats. *Methods*. *V. vinifera* seed ethanolic extract (250 and 500 mg/kg/day) or glibenclamide (600 μg/kg/day) was administered to streptozotocin-induced male diabetic rats for 28 consecutive days. At the end of treatment, liver was harvested and activity levels of various liver enzymes were determined. Levels of thiobarbituric acid reactive substances (TBARS) were measured in liver homogenates and liver histopathological changes were observed. *Results*. *V. vinifera* seed ethanolic extract was able to prevent the decrease in ICDH, SDH, MDH, and G-6-PDH and the increase in LDH activity levels in liver homogenates. The seed extract also caused serum levels of ALT, AST, ALP, ACP, GGT, and total bilirubin to decrease while causing total proteins to increase. Additionally, the levels of ALT, AST, and TBARS in liver homogenates were decreased. Histopathological changes in the liver were reduced. *Conclusion*. Near normal activity levels of various enzymes and histology of the liver following *V. vinifera* seed ethanolic extract administration may be due to decrease in liver oxidative stress in diabetes.

1. Introduction

Liver plays a central role in carbohydrate metabolism which function can be affected in diabetes [1]. Liver participates in the metabolic processes including glucose synthesis and storage [2]. The glycolytic and Krebs cycle enzymes play pivotal role in the ATP generation from glucose [3]. In glycolysis, few key liver enzymes are involved for example lactate dehydrogenase (LDH) (which converts pyruvate to lactate and *vice versa*) [4], isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (α-KDH), succinic dehydrogenase (SDH), fumarase, and malate dehydrogenase (MDH) which participate in interconversion of metabolites within the Krebs cycle [3]. Meanwhile, intermediary molecules formed in glycolytic pathway such as glucose-6-phosphate (G-6-PD) can be shunted into pentose phosphate (PPP) pathway and into the pathway that leads to glycogen or triglyceride (TG) syntheses involving 6-phospho-D-glucono-1,5-lactone. The latter process is catalyzed by glucose-6-phosphate dehydrogenase (G-6-PDH) enzyme [2]. In rats, the levels of carbohydrate metabolizing enzymes in the liver were decreased in diabetes [5].

The enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) which serve as biomarkers of hepatocyte damage are involved in various reactions in the liver. Plasma levels of AST and ALT were increased following hepatocyte injury while ALP, gamma glutamyl transferase (GGT), and total bilirubin levels were elevated in biliary tree obstruction [6]. Diabetes has been reported to induce pathological changes in the liver

[7] such as glycogen deposition, steatosis, and nonalcoholic steatohepatitis (NAFLD) which could ultimately lead to fibrosis and cirrhosis [8]. Clinical study has revealed that type 2 diabetic patients have higher incidence of liver function test abnormalities as compared to the healthy individuals [9].

Vitis vinifera (Linn.), which belongs to family Vitaceae, is one of the most widely grown fruit crops in the world. *V. vinifera* possesses wide range of pharmacological activities including inhibition of platelet aggregation and low density lipoprotein (LDL) oxidation [10], antidiabetic, antioxidant [11], antimicrobial [12], and anticarcinogenesis [13]. The seed extract has been reported to protect the liver against carbon tetrachloride- (CCl_4-) induced toxicity in rats [14]. So far, the effect of *V. vinifera* seed extract on diabetes-induced liver damage has not been fully revealed. In this study, we hypothesized that *V. vinifera* seed was capable of preserving activity levels of liver carbohydrate metabolizing enzymes and prevents liver damage in diabetes. We further hypothesized that the seed extract was able to reduce TBARS levels in the liver in diabetes. The aims of this study are therefore to investigate *V. vinifera* seed extract effect on liver carbohydrate metabolizing enzymes and other enzymes related to liver function, histopathological changes, and TBARS level in diabetes.

2. Materials and Methods

2.1. Chemicals and Reagents. Streptozotocin (STZ) and glibenclamide were purchased from Sigma Chemicals (St. Louis, MO, USA). ALT, AST, acid phosphatase (ACP), ALP, GGT, and total bilirubin estimation kits were purchased from Randox Laboratories Ltd. (Crumlin, County Antrim, UK). Other chemicals and reagents used in this study were of analytical grade.

2.2. Collection and Extraction of Plant Materials. The ripe fruits of *V. vinifera* (Bangalore Blue, Muscat variety) were collected from Tirupathi, Andhra Pradesh, India, during October 2012 and authenticated by Dr. K. Madhava Chetty, Botanist, Sri Venkateswara University, Tirupati, India. The seeds were deposited in Herbarium of Department of Botany, Sri Venkateswara University with the number 86783. The seeds were separated from pulp and shade dried. The dried powdered seed materials (2 kg) were subjected to maceration in 5 L of ethanol (95%) for 3 days at room temperature and filtered into a clean round bottom flask using an adsorbent cotton wool and a filter paper (Whatman no. A-1). The whole process was repeated five times to ensure maximum yield of ethanol soluble compounds from the seed powder. The combined ethanolic extract was concentrated at 40–50°C using a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) and lyophilized using a cryodos freeze dryer (Telstar, Barcelona, Spain) to yield 26.67 g of solid extract (1.28% W/W). These steps ensure complete evaporation of ethanol, leaving behind a solid mass [15].

2.3. Phytochemical Screening of V. vinifera Seed. The phytochemical composition of *V. vinifera* seeds was screened by using a standard method as described by Harborne [16]. The compounds analyzed include alkaloids, proteins, glycosides, tannin, steroids, phenol, lignins, saponins, monoterpenoids, flavonoids, carbohydrates, oils, and fats.

2.4. Animals. Male albino rats of Wistar strain with body weight between 175 to 200 g were procured from Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. The animals were maintained at room temperature of 25 ± 2°C and 12/12 hr light/dark cycle. Animals were given standard commercial rat chow diet (Harlan, UK) and tap water *ad libitum*. Experimental procedures were in accordance with ARRIVE guidelines (Animals in Research: Reporting In-Vivo Experiments) and European Community Guidelines/EEC Directive, 1986. This study was approved by the Faculty of Medicine, Animal Care and Use Committee, with ethics number: FIS/01/12/2013. Acute toxicity study was conducted according to Organization for Economic Cooperation and Development (OECD) revised up-and-down procedure for acute toxicity testing (OECD guideline 425) [17]. Thirty male Wistar rats were divided into five groups with each group received a single dose of 100, 500, 1000, and 3000 mg/kg bw of *V. vinifera* seed ethanolic extract. No signs of toxicity were observed at these tested doses.

2.5. Induction of Diabetes. Overnight (12 h) fasted animals were rendered diabetes via a single intraperitoneal (i.p) injection of a freshly prepared STZ (55 mg/kg bw) dissolved in 0.1 M citrate buffer (pH 4.5). STZ injected animals were given 5% glucose solution for 24 hr to overcome drug-induced hypoglycemia. Diabetes was confirmed by the presence of polydipsia, polyuria, and weight loss and only animals exhibiting fasting blood glucose (FBG) levels between 300–400 mg/dL three days following STZ injection were used [18]. Treatment was commenced on the fourth day of STZ injection which was considered as day one. *V. vinifera* seed ethanolic extract was administered orally at 250 and 500 mg/kg/day according to the previously reported doses [11], in a form of suspension in 1% sodium carboxy methyl cellulose (Na-CMC) in distilled water. The extract was administered by using oral gavage tube daily for 28 consecutive days.

2.6. Experimental Design. Rats were divided into five groups with six animals per group as follows: Group I, control rats, received 1% Na-CMC vehicle only; Group II, diabetic control rats, received 1% Na-CMC vehicle only; Group III, diabetic rats, was treated with *V. vinifera* seed ethanolic extract at 250 mg/kg bw; Group IV, diabetic rats, was treated with *V. vinifera* seed ethanolic extract at 500 mg/kg bw; and Group V, diabetic rats, was treated with standard drug, glibenclamide at 600 μg/kg bw as previously described [19].

At the end of 28-day treatment, animals were fasted overnight prior to sacrificed. Immediately after sacrificed, the liver was excised and was then stored at −80°C for later analysis or immediately used. In the meantime, blood was withdrawn via direct heart puncture and was then stored into tubes for serum analyses of total protein, total

Table 1: Effect of *V. vinifera* seed ethanolic extract on liver SDH, ICDH, MDH, LDH, and G-6-PPDH in streptozotocin-induced diabetic rats.

Parameters	Normal	Diabetic	Diabetic		
			250 mg/kg *V. vinifera*	500 mg/kg *V. vinifera*	600 μg/kg glibenclamide
SDH[#]	4.51 ± 0.08	2.15[*] ± 0.05	2.93[†] ± 0.06	3.29[†] ± 0.08	2.84[†] ± 0.07
ICDH[#]	0.78 ± 0.04	0.4[*] ± 0.04	0.57[†] ± 0.06	0.65[†] ± 0.07	0.68[†] ± 0.06
MDH[#]	0.62 ± 0.08	0.32[*] ± 0.08	0.48[†] ± 0.06	0.53[†] ± 0.08	0.56[†] ± 0.09
LDH[#]	0.94 ± 0.06	1.75[*] ± 0.08	1.26[†] ± 0.08	1.06[†] ± 0.07	1.18[†] ± 0.06
G-6-PDH[#]	1.78 ± 0.04	0.73[*] ± 0.06	1.27[†] ± 0.08	1.44[†] ± 0.05	1.38[†] ± 0.06

[#](μ moles of formazan formed/mg protein/h). Value represents mean ± SD for 6 rats per group. [*]$P < 0.05$ as compared to normal, nondiabetic rats group and [†]$P < 0.05$ as compared to nontreated diabetic rats.

bilirubin, ALT, AST, ACP, ALP, and GGT levels. Serum and liver homogenates were analyzed for the presence of ethyl glucoronide (EtG), a biomarker for ethanol consumption [20], using ELISA kit (Microgenics Corp., Thermo Fisher Scientific, Fremont, CA, USA). In all samples, no traces of EtG were detected.

2.7. Preparation of Liver Mitochondrial and Cytosolic Fractions. Liver was weighed and 10% tissue homogenate was prepared in phosphate buffer (0.1 M, pH 7.4) using a glass-Teflon homogenizer (Heidolph Silent Crusher M, Germany). Homogenates were centrifuged at 500 g at 4°C for 10 min. Supernatant was collected and recentrifuged at 2000 g for 10 min. Supernatant was again collected and recentrifuged at 12,000 g at 4°C for 10 min, and pellet was resuspended in 200 mM mannitol, 50 mM sucrose, 10 mmol/L Hepes-KOH (pH 7.4) and stored as mitochondrial fraction at −80°C. The final supernatant was taken and centrifuged for 1 hr at 40,000 g [21]. The resulting supernatant was used as cytosolic fraction and was stored at 4°C. In the present study, mitochondrial fraction was used to determine ICDH, SDH, and MDH enzymes activity levels while cytosolic fraction was used to determine LDH, G-6-PDH, ALT, and AST enzymes activity levels.

2.8. Estimation of Carbohydrate Metabolizing Enzymes Activity Levels. LDH (EC: 1.1.1.27) activity levels were measured following the method of Srikantan and Krishnamurti [22]. ICDH (EC: 1.1.1.41) activity levels were estimated according to the method of Kornberg and Pricer [23]. SDH (EC: 1.3.99.1) and MDH (EC: 1.1.1.37) activity levels were estimated according to the method of Nachlas et al. [24] while G-6-PDH (EC: 1.1.1.49) activity levels were measured according to the method of Bergmeyer and Bernt [25]. Enzyme activity levels were expressed as μmol of formazan formed/mg protein/hr.

2.9. Estimation of Liver Enzymes Levels in Serum and Liver Homogenates. The levels of AST (EC: 2.6.1.1) and ALT (EC: 2.6.1.2) in liver homogenates were estimated according to the method of Bergmeyer and Bernt [25]. Meanwhile, serum levels of AST, ALT, ALP, and total bilirubin were estimated according to the protocol of the manual of diagnostic kits (Randox Laboratories Ltd, Crumlin, UK).

2.10. Histopathological Changes of the Liver. Liver was excised immediately following sacrifice, washed with a phosphate buffer solution, and then fixed in 10% formalin. Tissues were dehydrated through graded series of alcohol, cleared in xylene, and embedded in paraffin wax. Tissues were then cut into sections of 5 μm in thickness using a microtome (Histoline laboratories, ARM-3600, Viabrembo, Milano, Italy) and stained with hematoxylin-eosin (H&E). Histopathological changes were examined under phase contrast microscope (Nikon H600L, Japan) and images were captured at magnification of 40× using a computer-assisted image analyzer (Nikon H600L, Nikon DS camera control Unit DS-U2, Version 4.4). Histopathological changes such as necrosis, sinusoidal hyperemia, and connective tissue inflammation of the portal region were scored by three independent observers following the description by Guven et al. [26] (0: normal, no changes, +: mild, ++: moderate, and +++: severe changes).

2.11. Estimation of TBARS Levels in Liver Homogenates. TBARS measures the malondialdehyde (MDA) levels, a lipid peroxidation (LPO) product present in the sample. Determination was made according to the method of Esterbauer and Cheeseman [27]. The rate of lipid peroxidation was expressed as μmoles of MDA formed/gram wet weight of tissue.

2.12. Statistical Analysis. The values were expressed as mean ± standard deviation (SD) ($n = 6$). Statistical analyses were performed by one way analysis of variance (ANOVA) and Student's t-test followed by *post hoc* statistical test. Significant difference was analyzed at P level <0.05.

3. Results

3.1. Phytochemical Screening. Preliminary phytochemical screening of the seed extract of *V. vinifera* revealed the presence of alkaloids, flavonoids, glycosides, saponins, steroids, lignins, phenols, tannins, and monoterpenoids (data was not shown).

3.2. Effects of V. vinifera Seed Ethanolic Extract on Liver LDH, ICDH, SDH, MDH, and G-6-PDH Activity Levels. Table 1 shows the effect of *V. vinifera* seed ethanolic extract on activity levels of liver carbohydrate metabolizing enzymes

Table 2: Effect of *V. vinifera* seed ethanolic extract on serum ALT, AST, ALP, ACP, GGT, total protein, total bilirubin, and liver ALT and AST in streptozotocin-induced diabetic rats.

Parameters	Normal	Diabetic	Diabetic		
			250 mg/kg *V. vinifera*	500 mg/kg *V. vinifera*	600 μg/kg glibenclamide
Serum					
ALT (U/L)	142.39 ± 6.32	236.18* ± 15.09	213.76† ± 9.18	184.13† ± 11.37	169.37† ± 9.43
AST (U/L)	103.78 ± 8.64	184.67* ± 7.15	145.91† ± 9.49	139.36† ± 9.62	125.17† ± 10.23
ALP (U/L)	46.75 ± 3.65	247.25* ± 8.46	166.92† ± 5.69	132.54† ± 3.73	112.73† ± 4.67
ACP (U/L)	11.54 ± 0.58	21.58* ± 0.84	18.64† ± 0.94	14.52† ± 0.87	12.68† ± 0.76
GGT (U/L)	10.23 ± 0.72	14.75* ± 0.14	8.65† ± 0.76	8.45† ± 0.89	8.79† ± 0.02
Total protein (U/L)	8.96 ± 0.75	4.22* ± 0.86	5.26† ± 0.34	7.45† ± 0.86	7.84† ± 0.73
Total bilirubin (U/L)	0.43 ± 0.05	4.58* ± 0.05	2.33† ± 0.06	1.58† ± 0.04	1.16† ± 0.05
Liver tissue					
ALT (μ moles of pyruvate formed/mg protein/h)	0.48 ± 0.08	0.78* ± 0.12	0.57† ± 0.13	0.52† ± 0.11	0.48† ± 0.09
AST (μ moles of pyruvate formed/mg protein/h)	0.37 ± 0.06	0.65* ± 0.09	0.56† ± 0.05	0.43† ± 0.09	0.36† ± 0.06

Value represents mean ± SD for 6 rats per group.

$^*P < 0.05$ as compared to normal, nondiabetic rats group and $^\dagger P < 0.05$ as compared to nontreated diabetic rats.

in different experimental groups. Our findings indicate that SDH activity was the highest followed by G-6-PDH, LDH, ICDH, and MDH. In diabetic rats, activity levels of ICDH, SDH, MDH, and G-6-PDH were significantly decreased while LDH activity level was markedly increased as compared to normal, nondiabetic rats. Administration of 250 mg/kg/day and 500 mg/kg/day *V. vinifera* seed ethanolic extract resulted in a significantly higher ICDH, SDH, MDH, and G-6-PDH activity levels and lower LDH activity levels as compared to nontreated diabetic rats. 500 mg/kg/day *V. vinifera* seed ethanolic extract had an almost similar potency to glibenclamide in preventing the changes in LDH, SDH, G-6-PDH, ICDH, and MDH activity levels in diabetic rats liver.

3.3. Effects of V. vinifera Seed Ethanolic Extract on Serum Levels of ALT, AST, ALP, ACP, and GGT. Table 2 shows the effect of *V. vinifera* seed extract or glibenclamide on serum ALT, AST, ALP, ACP, and GGT in different experimental groups. In nontreated diabetic rats, the levels of ALT, AST, ALP, ACP, and GGT were significantly higher than normal, nondiabetic rats. Treatment with 250 mg/kg/day and 500 mg/kg/day of the seed extract or glibenclamide resulted in lower serum level of these enzymes as compared to nontreated diabetic rats. 500 mg/kg/day *V. vinifera* seed had lesser effect than glibenclamide in preventing the increase in serum ALT, AST, ALP, and ACP levels in diabetic rats.

3.4. Effects of V. vinifera Seed Ethanolic Extract on Serum Levels of Bilirubin and Total Protein. Table 2 shows changes in total protein and bilirubin levels in the serum of different experimental groups. Our findings indicate that total protein levels were lower while total bilirubin levels were markedly higher in diabetic rats as compared to normal, nondiabetic rats. Treatment with 250 mg/kg/day and 500 mg/kg/day *V. vinifera* seed extract or glibenclamide resulted in higher total protein but lower total bilirubin levels as compared to nontreated diabetic rats. 500 mg/kg/day *V. vinifera* seed extract had lesser effect than glibenclamide in preventing the decrease in total protein and the increase in total bilirubin levels in the liver of diabetic rats.

3.5. Effect of V. vinifera Seed Ethanolic Extract on Liver ALT and AST Levels. Table 2 shows the levels of ALT and AST in liver homogenates of different experimental groups. Our findings indicate that the levels of these enzymes were markedly increased in diabetic rats as compared to normal, nondiabetic rats. Treatment with 250 mg/kg/day and 500 mg/kg/day *V. vinifera* seed extract or glibenclamide resulted in lower ALT and AST levels as compared to nontreated diabetic rats. 500 mg/kg/day *V. vinifera* seed produced a slightly lesser effect than glibenclamide in preventing the increase in ALT and AST levels of the liver of diabetic rats.

3.6. Effect of V. vinifera Seed Extract on Histopathological Changes of the Liver. Figure 1 shows histopathological changes while Table 3 shows semiquantitative analyses of inflammatory changes in the liver of diabetic rats receiving *V. vinifera* seed extract or glibenclamide treatment. In normal, nondiabetic rats, a distinct and well-arranged hepatocytes, sinusoids, and central vein could be seen (Figure 1(a)). Meanwhile, in diabetic rats, hepatocytes were disorganized with several areas of necrosis. Sinusoids were enlarged with the wall of veins thickened (Figure 1(b)). *V. vinifera* seed extract or glibenclamide treatments prevented these changes

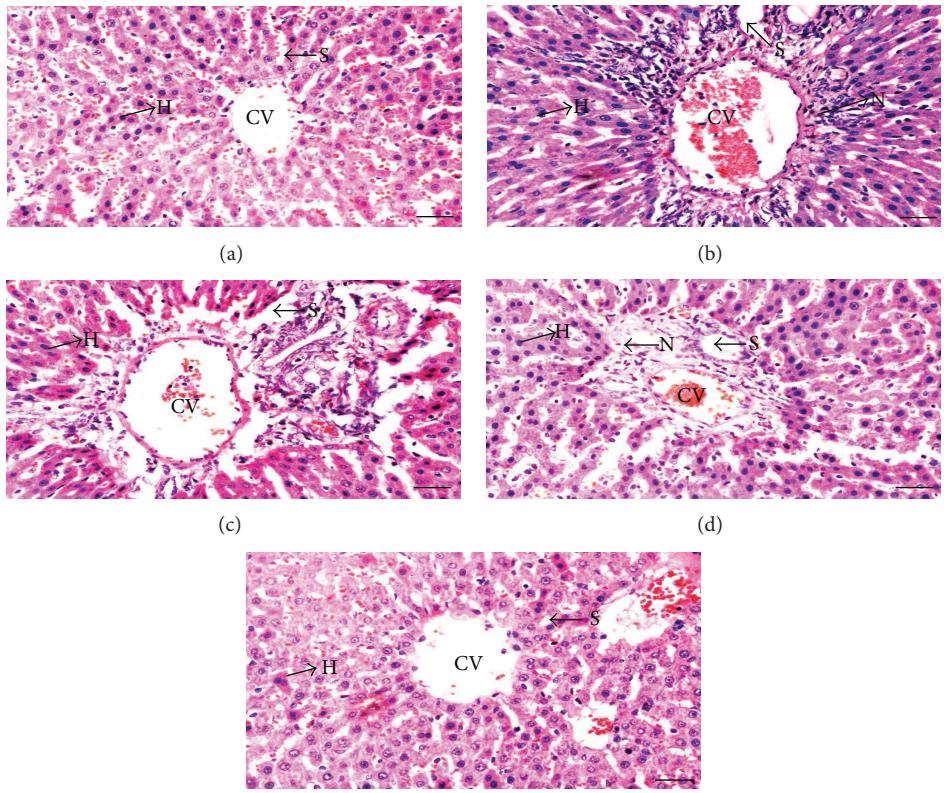

Figure 1: Effect of the seed ethanolic extract of *V. vinifera* on liver histology. Representative images of the liver in (a) normal, (b) STZ-induced diabetic rats, (c) diabetic rats treated with 250 mg/kg/day *V. vinifera* seed extract, (d) diabetic rats treated with 500 mg/kg/day *V. vinifera* seed extract, and (e) diabetic rats treated with 600 μg/kg/day glibenclamide. Images were taken under 20× magnification. Scale bar represents 50 μm. In diabetic rats, several areas of moderate to severe necrosis could be seen around the central vein. Mild to absence of necrotic changes could be seen following treatment with 500 mg/kg/day *V. vinifera* or glibenclamide to diabetic rats. H = hepatocytes, CV = central vein, S = sinusoid, and N = necrosis (40× magnification).

Table 3: Semiquantitative analyses of histopathological changes of the liver.

Parameters	Normal	Diabetic	Diabetic		
			250 mg/kg *V. vinifera*	500 mg/kg *V. vinifera*	600 μg/kg glibenclamide
Necrosis	0	++	+	+	+
Sinusoidal hyperemia	0	++	++	+	0
Connective tissue inflammation in portal region	0	++	+	0	0

0: no changes, +: mild changes, ++: moderate changes, and +++: severe changes.

as evidenced by lesser signs of necrosis, lack of central hemorrhagic necrosis, mild sinusoid hyperemia, and mild connective tissue inflammation in the portal region (Figures 1(c), 1(d), and 1(e)).

3.7. TBARS Levels in Liver Homogenates. In Figure 2, TBARS levels in liver homogenates in nontreated diabetic rats liver were markedly higher as compared to normal, nondiabetic control rats ($P < 0.05$). Administration of 250 and 500 mg/kg *V. vinifera* seed extract or glibenclamide resulted in a significant decrease in the level of liver TBARS as compared to nontreated diabetic rats.

4. Discussion

Chronic hyperglycemia and insulin deficiency can produce various disruptions to the metabolic processes in the liver. Additionally, diabetes has also been reported to cause liver

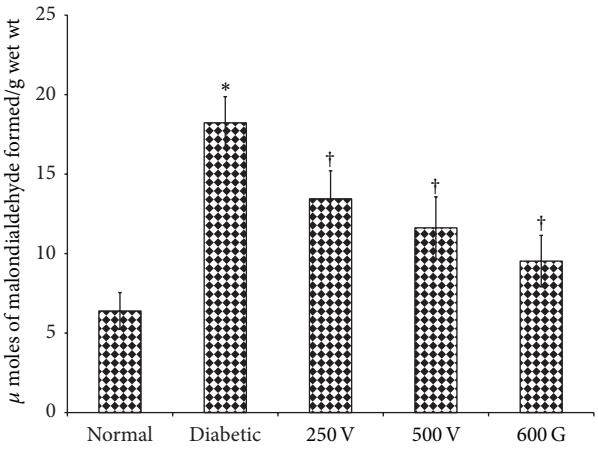

Figure 2: Effect of seed ethanolic extract of *V. vinifera* on TBARS levels. A significantly higher liver TBARS levels were observed in diabetic rats as compared to normal, nondiabetic rats. Administration of glibenclamide or seed ethanolic extract of *V. vinifera* prevented the increase in TBARS levels in diabetic rats. 250 V: 250 mg/kg/day *V. vinifera* seed extract; 500 V; 500 mg/kg/day *V. vinifera* seed extract; 600 G: 600 μg/kg/day glibenclamide. $n = 6$, $^{*}P < 0.05$ as compared to normal, nondiabetic rats, and $^{\dagger}P < 0.05$ as compared to nontreated diabetic rats.

damage [28]. In the present study, orally administered ethanolic seed extract of *V. vinifera* from Muscat variety to diabetic rats was able to prevent the decrease in activity levels of key enzymes involved in liver carbohydrate metabolism which include the G-6PDH, ICDH, SDH, and MDH. We have shown that administration of the seed extract to diabetic rats prevented hepatocyte destruction as evidenced from near normal serum levels of ALT, AST, ALP, ACP, GGT, and total bilirubin. The total protein level in the serum was also maintained near normal following supplementation with the seed extract.

In this study, activity levels of liver mitochondrial enzymes (ICDH, SDH, and MDH) were markedly reduced in diabetes. These enzymes are involved in ATP generation which yielded 36 moles of ATPs per mole of glucose [3]. SDH and MDH are the two Krebs cycle enzymes where the former has the highest activity as compared to other enzymes in the cycle [29]. In diabetes, activity of Krebs cycle enzymes was lower than normal [30, 31], resulting in impairment of ATP generation. These may compromise the liver biosynthetic, degradation, and detoxification functions. However, despite of diabetes-induced decrease in activity levels of liver Kreb cycle enzymes, LDH activity level was markedly increased. Similar findings were reported by others [29, 32]. LDH is the terminal glycolytic enzyme involved in pyruvate interconversion to lactate to produce energy under anaerobic condition [3]. The significance of LDH increase in diabetes is unknown; however this could be related to lower amount of insulin as insulin has been reported to affect the activity of LDH [33]. Recent evidence indicated that increased in cellular activity of LDH in diabetes was due to increase in peroxide (H_2O_2) levels [34]. Ability of the seed extract to lower the free radical levels in diabetes could explain the decrease in hepatic LDH activity levels; however this needs to be confirmed. In our study, lower activity levels of G-6-PDH were observed in the liver of diabetic rats which was consistent with other findings [29, 35]. G-6-PDH is a highly specific enzyme involved in NADPH generation in the pentose phosphate pathway [2]. Activity levels of pentose phosphate and glycolytic pathways enzymes were reported to decrease in experimental diabetic animals [36]. Besides liver, PPP enzymes activity levels in the brain were also reported to reduce in STZ-induced diabetic rats [37].

This study has provided evidences of hepatocyte damage from elevated levels of liver enzymes (ALT, AST, ACP, ALP, and GGT) in serum and liver homogenates in diabetes. The increase in serum levels of ALT and AST indicates hepatocellular injury where these enzymes were released into the circulation while the elevated levels of serum GGT and ALP indicates biliary tree obstruction most likely due to edematous compression. The serum levels of total bilirubin were also increased indicating either intra- or extrahepatic biliary tree obstruction. These findings were consistent with others who reported the rise in serum ALT [38], AST [39], ACP [40], GGT, and AST [41] in diabetic rodents and humans. In this study, the levels of AST and ALT were significantly higher than ALP consistent with the reports of a highly elevated ALT level in patient with type 2 diabetes [9].

The deranged liver function test (LFT) parameters in both serum and liver homogenates in diabetic rats indicative of hepatocellular damage were supported by histopathological changes of the liver as featured by moderate to severe necrosis. The structural changes might cause compression of the biliary trees, resulting in the rise in serum levels of ALP, GGT, and total bilirubin in diabetic animals. Administration of *V. vinifera* seed prevented the histopathological changes in the liver as well as lowered the serum levels of liver enzymes and total bilirubin. Lesser signs of necrosis, hyperemia, and connective tissue inflammation were seen in the liver of *V. vinifera*-treated diabetic rats. An elevated level of serum total proteins following *V. vinifera* seed extract treatment to diabetic rats suggested that function of the liver was restored to near normal following an insult caused by diabetes.

Our findings indicated that TBARS levels in the liver homogenates were reduced following *V. vinifera* seed ethanolic extract administration to diabetic rats. Previous study has shown that the red grape seed (*V. vinifera* variety Burgund mare) reduces the oxidative stress level in diabetic rats as evidence from the decrease in TBARS levels [42]. Phytochemical screening showed that the seed extract contains flavonoids and phenols, the two compounds known to possess antioxidant activities [43]. Therefore, the ethanolic extract of *V. vinifera* seed could help to reduce the levels of oxidative stress in the liver of diabetic rats most likely via scavenging the free radicals that are highly elevated in diabetes [44]. In addition, flavonoid may help to improve the activity of carbohydrate metabolizing enzymes in the liver from an observation that high flavonoid-rich green tea improves the activity of hepatic carbohydrate metabolizing enzymes [1]. Further works are needed to better characterize the active compounds present in *V. vinifera* seed that are

responsible for reducing the oxidative stress level in diabetes and to identify the levels of endogenous antioxidant enzymes following seed extract administration. Additionally, *in vitro* antioxidant assay of the seed extract will also be carried out in order to support its claimed free radicals scavenging activity.

5. Conclusions

Our study has shown for the first time the effect of ethanolic seed extract of Muscat variety of *V. vinifera* against deterioration of activity levels of key enzymes involved in liver carbohydrate metabolism in diabetes. The seed extract helps to prevent liver damage due to oxidative stress which may contribute towards improvement in liver function and histology. Further study will include identifying the effect of the seed extract on other key carbohydrate metabolizing enzymes such as hexokinase, pyruvate kinase, glycogen synthase, and phosphorylase. Taken into account various limitations, this study provides preliminary evidence that the seed extract of a Muscat variety of *V. vinifera* helps in liver protection in diabetes.

Conflict of Interests

The authors reported no conflict of interests in this study.

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Review Article

Phytochemical, Phytotherapeutical and Pharmacological Study of *Momordica dioica*

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Momordica dioica is a perennial, dioecious, cucurbitaceous climbing creeper (commonly known as kakrol, spiny gourd or teasle gourd). It is native to Asia with extensive distribution in India and Bangladesh. It is used not only as preventive and curative agent for various diseases but also as vegetable with a significant nutritional value over thousands of years. This review aims to take an attempt to evaluate the phytochemical, ethnobotanical, phytotherapeutical and pharmacological properties of kakrol according to the view of traditional medicinal plant based treatment including ayurveda along with recent scientific observations. Kakrol is considered as an underutilized vegetable, although having significant presence of certain compounds containing higher nutritional value than many frequently consumed vegetables. Moreover, as a traditional medicinal plant, it is still potential for its phytochemical components that increase the demand of further extensive evaluation to justify its other therapeutical roles. Therefore, this effort will be helpful to researchers who interested to disclose the unjustified phytotherapeutical role of Momordica dioica.

1. Introduction

Momordica dioica Roxb. is a perennial, dioecious ($2n = 28$) climber included in Cucurbitaceae family (Figure 1). Momordica genus contains about 80 species [1, 2]. According to the latest revision of Indian *Momordica*, there are six well identified species of which four are dioecious and two are monoecious [3]. Although this genus is originated from Indo-Malayan region, it is now found to grow in India, Bangladesh, Srilanka, Myanmar, China, Japan, South East Asia, Polynesia, Tropical Africa, and South America [4, 5]. Its cultivation up to an altitude of 1500 meters in Assam and Garo hills of Meghalaya is reported [6]. It is commonly known as spine gourd, teasel gourd or small bitter gourd worldwide whereas in Bangladesh it is known as kakrol and in India as kankro, kartoli, kantoli, kantola, kantroli, ban karola, or janglee karela [7–10]. Kakrol is about 5–7 meters in length, a popular summer vegetable of which its fruit, young twigs and leaves are used as vegetable [11–13].

2. Phytochemical and Nutrient Study

The fruit of *Momordica dioica* contains ashes: 9.1%, crude protein: 5.44%, crude lipid: 3.25%, crude fiber: 22.9%, and carbohydrate: 59.31%. Its fruit has high energy value (288.25 kcal/100 g) in dry weight. Its mineral ranges (mg/100 g dry weight,) are: potassium (4.63), sodium (1.62), calcium (7.37), iron (5.04), and zinc (3.83) [14]. In another investigation, its nutritional value of per 100 g edible fruit is reported to contain 84.1% moisture, 7.7 g carbohydrate, 3.1 g protein, 3.1 g fat, 3.0 g fiber and 1.1 g minerals and small quantities of essential vitamins like carotene, thiamin, riboflavin and niacin [15].

Ali and Deokule evaluated some of its micronutrient and secondary metabolites as follows: calcium: 0.5 mg/g, sodium: 1.5 mg/g, potassium: 8.3 mg/g, iron: 0.14 mg/g, zinc: 1.34 mg/g, protein: 19.38%, fat: 4.7%, total phenolic compound: 3.7 mg/g, phytic acid: 2.8 mg/g, and ash value: 6.7% [16]. Moreover, its fruit is recommended as nutritionally

Figure 1: *Momordica dioica* (Photo credit: https://www.flickr.com/, http://www.tropicalfruitandveg.com/).

rich source of protein and good source of lipid, crude fiber, carbohydrate, iron, calcium, phosphorous. Additionally, it is the highest amount of carotene (162 mg/100 g of edible portion) container amongst the cucurbitaceous vegetables [17–19]. The ash content is reported as 3-4% containing a trace of manganese [20].

Tirmizi et al. screened it as a potential source of chromium and zinc [21]. Whereas, *Momordica dioica* (peeled) contained 0.27 mg/kg of chromium and 4.91 mg/kg of zinc, *Momordica dioica* (unpeeled) contained 0.26 mg/kg of chromium and 11.0 mg/kg of zinc. The protein content of leaves and dry weight of aerial plant parts remained higher in male as compared to female defruited and monoecious plants [22]. The fruit contains higher amount of ascorbic acid and iodine [23, 24]. The presence of secondary metabolites of fruit including alkaloids, steroids, triterpenoids, and saponins was determined [25]. Among them, four compounds were isolated from ethyl acetate extract and five compounds were isolated from methanol extract consisting of alkaloids and flavonoids with NH and C=O functional groups, respectively. The alkaloids present in seed and root were called momordicin and *Momordica foetida*, respectively [26]. Phytochemical investigations summurized in Table 1 also showed the presence of lectins, β-sitosterol, saponin glycosides, triterpenes of ursolic acid, hederagenin, oleanolic acid, α-spinasterol, stearic acid, gypsogenin, momodicaursenol, and three new compounds named 3β-o-benzoyl-11-oxo-ursolic acid, 3β-o-benzoyl-6-oxo-ursolic acid, and 3-o-β-D-glucuronopyranosyl gypsogenin [27–32].

3. Ethnobotanical and Phytotherapeutical Study

According to Ayurveda (Table 2), not only its fruits have diuretic, laxative, hepatoprotective, antivenomous, antihypertensive, anti-inflammatory, antiasthmatic, antipyretic, antileprosy, antidiabetic, and antidepressant properties but also its leaves have antihelminthic, aphrodisiac, antihemorroidal, hepatoprotective, antibronchitic, antipyretic, antiasthmatic, and analgesic properties [33, 34]. Fresh fruit juice and cooked fruit in small amount of oil are prescribed for hypertension and diabetes, respectively. Oral administration of 50 mL of root juice is advised once a day with empty stomach to beat diabetes. The juice of root is a domestic remedy for the inflammation caused by contact with the urine of the house lizard. The juice of the leaves are mixed with coconut, pepper, red sandalwood, and so forth in order to form an ointment and applied to the head to relieve pain. Dried fruit powder applied into the nostrils produces a powerful errhine effect and provokes a copious discharge from the schneiderian mucous membrane [35]. Root juice has stimulant, astringent, antiseptic, antidiabetic, anti-inflammatory, and antiulcerant effect. The mucilaginous tubers act as antihelminthic, spermicidal, and antifertility abortifacient agent [36]. The root of the male plant is used in snake bites and scorpion sting [37]. The superficial use of root paste over the whole body is believed to act as a sedative in high fever with delirium [38, 39]. Beside the superficial and oral administration of leaf paste for skin disease, tender fruits are rubbed on skin for pimples and acne and roasted seeds are used for eczema and other skin problems [40]. Root powder is also applied for softening skin and reducing perspiration. The protective role of the leaves against chronic skin diseases is also reported. A preparation called "Panchatikta ghrita" is made by boiling 800 g each of neem bark, leaves of *Momordica dioica*, *Solanum surattense*, *Tinospora cordifolia,* and bark of *Adhatoda vasica*, in 5-6 liters of water up to its reduction to quarter and then adding of 3.5 liters of butter and about 3 kg myrobalans and is recommended as one tablespoonful with little hot milk internally twice daily in chronic skin diseases [41]. Mucilaginous tuber of female plant and toasted root are used in bleeding piles and bowel infections. The traditional use of *Momrdica dioica* against bleeding piles (hemorrhoids) is also reported [42, 43].

4. Pharmacological Study

4.1. Antioxidant Activity. Compounds derived from natural sources are capable of providing protection against free radicals [44]. The alcoholic extract inhibited the formation of oxygen derived free radicals (ODFR) *in vitro* with 4000 μg/mL ascorbic system [45]. In another work, the free radical scavenging potential of the tuberous roots was studied by different *in vitro* methods, namely, DPPH radical scavenging, ABTS radical scavenging, iron chelating activity, total antioxidant capacity, and haemoglobin glycosylation assay. Total antioxidant capacity of ethanolic extract was found to be 26 μg/mL which is equivalent to ascorbic acid.

Table 1: Nutrient and phytochemical study of *Momordica dioica* as described in this paper.

Plant part	Classification	Compound	Extract or preparation	Reference
Fruit	Crude protein	—	Quantitative analysis showed 5.44%	[14]
	Protein	—	Quantitative analysis showed 3.1/100 g	[15]
		—	Quantitative analysis showed 19.38%	[16]
	Crude lipid	—	Quantitative analysis showed 3.25%	[14]
	Fat	—	Quantitative analysis showed 3.1/100 g	[15]
		—	Quantitative analysis showed 4.7%	[16]
	Crude fiber	—	Quantitative analysis showed 22.9%	[14]
	Carbohydrate	—	Quantitative analysis showed 59.31%	[14]
		—	Quantitative analysis showed 7.7/100 g	[15]
	Niacin	—	Not specified	[15]
	Thiamin	—	Not specified	[15]
	Carotene	—	Not specified	[15]
		—	Quantitative analysis showed 162 mg/100 g of edible portion	[18, 19]
	Ascorbic acid	—	Not specified	[24]
	Potassium	—	Quantitative analysis showed 4.63 mg/100 g dry weight	[14]
		—	Quantitative analysis showed 8.3 mg/g	[16]
	Sodium	—	Quantitative analysis showed 1.62 mg/100 g dry weight	[14]
		—	Quantitative analysis showed 1.5 mg/g	[16]
	Calcium	—	Quantitative analysis showed 7.37 mg/100 g dry weight	[14]
		—	Quantitative analysis showed 0.5 mg/g	[16]
	Iron	—	Quantitative analysis showed 5.04 mg/100 g dry weight	[14]
		—	Quantitative analysis showed 0.14 mg/g	[16]
	Zinc	—	Quantitative analysis showed 3.83 mg/100 g dry weight	[14]
		—	Quantitative analysis showed 1.34 mg/g	[16]
		—	Not specified	[21]
		—	Quantitative analysis showed 4.91 mg/kg (peeled), 11.0 mg/g (unpeeled)	[22]
	Manganese	—	Not specified	[20]
	Iodine	—	Not specified	[23]
	Chromium	—	Quantitative analysis showed 0.27 mg/kg (peeled), 0.26 mg/kg (unpeeled)	[22]
		—	Not specified	[21]
	Phytic acid	—	Quantitative analysis showed 2.8 mg/g	[16]
	Total phenolic compound	—	Quantitative analysis showed 3.7 mg/g	[16]
	Alkaloids	—	Identified in ethyl acetate, methanol extract	[25]
	Flavonoid	—	Identified in methanol, hexane extract	[25]
	Steroids	—	Identified in ethyl acetate, methanol, aqueous extract	[25]
	Saponins	—	Identified in methanol, aqueous extract	[25]
	Triterpenoids	—	Identified in ethyl acetate, methanol, aqueous extract	[25]
Seed	Alkaloid	Momordicin	Identified in seed oil	[26]
	Lectin	Anti-H-Lectin	Not specified	[30]

Table 1: Continued.

Plant part	Classification	Compound	Extract or preparation	Reference
Root	Alkaloid	*Momordicafoetida*	Not specified	[26]
	Stearic acid	—	Identified in methanol extract	[31]
	Steroid	α-spinasterol octadecanoate	Identified in methanol extract	[31]
		α-spinasterol-3-O-β-D-glucopyranoside	Identified in methanol extract	[31]
	Triterpenoid	Oleanolic acid	Identified in methanol extract	[32]
		Gypsogenin	Identified in methanol extract	[32]
		Hederagenin	Identified in methanol extract	[32]
		3β-O-benzoyl-6-oxo-ursolic acid	Identified in methanol extract	[32]
		3β-O-benzoyl-11-oxo-ursolic acid	Identified in methanol extract	[32]
		3-O-β-D-glucopyranosyl hederagenin	Identified in methanol extract	[31]
		3-O-β-D-glucopyranosyl gypsogenin	Identified in methanol extract	[31]
		3-O-β-D-glucuronopyranosyl gypsogenin	Identified in methanol extract	[31]

Moreover, its ethanol extract showed percentage inhibition of haemoglobin glycosylation as 66.63 and 74.14 at conc. of 500 and 1000 μg/mL, respectively, while that of standard DL α-tocopherol was 61.53% and 86.68% inhibition at same concentration [46]. The antioxidant activities of methanol and aqueous extract of fruits were analyzed and the presence of phenolic compounds, flavonoids, sterol, alkaloids, amino acids, and so forth, were found [47]. Among those compounds, due to the presence of flavonoids, its fruit was reported as a potent antioxidant [48].

4.2. Analgesic Activity. Ilango et al. and Vaidya and Shreedhara reported that both hexane extract and soluble portion of methanolic extract of *Momordica dioica* fruit pulp exhibited analgesic activity when compared to standard drug [49, 50]. Petroleum ether, ethyl acetate, and methanol extracts exhibited significant analgesic activity in acetic acid induced writhing syndrome when compared to the vehicle treated control group. But among them petroleum ether and methanol extract gave more significant analgesic activity than ethyl acetate extract [51].

4.3. Nephroprotective Activity. The ethanol extract of seeds was screened and marked nephroprotective as well as curative activities was found without any toxicity caused by nephrotoxin-like gentamicin [52]. The nephroprotective and curative activities of its fruit extract ware also observed [53]. Gupta et al. evaluated the renal protective effect of *Momordica dioica* extract in streptozotocin-diabetic rats [54].

4.4. Neuroprotective Activity. The effect of methanol and aqueous extract of fruit pulp was observed on the central nervous system by using neuropharmacological experimental models in mice. These extracts were used for a dose-dependent reduction of the onset and duration of a reduction in locomotor activity. It was suggested that methanol and aqueous extract of fruit pulp (100 mg/kg and 200 mg/kg) had neuroprotective activities [55].

4.5. Antiallergic Activities. The antiallergic activity of its extract in mice was observed [56]. The alcoholic extract was evaluated and its efficacy to inhibit passive cutaneous anaphylaxis was found in mouse and rat [57].

4.6. Antiulcer Activity. Vijayakumar screened *Momordica dioica* extract mediated antiulcerogenic effect on ethanol-induced ulcer model of rat. A significant decrease occurred in the level of H^+-K^+ATPase, volume of gastric juice, and acid output. Gastric wall mucus, p^H, and catalase enzyme were increased significantly but antioxidant enzyme levels of superoxide dismutase were decreased [58]. Its gastroprotective and ulcer healing activities were also observed by Vijayakumar et al. [59].

4.7. Anticancer Activity. Luo et al. showed that the $CHCl_3$ extract of roots and five isolated constituents had anticancer activity during pharmacological testing on cancer cell (L1210). The growth inhibitory index (%) of α-spinasterol-3-o-β-D-glucopyranoside was shown to be 50%, at the dose of 4 μg/mL [31].

4.8. Antimicrobial Activity. Shrinivas et al. studied methanolic extract and aqueous extract of fruit and found that methanolic extract had more promising antimicrobial activity [47]. Arekar et al. screened antibacterial activities of ethyl acetate extract. The concentration of 200 μg/disc was more active against *E. coli* compared to *S. aureus, S. paratyphi*, and *P. mirabilis* bacteria. Ethyl Acetate extract of *in vitro* shoot culture (yield: 0.26%) showed maximum inhibition zone against *S. paratyphi* and *P. mirabilis* while ethyl acetate extract of *in vitro* callus culture (yield: 21.5%) showed maximum inhibition zone against *S. aureus* [60]. On the other hand,

Table 2: Ethnobotanical use of *Momordica dioica* as described in this paper.

Plant's part	Ethnobotanical use	Preparation or Mode of use	Reference
Fruit	Hypertension	Fresh fruit juice	[35]
	Diabetes	Cooked fruit in small amount of oil	[35]
	Pimple and acne protectant	Tender fruits are rubbed on skin for pimples and acne	[40]
	Diuretic	Not specified	[33, 34]
	Laxative	Not specified	[33, 34]
	Hepatoprotective agent	Not specified	[33, 34]
	Antihypertensive	Not specified	[33, 34]
	Anti-inflammatory agent	Not specified	[33, 34]
	Antipyretic	Not specified	[33, 34]
	Antivenomous agent	Not specified	[33, 34]
	Antiasthmatic agent	Not specified	[33, 34]
	Antidepressant	Not specified	[33, 34]
	Antileprosy agent	Not specified	[33, 34]
Root	Diabetes	Oral administration of 50 mL of root juice is advised once a day with empty stomach.	[35, 36]
	Anti-inflammatory agent	The juice of the root is a domestic remedy for the inflammation caused by contact with the urine of the house lizard.	[35, 36]
	Stimulant	Root juice	[36]
	Antiseptic	Root juice	[36]
	Antiulcerant	Root juice	[36]
	Antitoxic agent	The root of the male plant uses in snake bites and scorpion sting	[37]
	Antipyretic	The root paste smearing over the whole body act as a sedative fever with delirium	[38, 39]
	Skin softening agent	Root powder is applied for softening skin	[41]
	Antiperspirant	Root powder is applied for reducing perspiration.	[41]
	Antihemorroidal agent	Toasted roots are used in bleeding piles	[42, 43]
	Bowel infection reducer	Toasted roots are used in bowel infections	[42]
Mucilaginous tuber	Antihelminthic agent	Not specified	[36]
	Spermicidal agent	Not specified	[36]
	Antifertility agent	Not specified	[36]
	Antihemorroidal agent	Mucilaginous tuber of female plant are used in bleeding piles	[42, 43]
	Bowel infection reducer	Mucilaginous tuber of female plant are used in bowel infections	[42]
Seed	Eczema protectant	Roasted seeds are used for eczema and other skin problems	[40]
Leaf	Analgesic	Leaf juice is mixed with coconut, pepper, red sandalwood, and so forth in order to form an ointment to relieve pain.	[35]
	Antihelminthic	Not specified	[33, 34]
	Antihemorroidal	Not specified	[33, 34]
	Antibronchitic	Not specified	[33, 34]
	Skin disease reducer	A preparation called "Panchatikta ghrita" is made by boiling 800 g each of neem bark, leaves of *Momordica dioica, Solanum surattense, Tinospora cordifolia*, and bark of *Adhatoda vasica*, in 5-6 liters of water up to its reduction to quarter and then the addition of 3.5 liters of butter and 3 kg myrobalans, is recommended as one tablespoonful with little hot milk internally twice daily in chronic skin diseases	[40, 41]

Singh et al. found its no promising antimycobacterial activity [61].

4.9. Antidiabetic Activity. Antidiabetic specifically oral hypoglycemic effects of *Momordica dioica* in rat model was screened by Fernandopulle et al. [62]. Reddy et al. and Singh et al. showed aqueous, chloroform, ethyl acetate and ethanolic extract of fruit mediated antidiabetic activity in alloxan induced experimental rats [63, 64]. Moreover, Sharma and Arya reported ethyl acetate and ethanol extract containing steroids, triterpenoids had potential role in alloxan-induced diabetic rats and broadly type 2 diabetes [65]. Gupta et al. investigated the antidiabetic and renal protective effect of *Momordica dioica* methanolic extract (MDMtE) in streptozotocin-treated diabetic rats. MDMtE treatment markedly reduced serum glucose and increased serum insulin and urea levels. Furthermore, histologic observation of kidney of diabetic rats showed degenerative changes in glomerulus and renal tubules [54].

4.10. Antimalarial Activity. Misra et al. screened alcoholic extract *in vivo* and *in vitro* for antimalarial effect against NK65 strain of *Plasmodium berghei*, *Jurinea macrocephala*, and *Aegle marmelos* and found them to possess schizontocidal activity [66].

4.11. Anti-Inflammatory Activity. The anti-inflammatory effect of the alcoholic extract of roots was evaluated during CCl_4 induced hepatotoxicity [45]. Ilango et al. evaluated both hexane extract and methanolic extract of fruit pulp mediated anti-inflammatory activities [49].

4.12. Hepatoprotective and Antihepatotoxic Activity. CCl_4 induced hepatotoxicity prevention of methanol extract of *Momordica dioica* was observed by Chaudhary et al. [67]. Although Govind reported the hepatoprotective and antihepatotoxicity effect of leaf, Kumar et al. specifically mentioned the role of aqueous and methanol extract of leaves against it [68, 69]. Jain et al. examined leaf as a potent hepatoprotective agent against CCl_4 induced hepatic damage in rats by *in vivo* antioxidant and free radical scavenging activities. They were positive for both ethanolic and aqueous extracts although ethanolic extract was found more potent hepatoprotective [48]. Kushwaha et al. evaluated the flavonoidal fraction from ethanolic extract of fruit mediated hepatoprotective activity in wistar strain of albino rats of either sex against CCl_4 induced hepatic damage [70]. Rakh et al. reported that the alcoholic extract of roots significantly reduced CCl_4 induced hepatotoxicity in rats by inhibiting the formation of radicals *in vitro* [56]. The saponin fraction of *Momordica dioica* (27.5 and 55 mg/kg) administered to the CCl_4 treated rats to protect the liver cells from liver damages on hepatocytes and silymarin (100 mg/kg), a well-known natural antihepatotoxic drug was used as standard [71]. The hexane extract and ethyl acetate soluble fraction of the methanolic extract of the fruit pulp at a dose of 400 mg/kg administered for 7 days in rat exhibited a significant therapeutic effect [72]. Sato et al. observed significant lowering of liver cholesterol and triacylglycerol levels in rats. Fecal lipid excretion was increased and lymphatic transport of triacylglycerol and phospholipids were decreased in rats which were fed the kakrol after permanent lymph cannulation. Moreover, *n*-butanol extract caused a significant reduction in the pancreatic lipase activity *in vitro* and liver lipids by inhibiting lipid absorption [73].

4.13. Antifertility Activity. Shreedhar et al. reported the antifertility activity of ethanolic and aqueous extract of *Momordica dioica* root. The extracts showed moderate estrogenic activity and caused significant increase in uterine weight. Moreover, at a dose of 200 mg/kg, aqueous extract showed 83% and ethanolic extract showed 100% abortifacient activity [74]. Kudaravalli evaluated the ethanolic extract of fruit mediated antifertility activities of female rats but found no male antifertility activity at the dose of 250 mg/kg [75].

4.14. Antiedemic Activity. Shreedhara and Vaidya administered the alcoholic extract orally which significantly reduced carrageenan-induced paw edema. The activity was compared with ibuprofen (200 mg/kg) [45].

4.15. Antifeedant, Insecticidal, Grain Protectant, and Allelopathic Activity. Mishra et al. reported the role of *Momordica dioica* seed oil as insecticide and found satisfactory level of natural insecticidal activity up to 100% mortality at 4% conc. in 24 hours. Moreover, its lower conc. up to 2% was found to be effective but for 100% mortality longer time was required. They suggested the presence of alkaloid momordicin in oil was responsible for it [76]. In another work, Mishra et al. evaluated its seed oil's potential as grain protectant against *Callosobruchus chinensis* upon the stored legume-pulse grain. It was applied as a dose of 6–8 mL/kg to legume pulse grain sample for 60 days. As a result, appeared degree of dehusking was increased (%) from 40.00 to 72.59, 59.88 to 92.44, 63.39 to 87.50 and 57.00 to 79.43 for Pigeon pea (*Canjanas cajan*), Chickpea (*Cicer arietinum*), Urdbean (*Phaseolus mungo*), Mungbean (*Phaseolus radiatus*), respectively [77]. Narasimhan et al. and Meriga et al. reported that the hexane extract and ethyl acetate extract of the fruit pulp had moderate and concentration dependent antifeedant activity against *Spodoptera litura* [78, 79]. Allelopathy refers to the chemical inhibition of one species by another by releasing chemicals into the environment where it affects the development and growth of neighboring plants. Ahire and Deokule observed the leaf extract of *M. dioica* mediated allelopathic activity on seedling growth as well as seed germination of *P. aconitifolius* and found major toxicity at a dose of 2.0% and 2.5% w/v of phytoextracts [80]. These above information are summarized in Table 3.

5. Conclusion

The traditional use of medicinal plants has a long history. Ancient people as well as our ancestors were mainly dependent on plants for their recovery against diseases. But, the recent tendency to avoid natural sources rather than artificial

Table 3: Pharmacological evaluation of *Momordica dioica* described in the paper.

Pharmacological activity	Part of plant	Extract/preparation	Detail effect	Reference
Antioxidant activity	Root	Alcoholic extract	Inhibited the formation of oxygen derived free radicals (ODFR) *in vitro* with 4000 μg/mL ascorbic system.	[45]
	Root	Ethanol extract	DPPH radical scavenging, ABTS radical scavenging, iron chelating activity, total antioxidant capacity and haemoglobin glycosylation assay were studied. Total antioxidant capacity was 26 μg/mL equivalents to ascorbic acid.	[46]
	Fruit	Methanol, aqueous extract	Found the presence of phenolic compound, flavonoids, sterol, alkaloids and amino acids.	[47]
	Leaf	Ethanol, aqueous extracts	The presence of flavonoids was reported as a potent antioxidant	[48]
Analgesic activity	Fruit	Hexane, methanol extract	Exhibited analgesic activity when compared to standard drug	[49]
	Fruit	Petroleum ether, methanol, ethyl acetate extract	Petroleum ether and methanol extract gave more significant analgesic activity than ethyl acetate extract.	[51]
Nephroprotective activity	Seed	Ethanol extract	Found marked nephroprotective and curative activities without any toxicity caused by nephrotoxin-like gentamicin.	[52]
	Fruit	Ethanol extract	Observed significant reduction in GSH and an increase in malondialdehyde (MDA) production.	[53]
Neuroprotective activity	Fruit	Methanol and aqueous extract	Methanol and aqueous extract of fruit pulp (100 mg/kg and 200 mg/kg) had neuroprotective activities.	[55]
Antiallergic activities	Seed	Alcoholic extract	The antiallergic activity of extract in mice was observed.	[56]
	Not specified	Alcoholic extract	Found its efficacy to inhibit passive cutaneous anaphylaxis in mouse and rat.	[57]
Antiulcer activity	Fruit	Ethanol extract	Decreased the level of H^+-K^+ATPase, volume of gastric juice, and acid output. Gastric wall mucus, p^H and catalase enzyme were increased significantly. Antioxidant enzyme levels of superoxide dismutase were decreased.	[58]
	Fruit	Hydro alcohol extract	Gastroprotective and ulcer healing activities were observed.	[59]
Anticancer activity	Root	Methanol extract	The growth inhibitory index (%) of α-spinasterol-3-o-β-D-glucopyranoside was shown to be 50%, at the dose of 4 μg/mL while testing on cancer cell (L1210).	[31]
Antimicrobial activity	Fruit	Methanol, aqueous extract	Found methanolic extract had more promising antimicrobial activity.	[47]
	Root, Leaf	Ethyl acetate extract	The concentration of 200 μg/disc was more active against *E. coli* compared to, *S. paratyphi*, and *P. mirabilis* bacteria.	[60]
Antidiabetic activity	Fruit	Aqueous extract	Oral hypoglycemic effect of *Momordica dioica* in rat model was screened.	[62]
	Fruit	Chloroform, ethyl acetate, and alcohol extract	Ethyl acetate and ethanol showed significant antidiabetic activity at a dose of 200 mg/kg.	[63]
	Fruit	Aqueous, hexane, chloroform, and ethanol extract	Aqueous extract showed maximum fall (52.8%) in 0 to 1 h fasting blood glucose in glucose tolerance test compared to hexane (39%), chloroform (37.2%), and ethanol (37.7%) extract in normal healthy rats.	[64]
	Not specified	Ethyl acetate and ethanol extract	Screened potential role in alloxan-induced diabetic rats and broadly type 2 diabetes.	[65]
	Fruit	Methanol extract	Markedly reduced serum glucose and increased serum insulin and urea levels.	[54]

Table 3: Continued.

Pharmacological activity	Part of plant	Extract/preparation	Detail effect	Reference
Antimalarial activity	Not specified	Alcoholic extract	Misra screened extract *in vivo* and *in vitro* against NK65 strain of *Plasmodium berghei*, *Jurinea macrocephala*, *Aegle marmelos* and found to possess schizontocidal activity.	[66]
Anti-inflammatory activity	Root	Alcoholic extract	Significantly reduced carrageenan-induced paw edema when administered orally (200 mg/kg) and the activity was comparable with ibuprofen (200 mg/kg, p.o.)	[45]
	Fruit	Hexane, methanol extract.	Both extracts exhibited anti-inflammatory activities when compared to standard drug	[49]
Hepatoprotective and antihepatotoxic activity	Root	Ethanol extract	Prevented CCl_4 induced hepatotoxicity at a dose of 200 mg/kg	[67]
	Leaf	Aqueous, methanol extract	Reported hepatoprotective and antihepatotoxicity effect of leaf.	[68, 69]
	Fruit	Ethanol extract	*Evaluated* hepatoprotective activity in wistar strain of albino rats of either sex against CCl_4 induced hepatic damage.	[70]
	Leaf	Ethanol, aqueous extracts	Ethanol extract was found more potent hepatoprotective against CCl_4 induced hepatic damage in rats by *in vivo* free radical scavenging activities.	[48]
	Root	Alcohol extract	Reduced CCl_4 induced hepatotoxicity in rats by inhibiting the formation of radicals *in vitro* with ascorbic system.	[56]
	Fruit	Methanol extract	The saponin fraction of *Momordica dioica* (27.5 and 55 mg/kg) administered to the CCl_4 treated rats to protect the liver cells from liver damages on hepatocytes and silymarin (100 mg/kg).	[71]
	Fruit	Methanol extract	Exhibited a significant therapeutic effect at a dose of 400 mg/kg administered for 7 days in rat.	[72]
	Fruit	n-butanol extract	Observed significant lowering of liver cholesterol and triacylglycerol levels in rats. Moreover, n-butanol extract caused a significant reduction in the pancreatic lipase activity *in vitro*.	[73]
Antifertility activity	Root	Ethanol, aqueous extract	Found moderate estrogenic activity including significant increase in uterine weight and abortifacient activity.	[74]
	Fruit	Ethanolic extract	Found antifertility activities of female rats but no male antifertility activity at the dose of 250 mg/kg	[75]
Antiedemic activity	Root	Alcoholic extract	Showed significant reduction of carrageenan-induced paw edema.	[45]
Insecticidal activity	Seed	Seed oil	Alkaloid momordicin in seed oil was responsible for 100% mortality at 4% conc. in 24 hours.	[76]
Grain protectant activity	Seed	Seed oil	Seed oil was grain protectant against *Callosobruchus chinensis*	[77]
Antifeedant activity	Fruit	Hexane and ethyl acetate extract	Showed antifeedant activity against *Spodoptera litura*	[78, 79]
Allelopathic activity	Leaf	Aqueous extract	Leaf extract has allelopathic activity on seedling growth and seed germination of *P. aconitifolius*	[80]

sources against disease is frustrating. Because continuous reports of antibiotic resistance as well as the side effects of synthetic drugs all over the world are indicating a global health alert. The higher occurrence rate of worldwide diabetes, cancer, obesity, hypertension, and neurodegenerative diseases becomes alarming to all. Huge researches are carried out to find the causes and remedies of them. Therefore, to search for a better alternative than synthetic drug becomes the demand of time.

Medicinal plants may be a good option to play pivotal role against such complications. But, before that their previous use and curability should be justified. Medicinal plants are the source of enormous secondary metabolites. The diverse role of secondary metabolites may provide a key of the door

of undiscovered remedy against diseases. In that case, long term research on medicinal plant is essential to justify their potential. Moreover, the use of medicinal plants is important for its ecofriendly significance as well as its fewer side effects than other synthetic drugs. Additionally, it will be comparatively safer and cheaper than man-made drugs formulation.

South Asia, as one of the highest sources of medicinal plant in the world, provides enormous medicinal plants including kakrol, having several significant folk uses but not clinically evaluated till now. Therefore, vast chances have been created to justify the dynamic ethnobotanical and phytotherapeutical roles of several plants for future researchers. This paper has mainly focused on the phytotherapeutical and pharmacological potential of *Momordica dioica*. As it contains significant amount of antioxidant, vitamin, secondary metabolites, and other important ingredients, these may be helpful to fight against several diseases including diabetes, cancer, and neurodegenerative diseases. For example, ethyl acetate and ethanol extract of kakrol containing steroids, tritepenoids etc. have potential role in alloxan-induced diabetic rats and broadly type 2 diabetes. Similarly, methanol and aqueous extract of its fruit pulp have neuroprotective activities.

Therefore, this paper will be fruitful if it stimulates the researcher's emphasis to justify the unrevealed but potential therapeutic properties of *Momordica dioica* against diabetes, cancer, neurodegenerative disease, and other life threatening disorders.

Conflict of Interests

The authors declare that there is no conflict of interests in this paper.

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Research Article

Centella asiatica Attenuates Diabetes Induced Hippocampal Changes in Experimental Diabetic Rats

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Diabetes mellitus has been reported to affect functions of the hippocampus. We hypothesized that *Centella asiatica*, a herb traditionally being used to improve memory, prevents diabetes-related hippocampal dysfunction. Therefore, the aim of this study was to investigate the protective role of *C. asiatica* on the hippocampus in diabetes. *Methods*. Streptozotocin- (STZ-) induced adult male diabetic rats received 100 and 200 mg/kg/day body weight (b.w) *C. asiatica* leaf aqueous extract for four consecutive weeks. Following sacrifice, hippocampus was removed and hippocampal tissue homogenates were analyzed for Na^+/K^+-, Ca^{2+}- and Mg^{2+}-ATPases activity levels. Levels of the markers of inflammation (tumor necrosis factor, TNF-α; interleukin, IL-6; and interleukin, IL-1β) and oxidative stress (lipid peroxidation product: LPO, superoxide dismutase: SOD, catalase: CAT, and glutathione peroxidase: GPx) were determined. The hippocampal sections were visualized for histopathological changes. *Results*. Administration of *C. asiatica* leaf aqueous extract to diabetic rats maintained near normal ATPases activity levels and prevents the increase in the levels of inflammatory and oxidative stress markers in the hippocampus. Lesser signs of histopathological changes were observed in the hippocampus of *C. asiatica* leaf aqueous extract treated diabetic rats. *Conclusions*. *C. asiatica* leaf protects the hippocampus against diabetes-induced dysfunction which could help to preserve memory in this condition.

1. Introduction

The hippocampus is an area of the brain that is involved in short- [1] and long-term [2] memory. In rats, amnesia can be caused by hippocampal dysfunction [3], whereas, in higher animals including primates, memory loss could occur due to dysfunctions of both hippocampus and amygdala [4]. The loss of neurons or axonal degeneration secondary to ischemia can result in deficits of the hippocampus-dependent spatial memory [5]. Mild to moderate traumatic brain injury was capable of producing prolonged spatial memory deficit in rats without evidence of neuronal death [3]. Diabetes has been linked to memory impairment in rats [6] and humans [7]. On the other hand, hypoglycemia secondary to insulin overdose can also cause hippocampal injury which could result in anterograde amnesia [8].

Hyperglycemia could induce oxidative stress in the hippocampus [9] resulting in apoptosis of hippocampal synapses and neurons [10]. Hippocampal oxidative stress is associated with increased level of lipid peroxidation products [11] and diminution of activity levels of endogenous antioxidant enzymes [12]. Diabetes has been found to inhibit activity of hippocampal Na^+-K^+-ATPase or Na^+ pump [13]. Diabetes has also been reported to suppress activity of Mg^{2+}-ATPase [14] and Ca^{2+}-ATPase in rat whole brain [15]. The effect on Mg^{2+}-ATPase and Ca^{2+}-ATPase activity in the hippocampus of diabetic rats however remains unknown.

Centella asiatica, a herb from Mackinlayoideae family, is native to the wetlands in the tropical and subtropical regions of Asia. *C. asiatica* has been widely used in Ayurvedic, African, and Chinese traditional medicine [16]. This herb was found to promote wound healing via stimulating cellular

proliferation and collagen synthesis at the wound site [17], strengthening gastric mucosal barrier, and preventing ethanol-induced gastritis [18]. *C. asiatica* has also been shown to modulate the immune system [19], prevent blood coagulation [20], act as antioxidant [21], prevent alleviation of oxidative stress [22], act as anti-inflammatory agent [23], and inhibit proliferation of cancer cells [24]. Yet, the most widely reported health benefit of this herb is in improving the brain function, particularly related to memory and learning. In fact, *C. asiatica* has been reported to stimulate nerve regeneration *in vitro* [25] which could explain its brain protective effect. Administration of this herb to juvenile and young adult mice or rats was found to enhance their learning and memory performances [26, 27]. Meanwhile, in humans, *C. asiatica* has been reported to improve mental ability of the mentally retarded children [28] and could selectively decrease the amyloid-β levels in the hippocampus [29].

We hypothesized that *C. asiatica* was effective in protecting the hippocampus against oxidative stress and inflammation caused by diabetes. This study therefore investigated the effect of *C. asiatica* leaves on hippocampal function as reflected by the levels of ATPases activity. Additionally, evidence of alleviation of hippocampal destruction as indicated by decreased levels of oxidative stress and inflammation and lesser signs of histopathological changes in diabetic rats was also sought.

2. Material and Methods

2.1. Chemicals. Streptozotocin (STZ) was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Preparation of Leaf Aqueous Extract of C. asiatica. *C. asiatica* leaves were collected from Tirumala Hills, Andhra Pradesh, India. The taxonomic identification of the plant material was confirmed by the botanist at the Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. The leaves were dried in the shade and crushed into fine powder. The aqueous extract was prepared by soaking 1 kg of powder in 3 L of distilled water for 48 hrs. The extracted material was filtered through Whatman number 1 (0.45 μm Ref. HAWP04700, Bedford, MA, USA) filter paper. This process was repeated three or four times until the extract was rendered colorless. The extract was distilled and concentrated under reduced pressure in rotary evaporator (Rotavapor, R-210, Buchi Labortechnik, AG, Flawil, Switzerland) at 50 ± 5°C and lyophilized using freeze-dryer (Telstar, Barcelona, Spain), which yielded 7% (w/w) of freeze-dried material.

2.3. Phytochemical Screening. The freshly prepared aqueous leaf extract of *C. asiatica* was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed by using standard procedures [30] and phytochemical constituents were identified based on the characteristic color changes.

2.4. Experimental Animals. Adult male Wistar rats (12 weeks old, weighing 180 ± 10 g) were purchased from Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. The animals were maintained under standard laboratory conditions (temperature 22 ± 1°C; 12 h light/dark cycle) and had free access to water and commercial pellet diet (Harlan diet, UK). Experimental procedures were in accordance with ARRIVE guidelines (Animals in Research: Reporting *In Vivo* Experiments) and European Community Guidelines/EEC Directive, 1986. This study was approved by the Animal Care and Use Committee, Faculty of Medicine, University of Malaya, with ethics number 2013-07-15/FIS/R/NS.

2.5. Acute Toxicity Studies. Acute toxicity studies were conducted according to the guidelines by the Organization for Economic Cooperation and Development (OECD, 2001). The extract was dissolved in distilled water with 1% sodium carboxymethyl cellulose (Na-CMC) at 2 mL/kg and was administered to experimental animals by using oral gavage tube. Fifty male Wistar rats were divided into five groups with each group receiving a single dose of 100, 250, 500, 1000, and 2000 mg/kg b.w of *C. asiatica* leaf aqueous extract. Animals were continuously monitored for 4 hrs for behavioral (alertness, restlessness, irritability, vomiting, and fearfulness), neurological (spontaneous activity, convulsion, gait, bleeding orifices, and touch/pain response), and autonomic (defecation, micturition) changes. The number of demised rats in each group was recorded after 24–72 hrs. The extract was found to have no toxic effects when administered in doses up to 2000 mg/kg b.w. Hence, in this study, 100 and 200 mg/kg b.w of this extract were used in accordance with the previously reported doses which were safe [31].

2.6. Induction of Diabetes in Experimental Animals. Overnight-fasted adult male Wistar rats were rendered diabetic via a single intraperitoneal (i.p) injection of STZ (55 mg/kg) dissolved in 0.1 M cold citrate buffer (pH 4.5) [32]. The rats were allowed to drink 5% glucose solution overnight to overcome drug-induced hypoglycemia. Rats in control group received citrate buffer (i.p). Fasting blood glucose (FBG) levels were measured 3 days after STZ injection and only animals with FBG levels between 300 and 400 mg/kg/day were selected for the experiment. Treatment with the extract was started on the third day following STZ injection which was considered day one. *C. asiatica* leaf aqueous extract was administered in a form of suspension orally by using gavage tube daily for 28 consecutive days.

2.7. Experimental Design. A total of 30 rats were used and were divided into five experimental groups of six rats per group.

Group I: normal nondiabetic rats receiving vehicle (Na^+-CMC suspension) only.

Group II: nontreated diabetic rats receiving vehicle (Na^+-CMC suspension) only.

Group III: diabetic rats treated with *C. asiatica* leaf aqueous extract at 100 mg/kg b.w.

Group IV: diabetic rats treated with *C. asiatica* leaf aqueous extract at 200 mg/kg b.w.

Group V: diabetic rats treated with standard antidiabetic agent (glibenclamide) at 600 μg/kg b.w.

2.8. Collection of Blood and Hippocampus. 28 days after the starting of treatment, rats were weighed and overnight-fasted rats were sacrificed with i.p injection of pentobarbitone sodium (60 mg/kg b.w) anesthesia followed by cervical dislocation. Blood was collected from each rat via intracardiac puncture and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation (Thermo Scientific, Model 75005286, USA) at 3000 rpm for 15 minutes. The right hippocampus was immediately harvested, washed with ice-cold saline, and weighed prior to preparation of tissue homogenate. The left hippocampus was used for histological study. The tissue somatic index was determined by the following formula:

$$\frac{\text{weight of the tissue in grams}}{\text{weight of body in grams}} \times 100. \quad (1)$$

2.9. Histopathological Study. The left hippocampus was fixed with 10% neutral paraformaldehyde, dehydrated through ascending concentrations of ethyl alcohol, cleared in xylene, embedded in paraffin, and then cut manually using a microtome (Histo-Line Laboratories, ARM-3600, Via Brembo, Milan, Italy) to obtain 5 μm thick sections. The sections were deparaffinized and rehydrated through descending concentrations of ethyl alcohol and stained with hematoxylin and eosin (H&E). The stained tissues were dehydrated in 80% alcohol followed by 95% ethyl alcohol, placed in two changes of 100% ethyl alcohol, and cleansed with two changes of xylene. Histopathological examinations were carried out by using a phase contrast microscope with an attached camera (Nikon H600L, Tokyo, Japan).

2.10. Preparation of Tissue Homogenate. The excised right hippocampus was homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) by using a glass-teflon homogenizer (Heidolph Silent Crusher M, Germany). The supernatant was separated by means of centrifugation at 1000 ×g for 20 min at 4°C and was then frozen at −80°C until use for biochemical analysis.

2.11. Determination of Lipid Peroxidation (LPO) and Antioxidant Enzyme Activities. The malondialdehyde (MDA) content, a measure of LPO, was assayed in the form of thiobarbituric acid-reactive substances (TBARS) according to the method of Esterbauer and Cheeseman [33]. The rate of lipid peroxidation was expressed as μ moles of MDA formed/gram wet weight of tissue. SOD (EC 1.15.1.1) activity of the homogenates was assayed according to the method of Misra and Fridovich [34]. The assay procedure involves inhibition of epinephrine autoxidation in alkaline medium (pH 10.2) to adrenochrome, in the presence of this enzyme. SOD activity level was expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50%, which was equal to 1 U per milligram of protein. CAT (EC 1.11.1.6) activity was determined by decomposition of H_2O_2 at 240 nm for 3 min monitored spectrophotometrically [35]. The activity of this enzyme was expressed in μmol of hydrogen peroxide (H_2O_2) metabolized/mg protein/min. GPx (EC 1.11.1.9) activity level was determined according to the method by Rotruck et al. [36] and was expressed as μmol of GSH consumed/mg protein/min.

2.12. Determination of Inflammatory Markers in the Hippocampus. Tumor necrosis factor alpha (TNF-α) and interleukins (IL-1β, IL-6) were measured in the supernatant of homogenized hippocampal tissue by using ELISA kits (Biosource International Inc., Camarillo, CA). The procedures were carried out according to the manufacturer's guidelines. TNF-α, IL-1β, and IL-6 were determined from a standard curve and their levels were expressed in pg/mL.

2.13. Determination of ATPase Activity Levels. Na^+/K^+-ATPase (EC 3.6.1.3) activity was estimated in the supernatant of hippocampal tissue homogenates according to the method of Bonting [37]. The activity of calcium-dependent ATPase (Ca^{2+}-ATPase) was assayed according to the method of Hjertén and Pan [38]. Meanwhile, the activity of magnesium-dependent ATPase (Mg^{2+}-ATPase) was assayed according to the method of Ohnishi et al. [39]. Enzymes activities were expressed in μmol pi/min/mg protein. The protein concentration in hippocampal homogenate was determined according to the method of Lowry et al. [40].

2.14. Statistical Analyses. All data were expressed as mean ± S.D. of six determinants. Statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 7.5 (SPSS, Cary, NC, USA) and individual comparisons were made by Duncan's multiple range test (DMRT).

3. Results

3.1. Phytochemical Screening of Aqueous Leaf Extract of C. asiatica. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, glycosides, saponins, terpenoids, steroids, linens, phenols, and tannins (data not shown).

3.2. Effect of Aqueous Leaf Extract of C. asiatica on FBG Levels and Tissue Somatic Index of the Hippocampus. The effects of aqueous extract of *C. asiatica* leaves on FBG levels at days 0 and 28 and tissue somatic index of the hippocampus in rats from the different treatment groups are shown in Table 1. In nontreated diabetic rats, the FBG levels were significantly ($P < 0.001$) higher than normal, nondiabetic rats. However, diabetic rats treated with 100 and 200 mg/kg of *C. asiatica* leaf aqueous extract showed significantly ($P < 0.01$) lower FBG levels on day 28 as compared to nontreated diabetic rats.

Table 1: Effect of *C. asiatica* leaf aqueous extract on FBG levels and hippocampal tissue somatic index in experimental diabetic rats.

Parameters	Normal	Diabetic	Diabetic		
			100 mg/kg b.w *C. asiatica*	200 mg/kg b.w *C. asiatica*	600 μg/kg b.w glibenclamide
0th day blood glucose levels	94.38 ± 7.27	438.27^{*} ± 9.83	436.81^{ns} ± 7.69	437.68^{ns} ± 9.63	436.65^{ns} ± 9.76
28th day blood glucose levels	98.64 ± 8.67	432.16^{*} ± 7.16	$217.37^{\dagger}$ ± 8.36	$203.44^{\dagger}$ ± 8.42	$182.16^{\dagger}$ ± 8.71
Hippocampal tissue somatic index	0.04 ± 0.01	0.04^{ns} ± 0.01	0.04^{ns} ± 0.01	0.04^{ns} ± 0.01	0.04^{ns} ± 0.01

Value represents means ± S.D. for 6 rats per group. $^{*}P < 0.01$ as compared to normal, nondiabetic rats group; $^{\dagger}P < 0.01$ as compared to nontreated diabetic rats.

In diabetic rats, hippocampal tissue somatic index was not statistically different from normal, nondiabetic rats (Table 1).

3.3. Effect of Aqueous Extract of C. asiatica Leaves on MDA and Antioxidant Enzymes Activity Levels in the Hippocampus. MDA levels were significantly ($P < 0.01$) higher in the hippocampal homogenates of nontreated diabetic rats as compared to normal, nondiabetic rats (Figure 1). 28-day treatment of diabetic rats with 100 and 200 mg/kg b.w *C. asiatica* leaf aqueous extracts or glibenclamide resulted in a significantly ($P < 0.01$) lower MDA level in the hippocampal homogenates as compared to nontreated diabetic rats.

SOD activity was 60.93% lower in STZ-induced diabetic rats' hippocampus as compared to normal, nondiabetic rats. However, treatment of diabetic rats with 100 and 200 mg/kg b.w *C. asiatica* leaf aqueous extract or glibenclamide resulted in a significantly higher SOD activity in the hippocampal homogenate. CAT activity was significantly ($P < 0.01$) lower in nontreated diabetic rats as compared to normal, nondiabetic rats. Treatment of diabetic rats with *C. asiatica* leaf aqueous extract or glibenclamide resulted in a significantly higher CAT activity level as compared to nontreated diabetic rats (Figure 1). In nontreated diabetic rats, GPx activity in the hippocampus was significantly ($P < 0.01$) lower than that in normal, nondiabetic rats. Treatment of diabetic rats with 100 and 200 mg/kg b.w *C. asiatica* leaf aqueous extracts or glibenclamide resulted in a significantly higher GPx activity level in the hippocampus as compared to nontreated diabetic rats (Figure 1).

3.4. Effect of C. asiatica Leaf Extract on the Hippocampal Inflammatory Markers. The effect of *C. asiatica* leaf aqueous extract on inflammatory markers in the hippocampus of rats in different experimental groups is shown in Figure 2. In nontreated diabetic rats, the levels of TNF-α in the hippocampal homogenates were 252.38% higher than normal, nondiabetic rats. Diabetic rats treated with *C. asiatica* leaf aqueous extract at doses of 100 and 200 mg/kg b.w had a significantly lower TNF-α level in the hippocampal homogenates as compared to nontreated diabetic rats. A significantly higher IL-1β (306.31%) was observed in the hippocampus of nontreated diabetic rats as compared to normal, nondiabetic rats. Treatment of diabetic rats with 100 and 200 mg/kg b.w *C. asiatica* leaf aqueous extract resulted in a significantly lower levels of IL-1β in the hippocampus as compared to nontreated diabetic rats ($P < 0.01$). The hippocampal IL-6 levels were significantly higher in nontreated diabetic rats as compared to normal, nondiabetic rats ($P < 0.01$). Diabetic rats treated with 100 and 200 mg/kg b.w *C. asiatica* leaf aqueous extract had a significantly lower IL-6 level in the hippocampus as compared to nontreated diabetic rats ($P < 0.01$).

3.5. Effect of C. asiatica Leaf Extracts on the Hippocampal ATPase Activity Levels. Figure 3 shows the effect of *C. asiatica* leaf aqueous extract on ATPases activity of the hippocampus of rats in different experimental groups. The Na^{+}/K^{+}-ATPase activity was 65.95% lower in the hippocampus of nontreated diabetic rats as compared to normal, nondiabetic rats. Treatment of diabetic rats with 100 and 200 mg/kg b.w *C. asiatica* leaf aqueous extract or glibenclamide resulted in a significantly higher hippocampal Na^{+}/K^{+}-ATPase activity as compared to nontreated diabetic rats ($P < 0.01$). Mg^{2+}-ATPase activity was significantly lower (48.06%) in nontreated diabetic rats' hippocampus as compared to normal, nondiabetic rats. Diabetic rats treated with *C. asiatica* leaf aqueous extract at 100 and 200 mg/kg b.w or glibenclamide showed a significantly higher hippocampal Mg^{2+}-ATPase activity as compared to nontreated diabetic rats ($P < 0.01$). Meanwhile, Ca^{2+}-ATPase activity in the hippocampus of diabetic rats was significantly lower than normal, nondiabetic rats ($P < 0.01$). Ca^{2+}-ATPase activity in the hippocampus of diabetic rats treated with *C. asiatica* leaf aqueous extract at 100 and 200 mg/kg b.w or glibenclamide was significantly higher than nontreated diabetic rats ($P < 0.01$).

3.6. Histopathological Changes of the Hippocampus following Treatment with C. asiatica Leaf Aqueous Extract. Figure 4 shows histological sections of the hippocampus from normal nondiabetic, nontreated diabetic, and diabetic rats treated with *C. asiatica* leaf extract or glibenclamide. Our findings showed that there were signs of necrosis in the subiculum, presubiculum, and cornu ammonis (CA) areas of the hippocampus of diabetic rats. Administration of *C. asiatica* prevented the development of necrotic changes in these areas. Higher magnification images revealed a significant decrease in the number of neurons and glial cells in the hippocampus of diabetic rats. Administration of 200 mg/kg/day b.w *C. asiatica* leaf extract resulted in higher hippocampal neurons

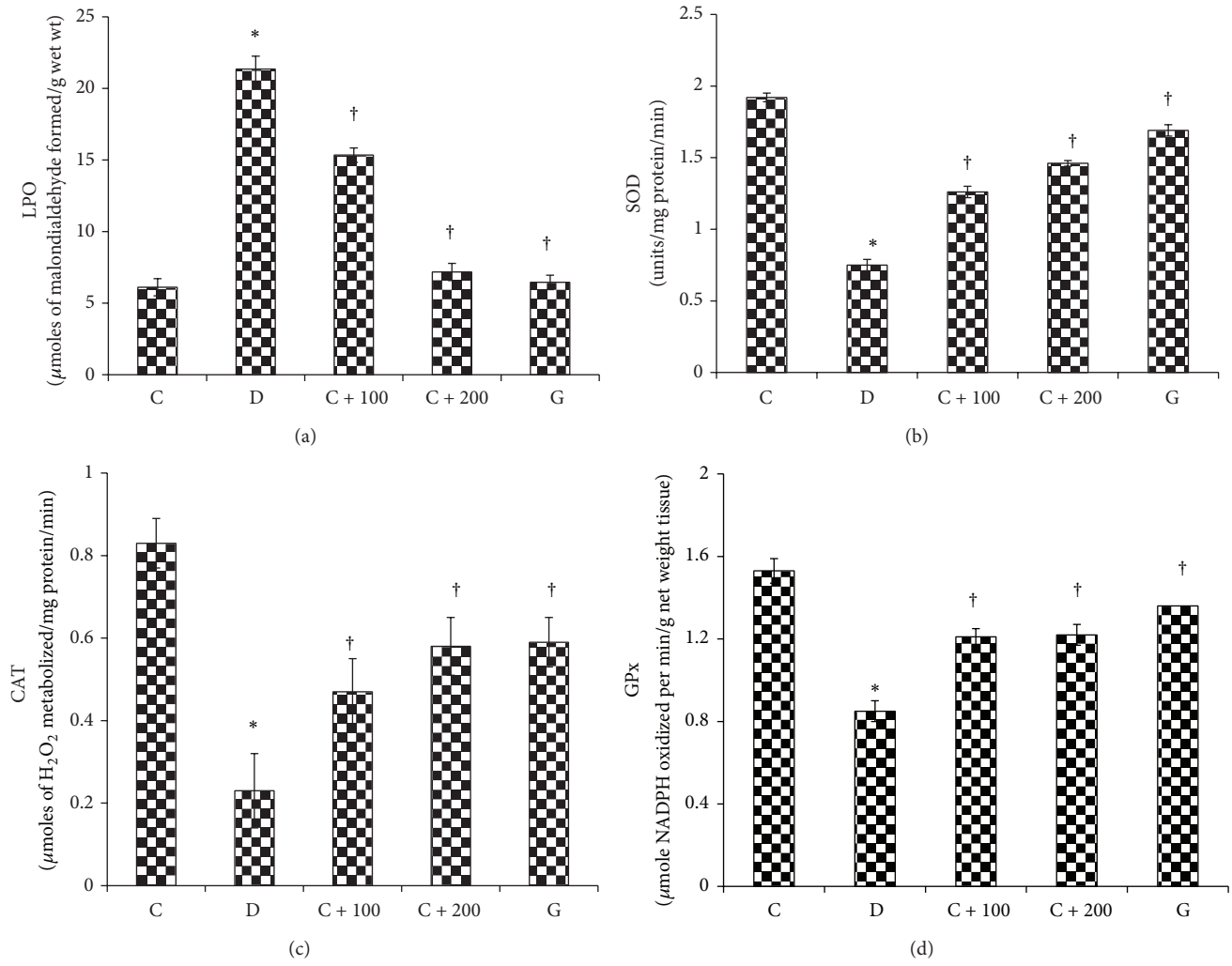

Figure 1: The levels of lipid peroxidation (LPO) product and antioxidant enzymes (SOD, CAT, and GPx) in hippocampal homogenates. LPO level was the highest in diabetic rats. Meanwhile, the levels of SOD, CAT, and GPx were the lowest in diabetic rat hippocampus. Treatment with *C. asiatica* leaf extract reversed changes in these parameters in diabetic rat hippocampus. $^{*}P < 0.05$ as compared to normal, nondiabetic rats; $^{\dagger}P < 0.05$ as compared to nontreated diabetic rats.

and glial cells number as compared to nontreated diabetic rats.

4. Discussion

Our findings have revealed the protective effect of *C. asiatica*, a herb widely consumed by Asians, against hippocampal destruction due to oxidative stress in diabetes. *C. asiatica* has been used in Ayurvedic medicine to improve the memory and learning abilities [41]. Administration of this herb during postnatal period in mice has been shown to enhance learning and memory [26]. Studies in humans have indicated that *C. asiatica* could improve memory in mentally retarded children [28]. In addition, *C. asiatica* combined with other herbal extracts has been shown to improve memory in patients with Alzheimer's disease [42]. These effects were attributed to the ability of this herb to preserve normal hippocampal function. We have provided evidence which showed that consumption of *C. asiatica* could help preserve memory function in diabetes, known to be associated with long- and short-term memory impairment [43]. Diabetes has been found to cause degenerative changes in the hippocampus [10] which contributed towards memory loss. Our histopathological findings including neuronal degeneration in diabetic rat hippocampus were consistent with the previous reports which indicate similar changes in the hippocampus under this condition [9, 44, 45].

Our findings showed that hippocampal ATPases activities were reduced in diabetes. Hippocampal Na^+/K^+-ATPase has been linked to memory function in rodents [46]. Hippocampal neurons [47] and interneurons express Na^+/K^+-ATPase in their axonal and dendritic membranes [48]. Insulin has been reported to stimulate activity of this pump in the rat hippocampus [49]. In conditions associated with lack of insulin such as diabetes, hippocampal Na^+/K^+-ATPase activity was reduced, indicating that insulin is essential for maintaining

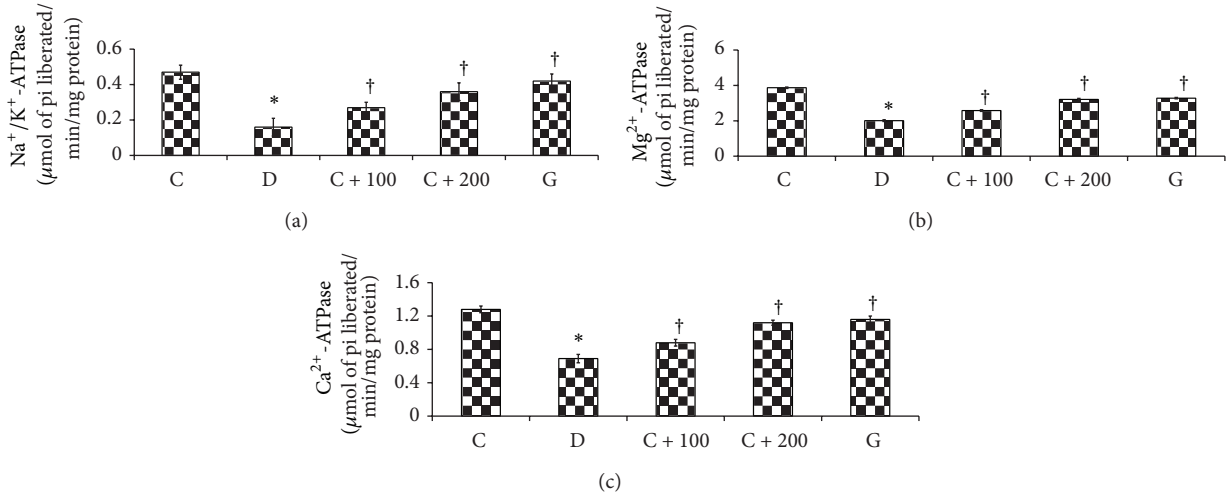

Figure 2: Activity levels of Na^+/K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase in hippocampal homogenates. ATPases activity levels were the lowest in diabetic rats. Treatment with *C. asiatica* leaf extract reversed changes in ATPases activity levels in diabetic rats. $^*P < 0.05$ as compared to normal, nondiabetic rats; $^\dagger P < 0.05$ as compared to nontreated diabetic rats.

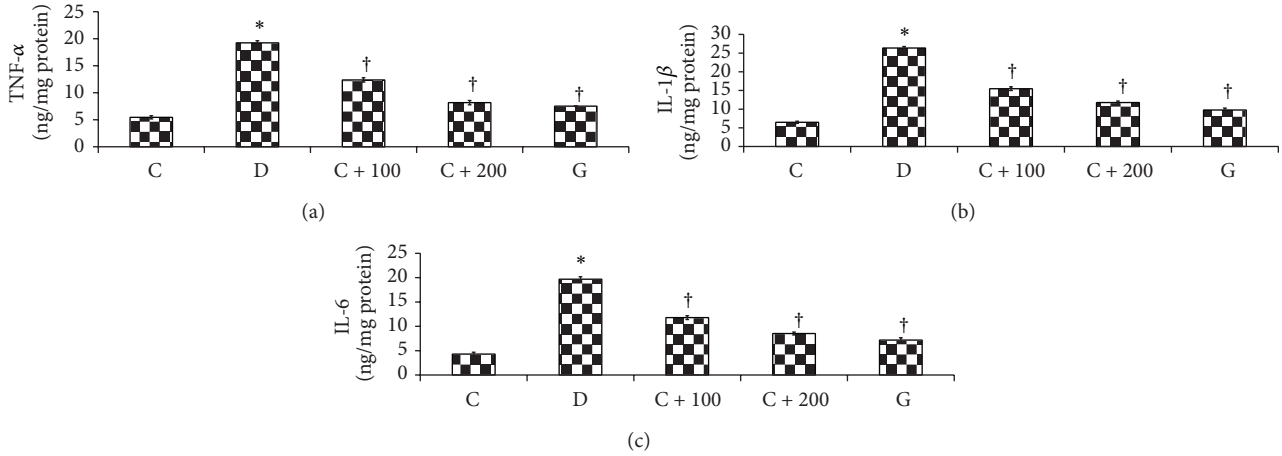

Figure 3: The levels of TNF-α, IL-1β, and IL-6 in hippocampal homogenates. The levels of inflammatory markers were the highest in diabetic rats. Treatment with *C. asiatica* leaf extract reversed changes of inflammatory markers levels in hippocampal homogenates of diabetic rats. $^*P < 0.05$ as compared to normal, nondiabetic rats; $^\dagger P < 0.05$ as compared to nontreated diabetic rats.

the normal function of this pump [50]. A marked decrease in Na^+/K^+-ATPase activity could adversely affect hippocampal function. Critical role of this pump in normal hippocampal function was evidence from impairment of hippocampal neuron activity in disease associated with mutation of this protein [51]. Besides hyperglycemia, hippocampal Na^+/K^+-ATPase activity was also inhibited by amino acids such as homocysteine [52] and drugs such as ouabain [53] and in ischaemia [54]. In our study, the decrease in activity of this pump in diabetes could be prevented by the administration of *C. asiatica* leaves, suggesting ability of this herb to prevent hyperglycemia-induced inhibition on the Na^+/K^+-ATPase activity.

The expression of Ca^{2+}-ATPase pump has been reported in rodent [55] and human [56] hippocampus. In this study, we have shown that hippocampal Ca^{2+}-ATPase activity was markedly reduced in diabetes. Besides diabetes, activity of this pump was also found to be reduced in seizure [57]. The expression of neuron-specific endoplasmic reticulum Mg^{2+}-ATPase was reported in the hippocampal neurons [58]. Our study has shown that activity of Mg^{2+}-ATPase pump was reduced in diabetic condition. Treatment with *C. asiatica* leaf extract in diabetic rats could help preserve both Ca^{2+}- and Mg^{2+}-ATPases activities of the hippocampus, therefore restoring normal hippocampal function. In view that activity of all three major hippocampal ATPases was conserved, *C. asiatica* leaves might be useful in preserving and enhancing memory in diabetes.

We have shown that the levels of LPO product in the hippocampus of diabetic rats were increased indicating oxidative stress. Oxidative stress has been linked to neurodegeneration [59]. Oxidative stress in the hippocampus could be increased

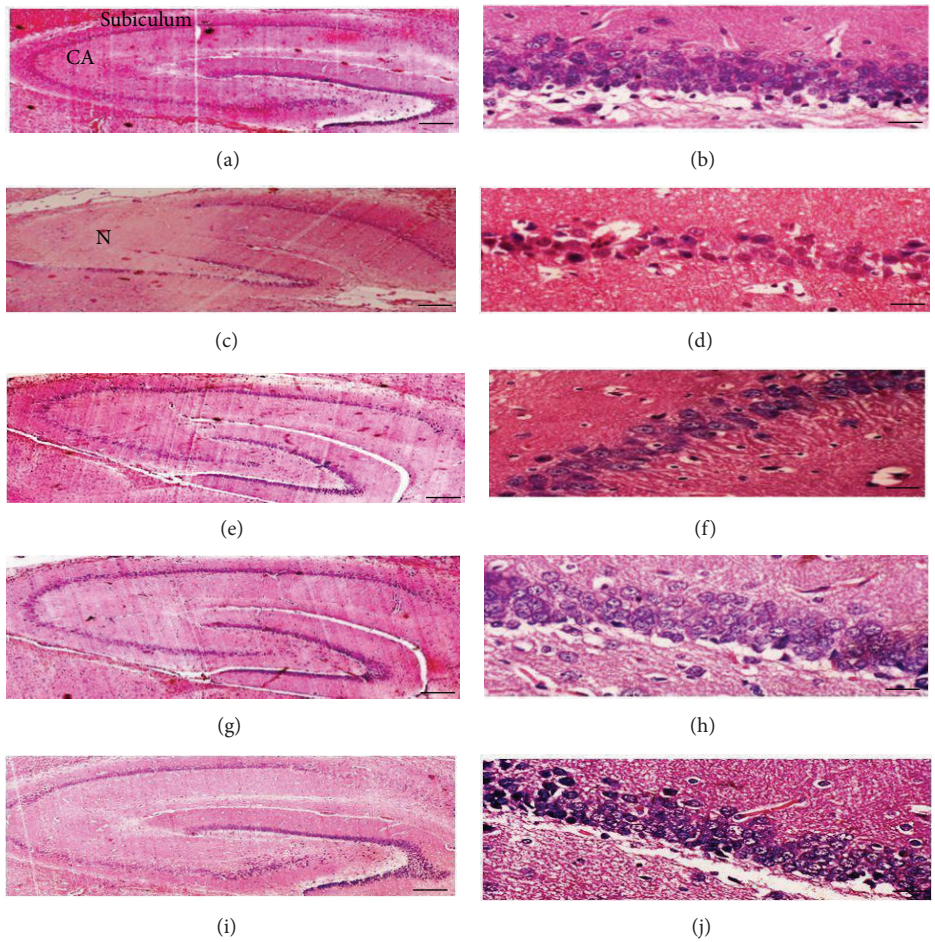

Figure 4: Photographs of hippocampal sections of rats in different experimental groups. There were signs of necrosis in diabetic rat hippocampus (c). Loss of neuronal and glial cells was observed in diabetic rat hippocampus (d). Administration of *C. asiatica* leaf extract or glibenclamide ((e)–(j)) preserved near normal hippocampal histology in diabetic rats. N = necrosis; CA = cornu amonis.

by guanylic acid [60] and could be prevented by melatonin [61]. Homocysteine was also reported to induce hippocampal oxidative stress [62]. In diabetes, increased formation of reactive oxygen species (ROS) in the hippocampus could cause oxidative stress resulting in neuronal apoptosis [63]. We have shown that activity of hippocampal antioxidant enzymes such as SOD, CAT, and GPx was decreased in diabetes which could contribute towards oxidative stress. A similar observation has been reported in homocystinuria where increased hippocampal oxidative stress was caused by decreased activity of endogenous antioxidant enzymes [62]. Antioxidant enzymes help to scavenge free radicals such as nitric oxide, superoxide, and peroxides, levels of which were markedly elevated in diabetes [59]. In our study, ability of *C. asiatica* to reduce hippocampal oxidative stress may be attributed to the free radical scavenging effect and the ability of this herb to preserve near normal activity levels of endogenous antioxidant enzymes.

Our findings have indicated that there were signs of hippocampal inflammation in diabetic rats from the histopathological changes and elevation of inflammatory markers (IL-1β, IL-6, and TNF-α) in hippocampal homogenates. IL-6 was reported to produce detrimental effect on the hippocampus [64]. Higher levels of interleukins (ILs) could activate the central inflammatory mechanisms that result in hippocampal neurodegeneration leading to memory impairment. An inverse correlation between peripheral IL-6 levels and memory has been reported in human adults during the midlife period [65], suggesting that IL levels could affect memory. We have shown that administration of *C. asiatica* leaf extract resulted in the levels of hippocampal inflammatory markers in diabetic rats to be reduced to near normal, which indicates decreased hippocampal inflammation. Meanwhile, normalization of FBG levels following administration of *C. asiatica* leaf might also contribute to the reduction in hippocampal inflammation in diabetes.

Evidence of necrotic changes was observed in cornu ammonis (CA) and subiculum areas of the hippocampus in diabetic rats with severe loss of pyramidal neurons and glial cells (Figure 4). A previous study reported that in diabetes, neuronal degeneration was due to apoptosis [66]. These changes were parallel with the reported decrease in hippocampal neurogenesis in mice with diabetes [67]. Administration of *C. asiatica* leaf extract to diabetic rats prevented

neuronal degeneration and therefore helps in maintaining normal hippocampal function in diabetes. In addition to *C. asiatica*, we have shown that antidiabetic drug glibenclamide could also prevent hippocampal neuronal degeneration in diabetes.

In conclusion, our study has provided mechanisms which could explain the protective role of *C. asiatica* leaf against hippocampal dysfunction which include preservation of near normal activity levels of the major hippocampal ATPases and the reduction in inflammatory changes and oxidative stress in the hippocampus in diabetes. Cumulatively, these effects justify the claims that *C. asiatica* contributes towards the preservation of memory function in diabetes.

Conflict of Interests

The authors declared that there was no conflict of interests with regard to the publication of this paper.

Acknowledgments

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Research Article

Behavioral and Histopathological Study of Changes in Spinal Cord Injured Rats Supplemented with *Spirulina platensis*

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Spinal cord injury (SCI) is a devastating disease that leads to permanent disability and causes great suffering. The resulting neurological dysfunction and paralysis is proportional to the severity of the trauma itself. Spirulina is widely used as a nutritional supplement due to its high protein and antioxidant content. In the present study, the protective effect of the Spirulina treatment on locomotor function and morphological damage after SCI was investigated. Seventy Sprague-Dawley (SD) rats were divided into three groups: Sham (laminectomy alone), Control (laminectomy with SCI), and Experimental (laminectomy with SCI +180 mg/kg per day *Spirulina platensis*). A laminectomy was performed at T12 and an Inox No.2 modified forceps was used to perform a partial crush injury on the spinal cord. The rats were then perfused at 3, 7, 14, 21, and 28 days after injury for morphological investigations. The injured rat spinal cord indicated a presence of hemorrhage, cavity, and necrosis. Pretreatment with Spirulina significantly improved the locomotor function and showed a significant reduction on the histological changes. The experimental results observed in this study suggest that treatment with *Spirulina platensis* possesses potential benefits in improving hind limb locomotor function and reducing morphological damage to the spinal cord.

1. Introduction

Spinal cord injury (SCI) is one of the leading clinical causes of disability in young adults for which no suitable natural remedies have been found for SCI management. As such, the exploration of novel therapeutic agents to enhance neuroprotection after SCI is needed [1]. The spinal cord does not have to be severed in order for loss of functioning to occur. In fact, in most people with SCI, the spinal cord is intact, but the damage to it results in loss of sensory or autonomic function and loss of normal motor function which may lead to paraplegia and quadriplegia. There are about 10,000 new SCI cases every year reported around the world, especially in the European countries. Males between the ages of 16 to 30 are among the majority who suffer from SCI and this occurs throughout the world with an annual incidence of 15 to 40 cases per million population [2].

There are some therapies available for SCI management such as erythropoietin, minocycline, inosine, riluzole, pioglitazone, and others [3]. At present, a high dose of methylprednisolone (MP) is the most common drug used for the treatment of spinal cord injury patients since the results of the landmark National Acute Spinal Cord Injury Studies (NASCIS) II trial in the 90s were published. However, many clinicians and scientists around the world have questioned the effectiveness of using MP due to conflicting results of experimental studies [4, 5] compared to the minor neurological improvements seen in patients [6, 7]. Furthermore, MP has been associated with certain side effects such as anxiety, dizziness, and mental depression and it can increase risk of infection both of wounds and at the site of trauma [8].

Spirulina is widely used as a nutritional supplement as it is complete with about sixty percent highly digestible protein, contains all the essential amino acids, and is rich in gamma-linolenic acid (GLA), minerals, trace elements, chlorophyll, and digestive and restriction enzymes [9]. In addition, it contains a wide range of antioxidants such as superoxide dismutase (SOD), provitamin-A (beta-carotene), vitamin C, E, selenium and phycocyanin, and flavonoids which have been proven in previous studies [10–15].

Spirulina is now attracting even more attention from medical scientists as it can be used as a nutraceutical and is a potential source of pharmaceuticals due to its ability to inhibit viral replication, strengthen both the cellular and humoral arms of the immune system, and aid in the regression and inhibition of cancer [16]. In addition to this, certain studies have reported that a dietary supplementation of Spirulina may reduce ischemic brain damage and provide a neuroprotective effect in cerebral ischemia-reperfusion injuries [17, 18]. This study is an initial attempt to investigate the effects of Spirulina supplementation on behavioral and morphological changes in traumatic injured rat spinal cords.

2. Materials and Methods

2.1. Animals. Seventy male Sprague-Dawley (SD) rats, weighing 200 ± 50 g, eight weeks old, were purchased from the Experimental Animal House, University of Malaya. All the rats were then divided into three groups: sham (laminectomy without SCI, $n = 10$), control (laminectomy with SCI, $n = 30$), and experimental (laminectomy with SCI +180 mg/kg per day *Spirulina platensis*, $n = 30$). For the control and experimental groups, all the rats were divided into five subgroups consisting of those sacrificed at 3, 7, 14, 21, and 28 postoperative days, respectively, with $n = 6$ for each group. The rats were kept at the standard conditions of temperature (23 ± 2°C) and humidity (50 ± 10%) with an alternating 12-hour light/dark cycle. The animals were kept in the Experimental Animal House, University of Malaya. The animals were acclimatized to the laboratory conditions prior to experimentation and the study was conducted according to the study protocol approved by the Animal Care and Use Committee (ACUC), Faculty of Medicine, University of Malaya, Kuala Lumpur, with the reference number ANA/27/01/2012/IA (R).

2.2. Surgical Procedures. All the rats were anesthetized using a mixture of 100 mg/kg of ketamil (100 mg/mL ketamine, Australia) and 10 mg/kg of xylazil (20 mg/mL xylazine, Australia) via intramuscular injection (IM). The fur overlying the thoracic vertebral column of the rat was removed using a shaver (Wahl, USA). The rats were then placed in a prone position on the operating table under sterile conditions. A midline dorsal incision was done on the rats and the paravertebral muscles were separated from the vertebrae. The vertebral was then removed with a microrongeurs to expose the underlying dura mater. A partial crush injury was extradurally performed for the control and experimental groups by compressing for 30 sec [19] at the twelfth thoracic spinal cord segment (T12) using Inox number 2 modified forceps (Dumont, Switzerland). The wounds were cleansed with saline and the muscle and connective tissue were closed with a 4-0 nylon. Finally, the skin was closed with surgical staples. The rats were then kept in the experimental animal house during recovery.

2.3. Postinjury Care. Each rat received 1 mL lactated ringers subcutaneously and 0.4 mg/kg Baytril (Bayer, Korea) intraperitoneally (IP) immediately after the surgery until day 7 following operation. The rats were carefully monitored for evidence of urinary tract infection or any sign of systemic disease. Additional Baytril was given for any evidence of hematuria/urinary infection. Immediately after the surgery, the animals exhibited hindlimb paralysis and a loss of bladder function. Following this, manual bladder expression was performed twice daily for the first week and once daily after that until the spontaneous emptying of the bladder had recovered. Animals were housed in individual cages with bedding.

2.4. Diet and Supplementation. All rats were fed with the standard rat chow. Animals were given access to food and water *ad libitum*. For the experimental group, the rats were supplemented with 180 mg/kg per day of *S. platensis* (Mutiara Saintifik, Kuala Lumpur) through an esophageal feeding tube starting from day 1 after the injury up to 3, 7, 14, 21, and 28 postoperative days according to their subgroup.

2.5. Behavioral Analysis. The right and left hindlimb locomotor functions were evaluated and graded using Basso, Beattie & Bresnahan's (BBB) locomotor rating score [20]. The locomotor evaluation was done in an open field 1.2 m diameter, circular, smooth-surfaced activity chamber for 4 mins. In this test, specific components of functional behavior were analyzed such as limb movement, paw placement/position, stepping, coordination, toe clearance, and tail position. A score of 0 was given when no spontaneous hindlimb movement was observed, while a score of 21 indicated normal locomotion. Hindlimb movement was scored by 2 investigators that were blinded to the experiment.

2.6. Histological Procedure. At the time of sacrifice, animals were overdose anesthetized with a mixture of 0.35 mL of ketamil (100 mg/mL ketamine, Australia) and 0.15 mL of xylazil (20 mg/mL xylazine, Australia). Then, the rat was perfused through the heart with 300 mL of normal saline followed by 300 mL of 4% paraformaldehyde. A 3 cm section including the T12 level of the spinal cord was then exposed and removed from the vertebral column and then fixed in the same fixative overnight. The tissue was embedded in paraffin and sectioned sagittally (4 μm thickness). The sections were stained with Haematoxylin and Eosin (H and E) to help visualize the morphology of the spinal cord.

2.7. Quantitative Image Analysis Procedure. The H and E stained sections were used for image analysis using the NIS-Elements AR 3.2 software. The area containing the spinal cord lesions was captured through a camera (Nikon, Japan) connected to the microscope (Nikon eclipse 80*i*, Japan). The area of the lesion was distinguished from the surrounding normal spinal cord tissue by the presence of necrosis, inflammatory cells, cysts, or cavities and also the appearance of pale staining. The lesions were measured according to two parameters: the lesion size from rostral to caudal and the lesion size from lateral to lateral.

2.8. Statistical Analysis. All the data collected from the experiment was recorded and analyzed using SPSS software

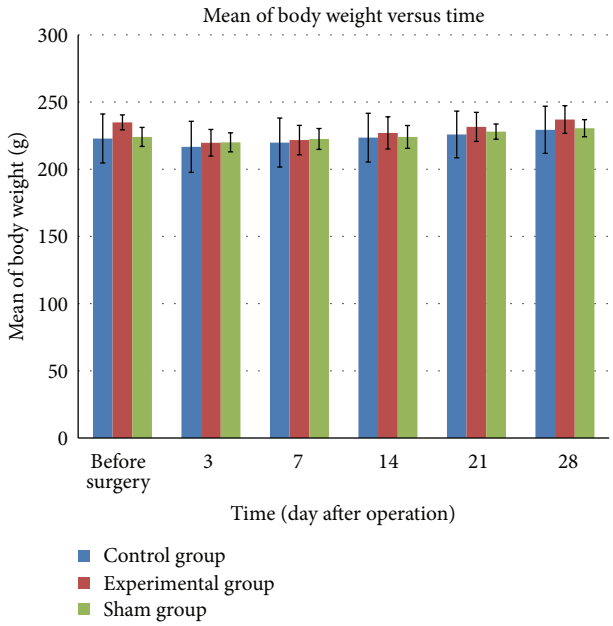

Figure 1: Changes in body weight in rats before and after surgery (postsurvival interval). The bar graphs indicate the mean, and the bars indicate SD of the measurements.

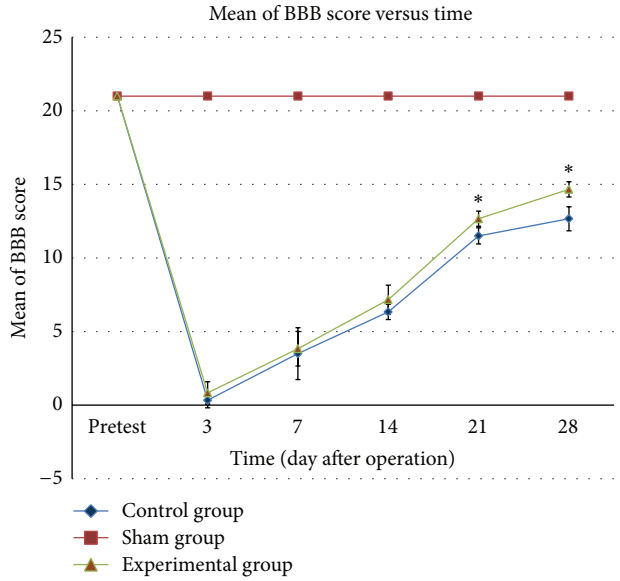

Figure 2: BBB score among the three groups of rats one day before surgery (pretest) and after surgery. The line graphs indicate mean of BBB score taken while the bars indicate SD of the measurements. $^{*}P < 0.05$ for experimental group versus control group.

for Windows (version 16.0, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with Tukey's post hoc analysis was used to compare differences among the groups. In each test, the data was expressed as the mean value ± standard deviation (SD) and differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Changes in Body Weight. The body weight of all rats in the control, experimental, and also sham groups decreased immediately within 3 days after surgery (Figure 1). Although the rats showed a decrease in body weight after the surgery, this loss was not significantly different. On day 7 after operation onwards, all the rats demonstrated an increase in body weight and on day 28 after operation, the weight exceeded their presurgery weight.

3.2. Locomotor Function Assessment. All the rats in the control and experimental groups demonstrated signs of paraplegia but the rats then showed a slight improvement throughout the 4 weeks after the surgery. Spontaneous emptying of the bladder was recovered a week after the surgery. The locomotor function of all the rats that had undergone the laminectomy alone (i.e., the sham group) was found to be unaffected, as shown by the BBB score (a score of 21). Before the surgery, all the rats in the control group had normal BBB scores of 21. Three days immediately after surgery, the paralysis of their hindlimbs resulted in a reduction in the BBB score (Figure 2) but the rats then recovered widespread movement of their locomotor hindlimb joints, which meant that the BBB score increased up to day 28 after the surgery. The BBB score for all the rats in the experimental group also displayed a similar score pattern when the score was reduced at day 3 after operation and then increased at 7, 14, 21, and 28 days following operation. At days 21 and 28 after operation, all the rats demonstrated an improvement in their locomotor functions, during which the BBB score showed a significant difference between the control group and the experimental group. This study found that the BBB score for all the rats in the experimental group was higher compared to those in the control group at day 3 up to day 28 after operation.

3.3. Histopathology Study. A histopathology study done using H and E staining on the spinal cord tissue showed the appearance of lesions at the crush site in the control (Figure 3(a)) and experimental (Figure 3(b)) groups at day 3 after operation. Meanwhile, in the sham group, no lesions were observed in the tissue. The lesion area in the control and experimental groups was observed to lead to areas of progressive necrosis and cavitation (Figures 3(c) and 3(d)) which extended farther rostrally and caudally until day 7 following operation. At day 14 after operation onwards, the size of the cavity and lesion decreased for both control and experimental groups. The presence of hemorrhagic foci was observed in the middle of the crushed edge. In all the tissue observed, a number of erythrocytes and neutrophils (Figures 3(e) and 3(f)) had emerged at the primary site. There was a difference in the histological appearance in certain important aspects among the control and experimental groups. In this study, the increase in lesion size in the H and E stained sections for the experimental group was significantly less than that in the control group. The lesion size was small and was limited to the area adjacent to the crush site only while the presence of neutrophils and erythrocytes in this tissue was

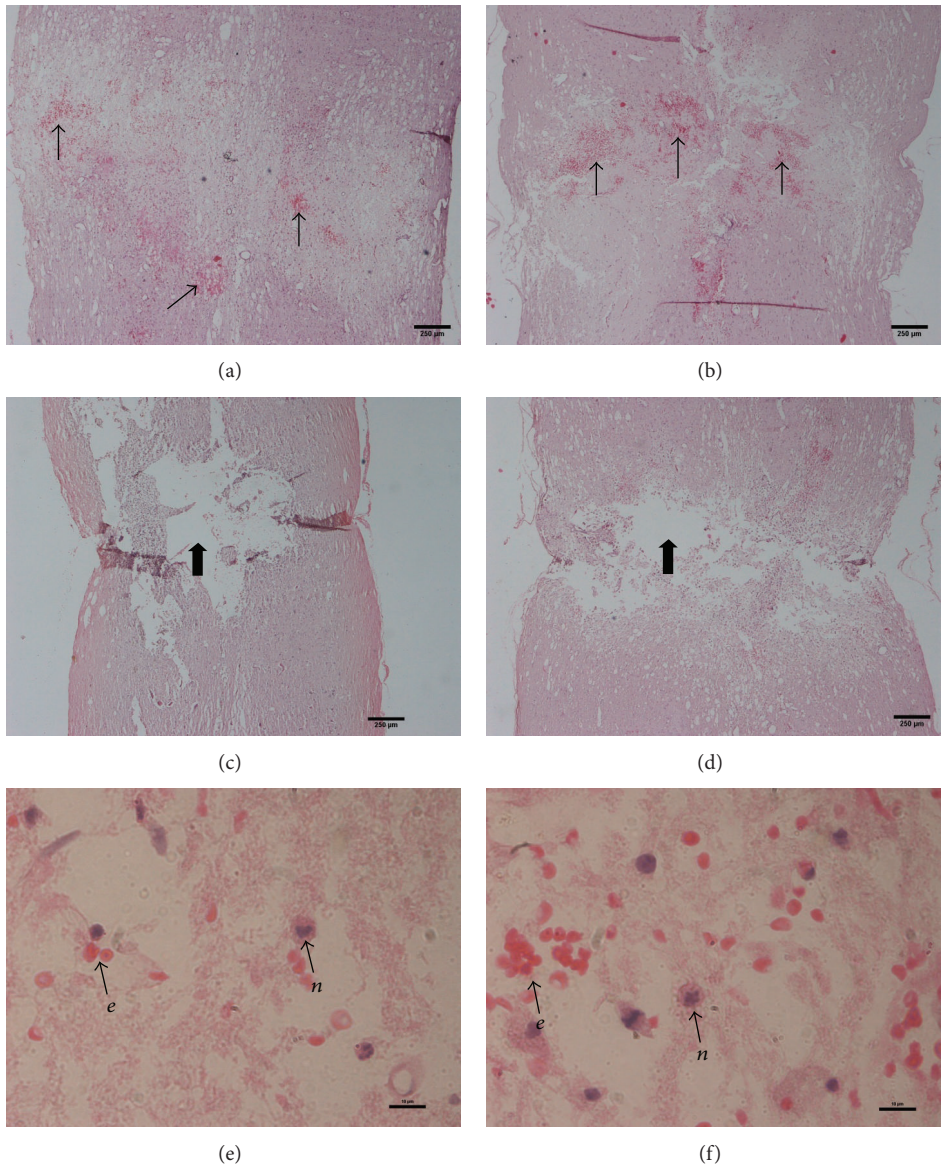

Figure 3: Histopathology slaids for control (a) and experimental (b) group 3 days after injury and control (c) and experimental (d) group 7 days after injury. The photographs illustrate H and E stained sections at the crush site. Arrows showed the area of hemorrhage that contains pockets of erythrocytes. Block arrows indicate cavity (empty lesion) with the cavity in the experimental group (d) being smaller than that in the control group (c). The difference of erythrocytes and neutrophils appearance in control (e) and experimental (f) group where (*e*) indicate erythrocyte while (*n*) indicate neutrophil. Scale bar, 250 μm for panels (a)–(d) under ×4 magnification and 10 μm for panels (e) and (f) under ×100 magnification.

higher (Figure 3(f)). There were less necrosis and a smaller cavity (Figure 3(d)) along the dorsal column.

3.4. Quantitative Analysis

3.4.1. Lesion Size from Rostral to Caudal. A quantitative study revealed differences between the lesion size in the control and experimental groups throughout the postinjury survival interval (Figure 4). The lesion sizes in the animals supplemented with 180 mg/kg per day of *S. platensis* were significantly ($P < 0.05$) smaller compared to those in the control group when they were measured at day 3 after operation. The lesion size was found to have increased at day 7 following operation in both groups but the lesion sizes in the experimental group were significantly ($P < 0.05$) smaller compared to those in the control group. Image analysis showed that the lesion size was smaller starting from day 14 onwards in both the control and experimental groups. The measurement taken from the experimental groups showed the efficacy of *S. platensis* in reducing neurological deficit when there was a significantly ($P < 0.05$) smaller lesion size in the animals supplemented with *S. platensis* at days 3, 7, and 14 compared to those that were not supplemented with it.

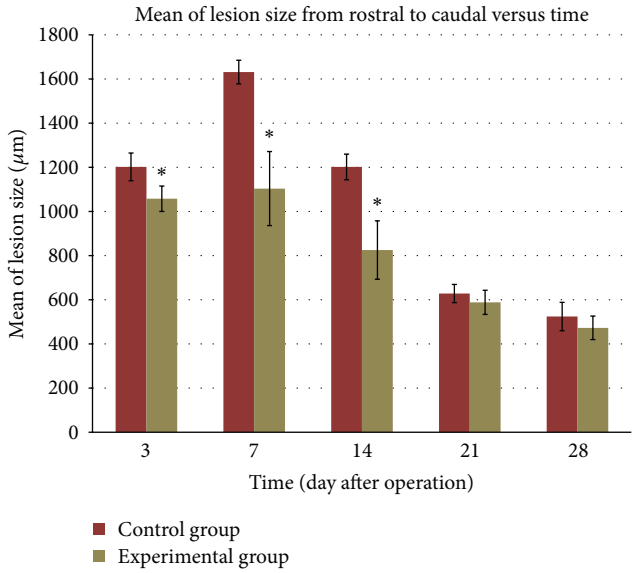

Figure 4: Effects of *S. platensis* on the lesion size from rostral to caudal after spinal cord injury ($n = 6$ per time point). The bar graphs represent mean of the measurement while the bars represent SD. $^{*}P < 0.05$ for experimental group versus control group.

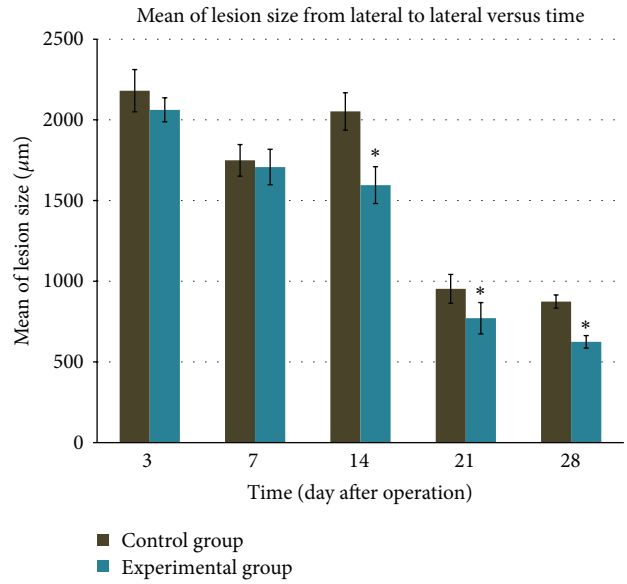

Figure 5: Difference in lesion size from lateral to lateral between animals without supplementation of *S. platensis* (control group) and animals supplemented with *S. platensis* (experimental group). The bar graphs indicate mean while the bars indicate SD of the measurement. $^{*}P < 0.05$ for experimental group versus control group.

3.4.2. Lesion Size from Lateral to Lateral. From the analysis of the spinal cord tissue, a difference of the lesion size when measured from lateral to lateral end was found (Figure 5). The tissue was ruptured in both the experimental and control groups at day 3 following operation but the lesion size measured in the experimental group was smaller compared to the control group, even though it was not significant. At 7th postoperative day, there was a reduction in lesion size but the difference of the size in the control and experimental group was not significant. The lesion size in the experimental group was significantly smaller compared to the control group at days 14, 21, and 28 after surgery.

4. Discussion

S. platensis at a dose of 180 mg/kg per day as used in this study showed its capacity to reduce the neurological deficits in the spinal cord injured rats. This dose is equivalent to the normal human dose because the normal dose of *S. platensis* taken by human is essentially around 500 to 2000 mg per day. Moreover, a previous study [18] proved that *S. platensis* at a dose of 180 mg/kg per day showed a significant reduction in cerebral neurological deficits.

In the present study, we tested whether the supplementation of *S. platensis* in the traumatic injured rat spinal cord has a protective effect. The current study is the first to investigate the effects of *S. platensis* on traumatic SCI. Based on the findings that have been demonstrated by both the behavioral and histopathological outcomes, the most significant finding in this study is that *S. platensis* possesses potential benefits in improving locomotor function and reducing morphological damage to the spinal cord. Many techniques have been developed by researchers and scientists around the world to induce crushes on SCI models. The most commonly used model is the contusion model [21–23]. This induces a SCI by weight drop [24] and an impactor rod [25]. In addition to this, the compression model has also been used in SCI research by dropping weight [26], balloon angioplasty catheters [27], and also cerebral vascular clips [28].

In this study, we used a pair of forceps to induce a crush injury to the spinal cord as it is easy to use, is affordable, and proved to be clinically relevant. From our study, we found that even with using forceps, the spinal cord crush still produced proportional tissue loss, necrosis, cavitation, and corresponding locomotor function deficits. Our results are similar to the findings of researchers who have also used forceps to induce crushes in SCI models and reported to perfectly reflect the pathological and physiological features of SCI [29, 30].

Body weight changes in this study were analyzed as it is a general indication of health. From the observations of this study, all animals in the sham, control, and also experimental groups which had undergone the surgical procedure had a slight drop in their body weight 3 days after surgery. This weight loss might be induced by trauma and loss of fluid during the surgical procedure [6].

From our functional study, the locomotor function assessment revealed significant ($P < 0.05$) neurological deficits in SCI rats. The rats become paralyzed immediately after the SCI procedure and neurological recovery was observed after one week. The BBB locomotor rating scale [31] was employed in this study as it has been shown to produce reproducible results and remains the gold standard in evaluating the functional assessment of the spinal cord

injury model. The results of the BBB score obtained from this study indicated that *S. platensis* supplementation prevented neurological deficits in the SCI rats. This was proved by the BBB score analysis which showed that the BBB score in the experimental group was significantly higher ($P < 0.05$) than that in the control group at days 21 and 28 after operation. As such, the behavioral assessment supported the beneficial effect of the *S. platensis* supplementation.

The morphological study was further analyzed as it supported the functional study outcomes. The spinal cord undergoes a sequence of pathological changes after a traumatic injury that causes the appearance of edema, hemorrhage, neuronal necrosis, axonal necrosis, demyelination, cyst formation, and also cavitation [32–34]. The histopathological outcomes in the animals in the control group in this study which had undergone the crush injury showed a presence of lesions in the tissue and there was an extended area of hemorrhage along the dorsal length of the cord. After a week, the crush site and the surrounding hemorrhagic zone had developed to progressive necrosis and cavitation. This finding was comparable with that of previous studies which have been well-characterized in rats [30, 35–37]. Furthermore, a previous study by Ducker et al. [38] also showed that the pathological changes worsened with time when necrosis is found in the tissue 6 days after an injury, which is quite similar to what we found at day 7 after the injury. The present study showed areas of empty lesions in the tissue where the large cavities developed are bordered by glial connective tissue scarring. This is similar to the results in the previous study conducted by Fujiki et al. and Zhang et al. indicating that a progressive series of cavitation resulted in an empty lesion [39, 40].

The presence of leukocytes in the spinal cord injury tissue represents the pathological changes towards the injury. From the microscopic observation in this study, we found that neutrophils are present at the injury site in response to the trauma. This observation is consistent with a previous study [41], where neutrophils accumulated at the site of compression. The accumulation of neutrophils in the areas of the hemorrhagic zone after a spinal cord injury is believed to act as phagocytes of red blood cells and necrotic tissue [41, 42]. Neutrophils play a vital role in secondary injury processes in traumatic injured rat spinal cords as the interaction between activated neutrophils and endothelial cells leads to spinal cord injury. These activated neutrophils release reactive oxygen species that can damage endothelial cells. This means that both the reactive oxygen species and the neutrophils are the main components in damaging the endothelial cells involved in the pathological changes which then result in the spinal cord injury. In this study, even though the *S. platensis* did not inhibit the accumulation of the neutrophils, it is not the only main feature as a new strategy for reducing the severity of spinal cord injury. To date, MP is still being used as a pharmacological treatment of spinal cord injury patients even though it does not inhibit neutrophil activation [43].

From the quantitative analysis, we found that *S. platensis* showed its efficacy to inhibit the morphological damage in the spinal cord tissue. *S. platensis* has been proven to be a neuroprotective agent in cerebral-ischemia injury rats [17, 18], but no previous research has tested it in rats with spinal cord injuries. In this study, we found that the lesion and cavity size remained smaller in the experimental group compared to the control group throughout the period following the injury. The *S. platensis* supplementation in this study demonstrated its capacity to reduce neurological deficits in the spinal cord. We found that the pattern of recovery in both control and experimental groups was the same but the size of the lesion and cavity in the experimental group was smaller than that in the control group, which indicate that *S. platensis* may inhibit the neurological deficits in the rat from that particular group. The reduction of lesion and cavity size is among indicators of the beneficial effects of the treatment, as was demonstrated in a previous study [30], which also reported that a reduction in lesion and cavity size was seen in the treated group.

5. Conclusion

The supplementation of *S. platensis* may inhibit progressive damage on the spinal cord injury tissue and can improve hindlimb locomotor function in traumatic injured rat spinal cords. More research is needed to confirm the efficacy of *S. platensis* in spinal cord injury studies as it may offer alternatives to SCI management.

Conflict of Interests

All authors have nothing to disclose and have no commercial or financial conflict of interests in the products described in this research paper.

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Research Article

Neuroprotective Effects of Cuscutae Semen in a Mouse Model of Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative movement disorder that is characterized by the progressive degeneration of the dopaminergic (DA) pathway. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes damage to the DA neurons, and 1-4-methyl-4-phenylpyridinium (MPP^+) causes cell death in differentiated PC12 cells that is similar to the degeneration that occurs in PD. Moreover, MPTP treatment increases the activity of the brain's immune cells, reactive oxygen species- (ROS-) generating processes, and glutathione peroxidase. We recently reported that Cuscutae Semen (CS), a widely used traditional herbal medicine, increases cell viability in a yeast model of PD. In the present study, we examined the inhibitory effect of CS on the neurotoxicity of MPTP in mice and on the MPP+-induced cell death in differentiated PC12 cells. The MPTP-induced loss of nigral DA neurons was partly inhibited by CS-mediated decreases in ROS generation. The activation of microglia was slightly inhibited by CS, although this effect did not reach statistical significance. Furthermore, CS may reduce the MPP+ toxicity in PC12 cells by suppressing glutathione peroxidase activation. These results suggest that CS may be beneficial for the treatment of neurodegenerative diseases such as PD.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by resting tremor, rigidity, slowed movements, and bradykinesia, which result from the loss of substantia nigra (SN) dopaminergic (DA) neurons and their projections to the striatum (STR) [1–4]. The etiology of this disease is unknown, but genetic, environmental, age-related, and inflammatory processes are factors that affect disease onset and progression [5–8]. The pathological hallmark of PD is the loss of DA neurons in the SN that project to the STR, which plays a fundamental role in normal motor function [4]. Clinical parkinsonian symptoms occur when 60 to 70 percent of SN DA neurons are lost, and losses in this range result in decreases in dopamine levels in the nigrostriatal system [9–11]. Although PD is a sporadic disease of unknown pathogenesis, accumulating evidence suggests that glial activation-derived oxidative stress increases the risk of developing PD [12]. *In vivo* and *in vitro* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of PD have shown that key enzymes involved in the production of reactive oxygen species (ROS) are upregulated in damaged areas and contribute to the death of DA neurons [13–16].

Cuscutae Semen (CS) has been widely used in traditional herbal medicine to modulate immune-related diseases such as hepatotoxicity and osteoblast differentiation [17, 18]. Several studies have suggested that CS invigorates the reproductive system and improves kidney deficits in animal models [19, 20]. Recent research has shown that CS affects oxidative stress in the brains of aged rodents [1, 2]. Additionally, CS has been shown to enhance lymphocyte proliferation both *in vitro* and *in vivo* [21, 22]. These reports suggest that CS has the potential to modulate immune responses. Although a number of the components of CS have been identified,

the mechanisms of and ingredients responsible for the immunomodulatory effects of CS remain to be illuminated.

In the present study, we sought to determine whether CS promotes the survival of DA neurons both in neuronal differentiated PC12 cells and the MPTP-induced mouse model of PD. We also investigated whether CS was ultimately associated with reductions in ROS generation and oxidative stress.

2. Materials and Methods

2.1. Reagents. Water-extracted dried Cuscutae Semen (CS) was purchased from Sun Ten Pharmaceutical (Taipei, Taiwan). Water extraction of Cuscutae Semen was performed following the manufacturer's protocol. First, the Cuscutae Semen was water-extracted at 100°C for 60 min. Then, the remains of the herbs and impurities were separated from the extracted liquid in a filtration separation process. The water extracts were then spray dried, and corn starch was added as an excipient to stabilize the concentrated herbal products (final ratio of water extracts versus starch of 7 : 3). After these procedures, the final product of Cuscutae Semen extract (ST) was produced in Sun Ten. The Cuscutae Semen extract powder was dissolved in PBS. MPTP-HCl (20 mg/kg, free base in saline; Sigma-Aldrich, St. Louis, MO) was also dissolved in PBS.

For the phytochemical analysis of CS, high-performance liquid chromatography (HPLC) was performed. The CS was accurately weighed to 500 mg and then dissolved in 50 mL 100% methanol. The sample was extracted in an ultrasonic bath for 60 min at 50°C. The suspension was filtered, and the filtrate was evaporated in vacuo. The residue was dissolved in methanol (500 μL), and the obtained solution was filtered through a membrane filter (0.45 μm pore size) prior to injection. For the quantitative analysis of CS, kaempferol, one of the known flavonol constituents of CS, was diluted in acetonitrile to five different concentrations (25, 50, 100, 200, and 500 ppm). Each solution was filtered through a membrane filter (0.45 μm pore size) prior to injection. The HPLC analysis was performed using a Waters Alliance system equipped with an in-line degasser and a photodiode array detector. The UV spectra were collected across the range of 200 to 500 nm and monitored at 360 nm for chromatograms. Empower software was used for instrument control, data collection, and data processing. The HPLC column was an Inspire C18 (4.6 × 250 mm, 5 μm). The mobile phase was an isocratic system with acetonitrile (A) phosphoric acid 0.25% (B) run for 15 min. The ratio of A : B was 39 : 61. The flow rate was 1 mL/min. The injection volume for all samples and standard solutions was 10 μL. Using this method, the concentration of kaempferol in CS was calculated to be 0.24 ± 0.01 μg/g as described in Figure 1.

2.2. Cell Culture and Drug Treatment. PC12 cells were obtained from the Korean Cell Line Bank. PC12 cells were cultured in Dulbecco's modified Eagle's media (DMEM, Welgene) supplemented with 10% fetal bovine serum (FBS, Welgene), 5% horse serum (HS, Welgene), 1% penicillin, and 1% streptomycin in a water-saturated atmosphere of 5% CO_2 at 37°C. PC12 cells were incubated in 50 ng/mL nerve growth factor (NGF) for differentiation. MPP+ (Sigma-Aldrich, St. Louis, MO) and CS (Sun Ten Pharmaceutical, Taipei, Taiwan) were dissolved in PBS and prepared as a 10 mmol/L stock immediately before use. The drugs were diluted in culture medium to the indicated final concentrations. The total volume of the reaction system in the following experiments was 5 mL.

2.3. Measurement of Cell Viability. Intracellular ROS was evaluated using the fluorescent probe 2,7-dichlorofluorescein diacetate (H_2CDFDA). H_2CDFDA is oxidized to the highly fluorescent compound dichlorofluorescein (DCF) by intracellular hydroperoxides. The generation of DCF is proportional to intracellular ROS levels. After exposure to 75 μmol/L MPP+ in the presence or absence of CS for 24 h, PC12 cells were incubated with 10 μmol/L H_2CDFDA (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h. The cells were washed twice with FBS-free media. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm using a fluorescence microplate reader.

2.4. Measurement of Intracellular Reactive Oxygen Species Formation. Intracellular ROS was evaluated using the fluorescent probe 2, 7-dichlorofluorescein diacetate (H_2CDFDA). H_2CDFDA is oxidized to the highly fluorescent compound dichlorofluorescein (DCF) by intracellular hydroperoxides. The generation of DCF is proportional to intracellular ROS levels. After exposure to 75 μmol/L MPP+ in the presence or absence of CS for 24 h, PC12 cells were incubated with 10 μmol/L H_2CDFDA (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h. The cells were washed twice with FBS free media. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm using a fluorescence microplate reader.

2.5. Measurement of Glutathione Peroxidase Activity. The effect of CS treatment on GSH level was measured using a commercial kit (Cayman Chemical Co., Ann Arbor, MI, USA). The homogenate of PC12 cells was used for GSH measurement with the GSH Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer's protocol. The GSH content was determined by comparison with the standards and normalized to protein content.

2.6. Animals and MPTP Administration. All of the experiments were conducted with 7- to 8-week-old male C57BL/6J mice (weighing 21-22 g; Japan SLC Inc., Hamamatsu, Japan). The mice were maintained in a room at 20–22°C on a 12 h light/dark cycle with food supplied ad libitum. The study was approved by the University of Kyung Hee Animal Care and Use Committee. Mice were randomly divided into five groups of six mice each. For MPTP intoxication, the mice received four intraperitoneal (i.p.) injections of MPTP-HCl (20 mg/kg, free base in saline; Sigma-Aldrich, St. Louis, MO) dissolved in PBS at 2 h intervals. Twelve hours after the last MPTP injection, the MPTP-injected mice received single daily p.o. administrations of CS (50, 100, or 200 mg/kg) or PBS for 6

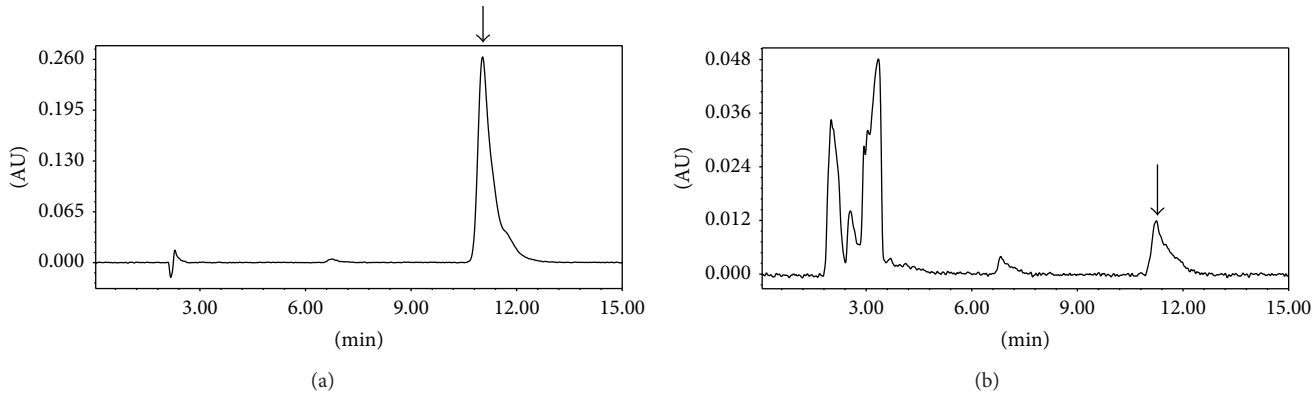

Figure 1: The HPLC analysis of the standard material to CS. Phytochemical analysis was performed by HPLC as described in the materials and methods section. Kaempferol was utilized as an authentic standard (arrows) and it was found that the concentration of kaempferol in CS was calculated to be $0.24 \pm 0.01\,\mu$g/g. (a) HPLC chromatogram of kaempferol standard solution, (b) methanol extract of Cuscutae Semen granule.

days. Control mice were administered either CS or vehicle alone.

2.7. Measurement of Motor Activity. The pole test has been utilized to measure motor coordination and balance in mouse models of PD [3]. We performed the pole test on the 7th day after the last MPTP injection. In this test, animals were placed on top of a rough-surfaced iron pole (50 cm in length and 0.8 cm in diameter) and allowed to climb down to the base of the pole. The times that it took for the mouse to turn completely downward (time to turn; T-turn) and then to reach the floor (locomotion activity time; T-LA) were recorded, with a cut-off limit of 30 s. The average of the best three measurements was used as the result.

2.8. Tissue Preparation and Immunostaining. The mice were transcardially perfused with a saline solution containing 0.5% sodium nitrate and heparin (10 U/mL) and then fixed with 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer (PB). The brains were dissected from the skulls, postfixed overnight in buffered 4% paraformaldehyde at 4°C, stored in a 30% sucrose solution at 4°C until they sank, and frozen sectioned on a sliding microtome in 30 μm thick coronal sections. All sections were collected in six separate series and processed for immunostaining as described previously [20]. In brief, the brain sections were rinsed in PBS and then incubated overnight at room temperature with the primary antibodies. The following day, the brain sections were rinsed with PBS and 0.5% bovine serum albumin (BSA), incubated with the appropriate biotinylated secondary antibody, and processed with an avidin-biotin complex kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). The bound antiserum was visualized by incubation with 0.05% diaminobenzidine-HCl (DAB) and 0.003% hydrogen peroxide in 0.1 M PB. The DAB reaction was stopped by rinsing the tissues in 0.1 M PB. The primary antibodies included antibodies directed against tyrosine hydroxylase (TH; 1 : 2000, Pel-freez, Brown Deer, WI) and CD11b (1 : 200; Serotec, Oxford, UK). Labeled tissue sections were then mounted on gelatin-coated slides and analyzed under a bright-field microscope (Nikon, Tokyo, Japan).

2.9. Stereological Cell Counting. Unbiased stereological estimations of the total numbers of TH-positive DA neurons and CD11b-positive microglia/macrophages in the SN were made using the optical fractionator method, which was performed on an Olympus computer assisted stereological toolbox (CAST) system (version 2.1.4, Olympus, Ballerup, Denmark) as previously described [21]. The sections used for counting covered the entire SN from the rostral tip of the pars compacta to the caudal end of the pars reticulata (anterioposterior: −2.06 to −4.16 mm from bregma). The actual counting was performed using a 1003 oil objective. The total number of cells was estimated according to the optical fractionator equation [22]. More than 300 points across all sections from each specimen were analyzed.

2.10. Densitometry Analysis. As previously described [5], an average of 17 coronal sections of the STR, starting rostrally from +1.60 mm anteroposterior and extending caudally to 0.00 mm, were examined at a 5x magnification using the IMAGE PRO PLUS system (version 4.0; Media Cybernetics, Silver Spring, MD) on a computer attached to a light microscope (Zeiss Axioskop, Oberkochen, Germany) and interfaced with a video camera with a charge-coupled device (Kodak Mega Plus model 1.4 I). To determine the density of the TH-immunoreactive staining in the STR, a square frame of $700 \times 700\,\mu$m was placed in the dorsal part of the STR. A second square frame of $200 \times 200\,\mu$m was placed in the region of the corpus callosum to measure background values. To control for variations in background illumination, the average of the background density readings from the corpus callosum was subtracted from the average of the density readings of the STR for each section as described previously [5]. Next, the average of all sections was calculated individually for each animal before the data were statistically processed.

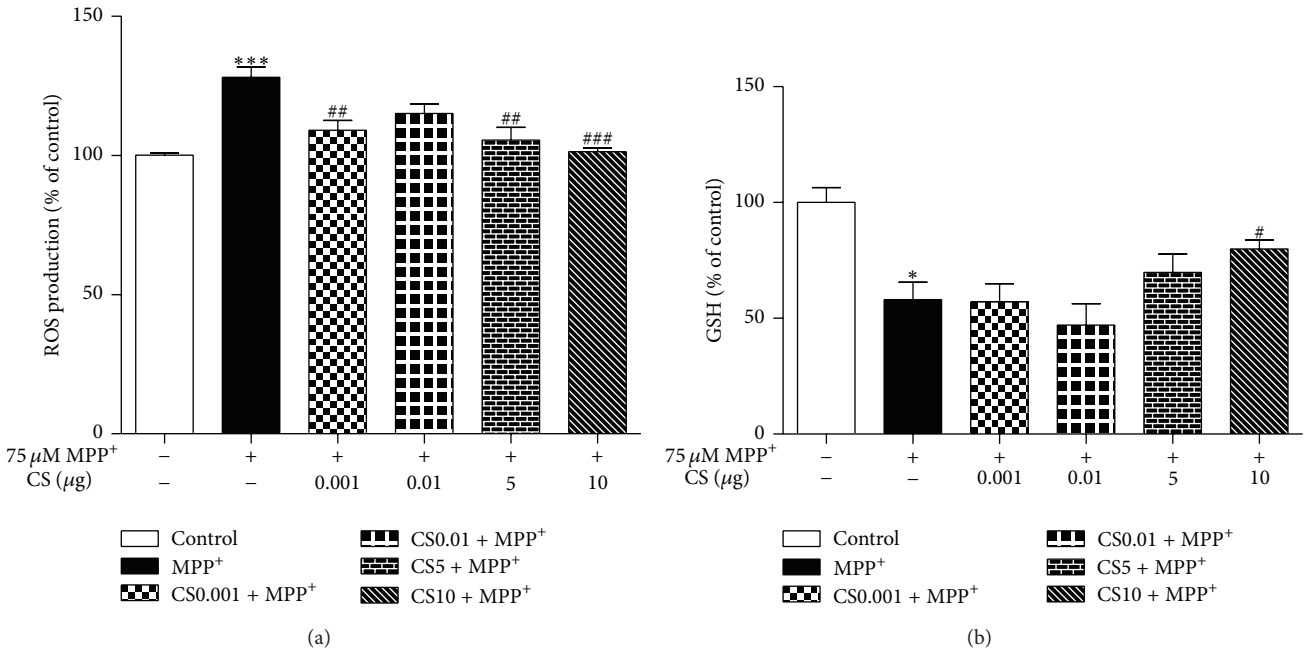

Figure 2: Effects of CS on the formation of reactive oxygen species (ROS) and glutathione (GSH) depletion due to MPP+. The PC12 cells (1×10^5 cells/mL) were treated with 1 mM MPP+ in the presence of CS (0.001, 0.01, 5, and 10 μg) for 24 h at 37°C. Formation of ROS was assayed by measuring fluorescence of dichlorofluorescein (DCF, a); GSH contents (μmol/mg protein) were determined using GSH reductase (b). Data are expressed as the mean ± S.E.M. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with MPP^+.

2.11. In Situ Detection of O_2^-- and O_2^--Derived Oxidants. Three days after final MPTP injection, hydroethidine (Molecular Probes; 1 mg/mL in PBS containing 1% dimethyl sulfoxide) was administered intraperitoneally. After 15 min, the animals were transcardially perfused and postfixed, and the brains were cut into 30 μm sections. Hydroethidine histochemistry was performed for the in situ visualization of O_2^-- and O_2^--derived oxidants as previously described [23]. The oxidized hydroethidine product, ethidium, was examined by confocal microscopy (Olympus).

2.12. Statistical Analyses. All values are expressed as the means ± the SEMs. The statistical significance ($P < 0.05$ for all analyses) of comparisons across multiple groups was assessed with one-way or two-way ANOVAs, and Student's t-tests were used for single comparisons. All statistical analyses were performed with Prism 5.01 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effects of CS on ROS Formation and GSH Depletion in MPP+-Induced PC12 Cells. After exposure to 75 μmol/L MPP+, the fluorescence intensity of DCF increased to about twice the control value. CS prevented the MPP+-induced increase in DCF fluorescence and showed a maximal inhibitory effect at 10 μg (Figure 2). ROS formation was not further attenuated by 0.01 μg concentrations of CS.

A reduction in GSH levels increases the sensitivity of neurons to the toxic effect of neurotoxins [24] and is associated with mitochondrial dysfunction [25]. In the present study, we assessed the effect of CS on the MPP+-induced decrease in GSH. CS prevented the MPP+-induced decrease in GSH (Figure 2).

3.2. Inhibitory Effects of CS on Behavioral Impairment by MPTP in Mice. To confirm the effect of AJW on dopaminergic neurons in an *in vivo* PD model, we treated mice with CS and/or MPTP and carried out the pole test. As a result, the T-turn and T-LA of MPTP-only treated mice were markedly prolonged at 12.35 ± 1.85 sec and 20.02 ± 1.49 sec compared with the control group (T-turn: 4.47 ± 1.46 sec; T-LA: 12.07 ± 1.38 sec) (Figure 3). However, the times of the CS 50 mg/kg/day treated group were shortened to 16.47 ± 2.5 sec for the T-turn, and the CS 200 mg/kg/day treated group showed a more shortened T-turn at 4.61 ± 1.53 sec and T-LA at 11.64 ± 1.74 sec (Figure 3).

3.3. CS Protects DA Neurons in the SN against MPTP Neurotoxicity. In the brain, MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP+), which is the active toxic compound that is primarily responsible for MPTP-induced neurotoxicity [24]. MPTP was rapidly eliminated and nearly undetectable at 6 h, and striatal MPP+ levels peaked within 30 min and subsequently declined steadily until levels no longer differed significantly from the control after 12 h [21]. Accordingly, CS was administered 12 h after the last injection of MPTP for all *in vivo* experiments to avoid interference with the metabolism of MPTP. To examine the neuroprotective effects of CS, the MPTP-injected mice

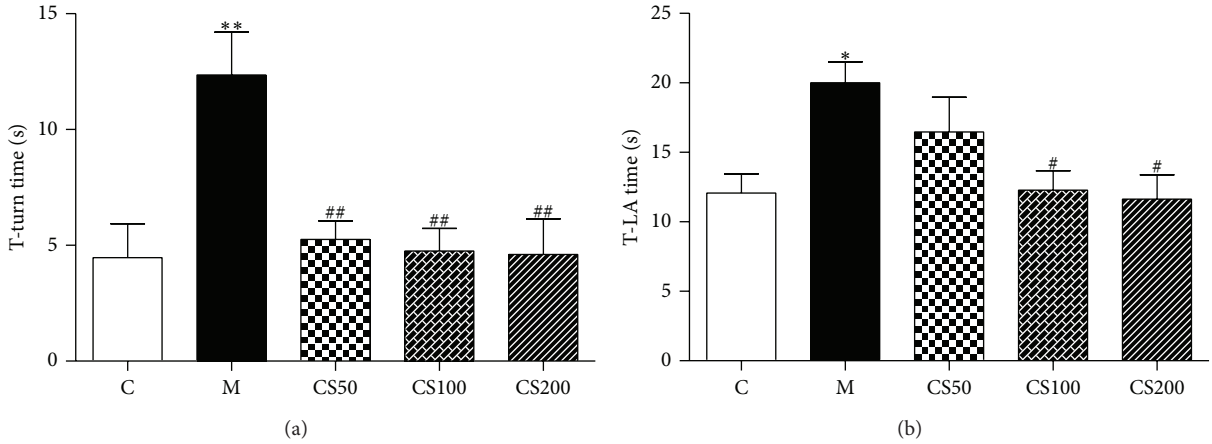

FIGURE 3: Effect of CS on MPTP-induced behavioral deficits in mice. Mice were pretreated with CS (50, 100, or 200 mg/kg) for six days and then injected with MPTP (20 mg/kg i.p.). The time taken for the mice to turn completely downward ((a); T-turn) and the time taken to reach the floor ((b); T-LA) were recorded. Values are indicated as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the MPTP-only treated group.

were treated with CS (50, 100, or 200 mg/kg) or PBS for 6 days. Seven days after the MPTP injections, brain sections were immunostained with an anti-TH antibody to detect DA neurons (Figure 4(a)). In MPTP-injected mice, there was a significant loss of TH^+ neurons in the SN (56%; $P < 0.001$; Figure 4(b)), and the density of TH^+ fibers in the STR was reduced by 51% ($P < 0.001$; Figure 4(b)) compared to the PBS-injected control mice. CS treatment effectively blocked DA neuronal damage in the SN by 17% ($P < 0.05$; Figure 2(b)) compared to the PBS-injected control mice.

3.4. CS Protects DA Fibers in the STR against MPTP Neurotoxicity. In the brain, MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP+), which is the active toxic compound that is primarily responsible for the neurotoxicity of MPTP [4]. MPTP was rapidly eliminated and nearly undetectable at 6 h, and striatal MPP+ levels peaked within 30 min and subsequently declined steadily until they were no longer significantly different from the controls after 12 h [5]. Accordingly, CS was administered 12 h after the final injection of MPTP in all *in vivo* experiments to avoid interfering with the metabolism of MPTP. To examine the neuroprotective effect of CS, MPTP-injected mice were treated with CS (50, 100, or 200 mg/kg) or PBS for 6 days. Seven days after the MPTP injection, brain sections were immunostained with an anti-TH antibody to detect DA neurons (Figure 5(a)). In the MPTP-injected mice, there was a significant reduction in the density of TH^+ fibers in the STR (55%; $P < 0.001$; Figure 5(b)) compared to the PBS-injected control mice. CS treatment effectively blocked the reduction in the density of TH^+ fibers in the STR by 23% ($P < 0.001$; Figure 5(b)) compared to the MPTP mice.

3.5. Effectiveness of CS against Microglia-Derived Neurotoxicity. Activated microglia play important roles in the death of DA neurons in PD patients [6, 7] and the MPTP-induced animal model of PD [5]. To determine whether the neuroprotective effect of CS resulted from the inhibition of microglial activation in the SN, we performed immunostaining with anti-CD11b to detect microglia/macrophages in brain sections that were prepared 7 days after the final MPTP injection (Figure 6). In saline-treated control mice, few $CD11b^+$ microglia/macrophages with resting morphologies (i.e., small cell bodies and thin processes) were observed in the SN on the 7th day. In contrast, numerous $CD11b^+$ microglia/macrophages with activated morphologies (i.e., larger cell bodies and thick processes) were apparent in the SN of the MPTP-injected mice on the 7th day. In the MPTP-injected mice treated with CS, activated microglia/macrophages also appeared in the SN on the 7th day after MPTP injection. Thus, CS had no effect on $CD11b^+$ microglia/macrophage activation in the SN.

3.6. CS Reduces Oxidant Production in the SN of MPTP-Injected Mice. Recent studies have suggested that activated microglia produce O_2^-- and O_2^--derived oxidants [8, 12]. We investigated whether CS enhanced DA neuron survival by inhibiting MPTP-induced ROS production. The fluorescent products of oxidized hydroethidine (i.e., ethidium accumulation) were significantly increased at 48 h in the SNs of the MPTP-injected mice (Figure 7) compared to the PBS-injected controls (Figure 7). This MPTP-induced oxidant production was dramatically decreased by CS (Figure 7).

4. Discussion

In this study, the protective effects of CS were verified *in vitro* using a PD cellular model induced by MPP+ in PC12 cells and *in vivo* using a PD model induced by MPTP administration in mice. We showed that CS suppressed MPTP-induced ROS generation and reduced oxidative stress, which led to the

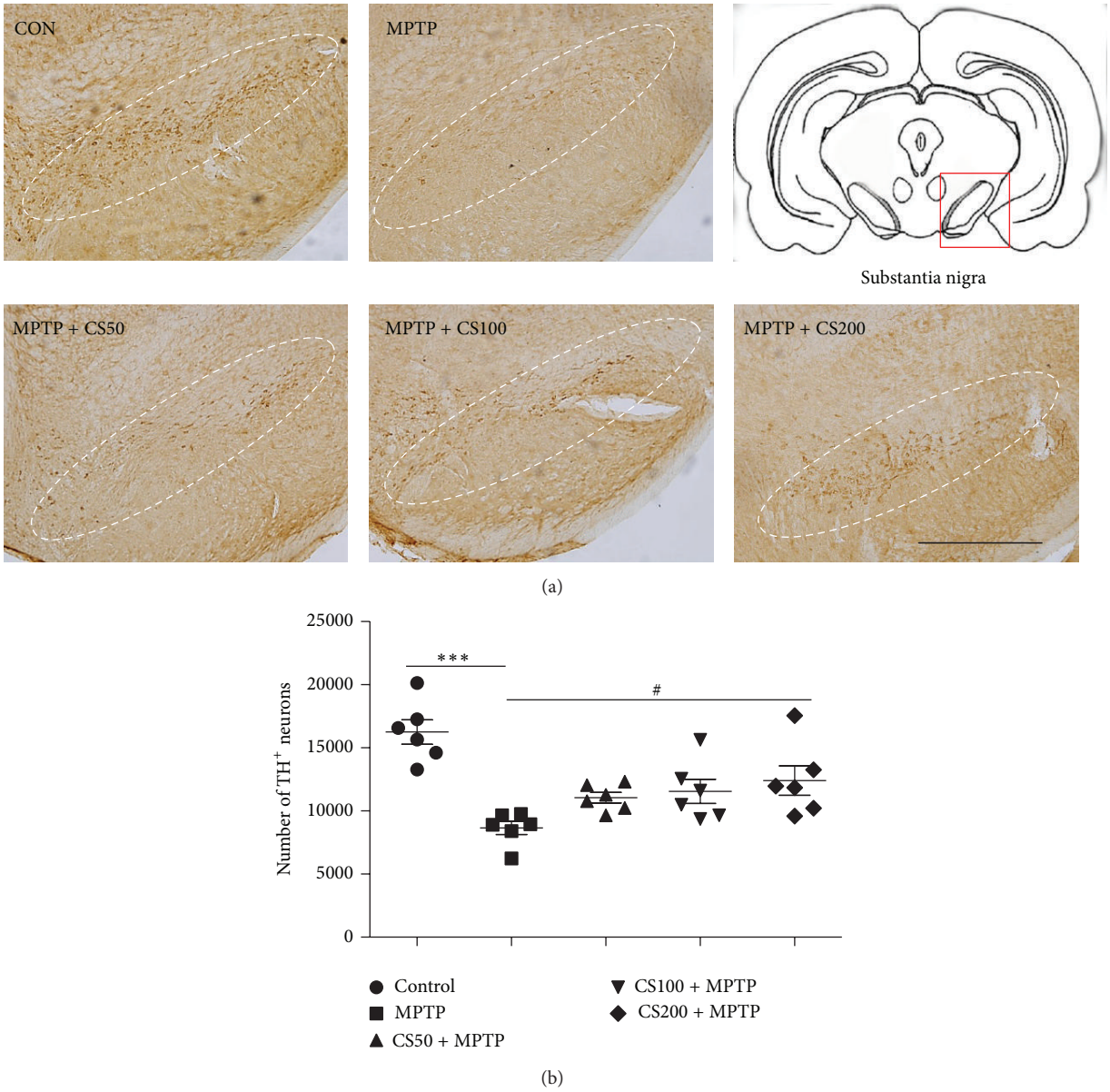

Figure 4: CS protects against DA neuronal death due to MPTP-induced neurotoxicity. At 7 days after the final MPTP injection, the animals were sacrificed, and the brains were prepared for immunohistochemistry. Tyrosine hydroxylase (TH) immunostaining of the SNs of the mice treated with PBS (control), MPTP, and vehicle or MPTP and CS (50, 100, or 200 mg/kg). The representative images were displayed (a). The scale bars represent 500 μm. The number of TH-positive neurons in the SN measured (b). At 7 days after the final MPTP injection, the animals were sacrificed, and the brains were prepared for immunohistochemistry. The error bars represent the SEMs. $^{\#}P < 0.05$, significantly different from the MPTP/PBS treated control group. $^{***}P < 0.001$, significantly different from the saline with PBS group (one-way ANOVA and Student-Newman-Keuls analyses). Dotted lines indicate the SN. The schematic brain sections are from the atlas (Paxinos and Watson, 1986). The square represents SN in the mouse brain. C: control; M: MPTP; CS50: CS50+MPTP; CS100: CS100+MPTP; CS200: CS200+MPTP.

survival of nigrostriatal DA neurons. At the cellular level, CS seems to reduce the MPP+-induced cell viability as well as ROS and GSH levels. To our knowledge, this is the first study to show that CS prevents nigrostriatal DA neuronal death via the blockade of ROS generation in the MPTP model of PD.

Previous studies have described the components of CS, such as quercetin, kaempferol, and hyperoside, as well as their various pharmacological activities [19, 26, 27]. In one of these studies, kaempferol was shown to attenuate the immune function of dendritic cells [26]. Some studies have suggested that CS acts on defective kidneys and the reproductive system in animal models [19, 20] and induces neuronal differentiation. Moreover, CS has hepatoprotective and antioxidant effects against acetaminophen-induced hepatotoxicity [18]. CS has also been shown to be neuroprotective in a psychological stress-induced rat due to its actions on

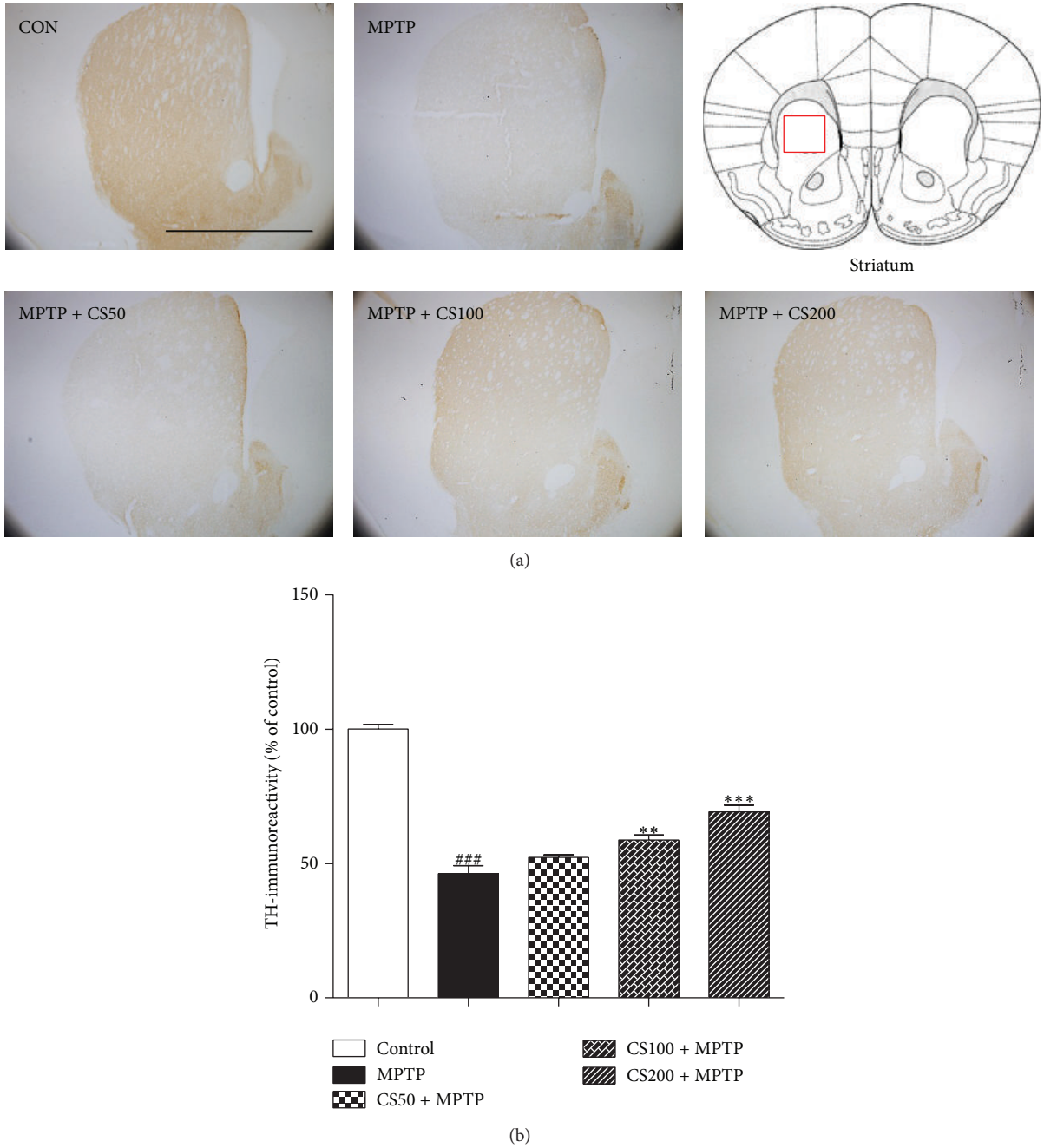

Figure 5: *In vivo* MPTP-induced DA fiber neurotoxicity in the STR. The treatment of the MPTP-injected mice with CS prevented DA neuronal death in the STR. MPTP-injected mice were treated with CS (50, 100, or 200 mg/kg) or PBS for 6 days beginning 12 h after the final MPTP injection. Seven days after the MPTP injections, brain sections were prepared and immunostained with TH antibody to identify DA fibers. The representative images were displayed (a). Scale bar was 5 mm. The densities of the TH-fibers in the STR were stereologically counted (b). The error bars represent the SEMs. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from the MPTP with PBS group. $^{###}P < 0.001$, significantly different from the saline with PBS group (one-way ANOVA and Student-Newman-Keuls analyses). The schematic brain sections are from the atlas (Paxinos and Franklin, 1997). The square represents STR in the mouse brain. C: control; M: MPTP; CS50: CS50+MPTP; CS100: CS100+MPTP; CS200: CS200+MPTP.

the hypothalamus-pituitary-ovary axis [28, 29]. Additionally, CS has been demonstrated to have protective effects in D-galactose-induced mimetic aging mice [1] and aging rats [2] that are mediated by improvements in the antioxidant statuses of these animals. Based on these findings, CS is relevant to PD because of its neuroprotective effects and its suppression of ROS.

The administration of CS had protective effects against the severe reduction in the levels of TH immunoreactivity in the striatum and SN after MPTP treatment. These results

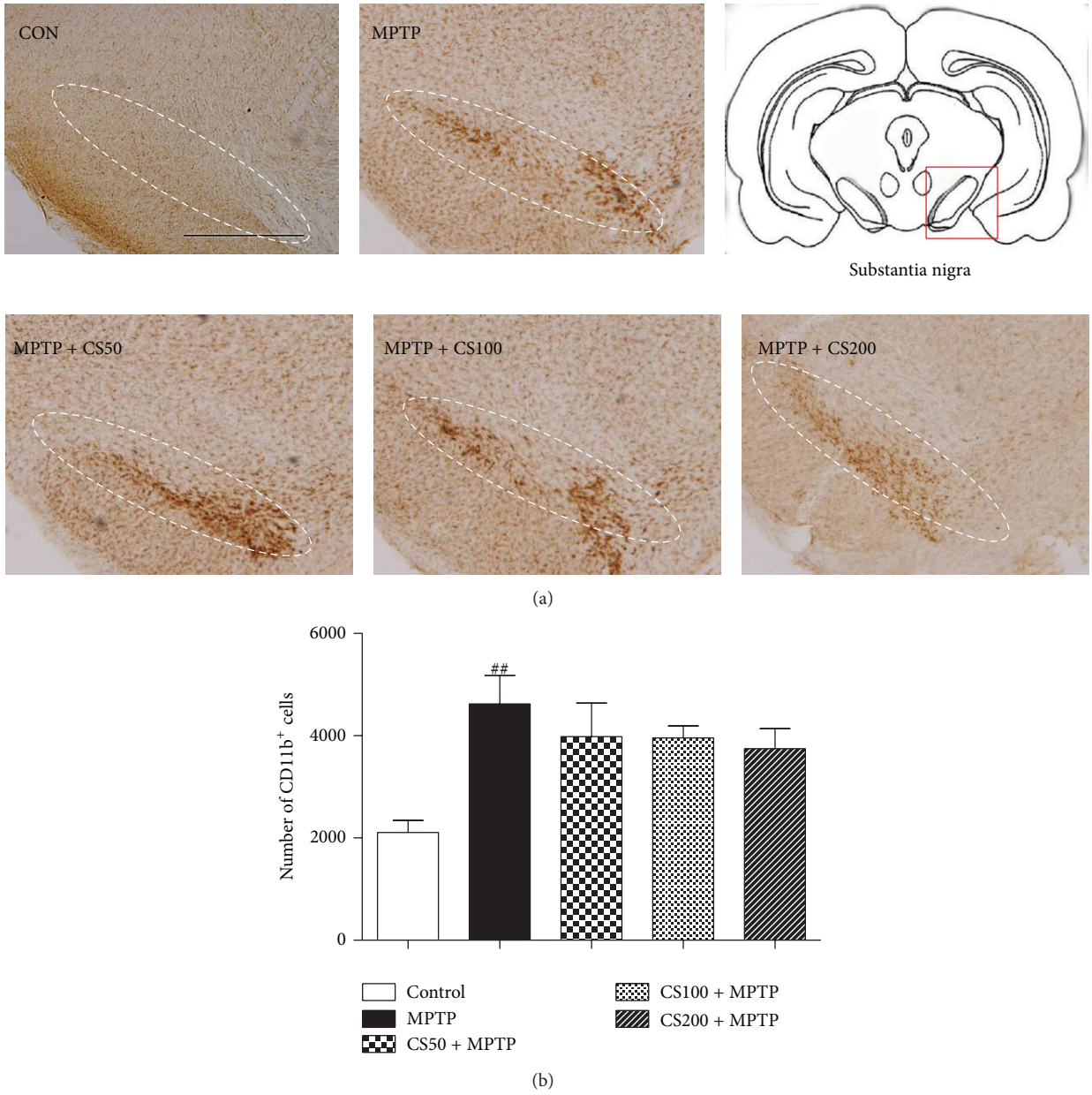

Figure 6: MPTP-induced microglial activation is inhibited by CS. Seven days after the final MPTP injection, the animals were sacrificed, and the brains were prepared for immunohistochemistry. CD11b immunostainings in the SN of mice treated with PBS (control), MPTP, and vehicle or MPTP and CS (50, 100, or 200 mg/kg) are shown. The representative images were displayed (a). The scale bars represent 500 μm. The numbers of CD11b-positive cells in the SN were stereologically counted (b). The error bars represent the SEMs. $^{\#\#}P < 0.001$, significantly different from saline with PBS (one-way ANOVA and Student-Newman-Keuls analyses). Dotted lines indicate the SN. The schematic brain sections are from the atlas (Paxinos and Watson, 1986). The square represents SN in the mouse brain. C: control; M: MPTP; CS50: CS50+MPTP; CS100: CS100+MPTP; CS200: CS200+MPTP.

seem to suggest that CS can protect against the neuronal damage caused by MPTP toxicity.

Microglia are the resident macrophages of the brain and play crucial roles in the development and preservation of the neural environment [30]. However, in the presence of reverse stimuli, microglia can induce chronic, damaging inflammation that ultimately leads to neuronal cell death. Microglia are the intrinsic immune effector cells that are activated in response to neuronal damage [31]. Several direct neurotoxins can activate microglia through the production of various factors, including ROS and proinflammatory cytokines [8, 32].

The conversion of MPTP to MPP+ by monoamine oxidase-B (MAO-B) in astrocytes is followed by the accumulation of MPP+ in the DA neurons of the SN due to the activity of DAT. This accumulation within the DA neurons

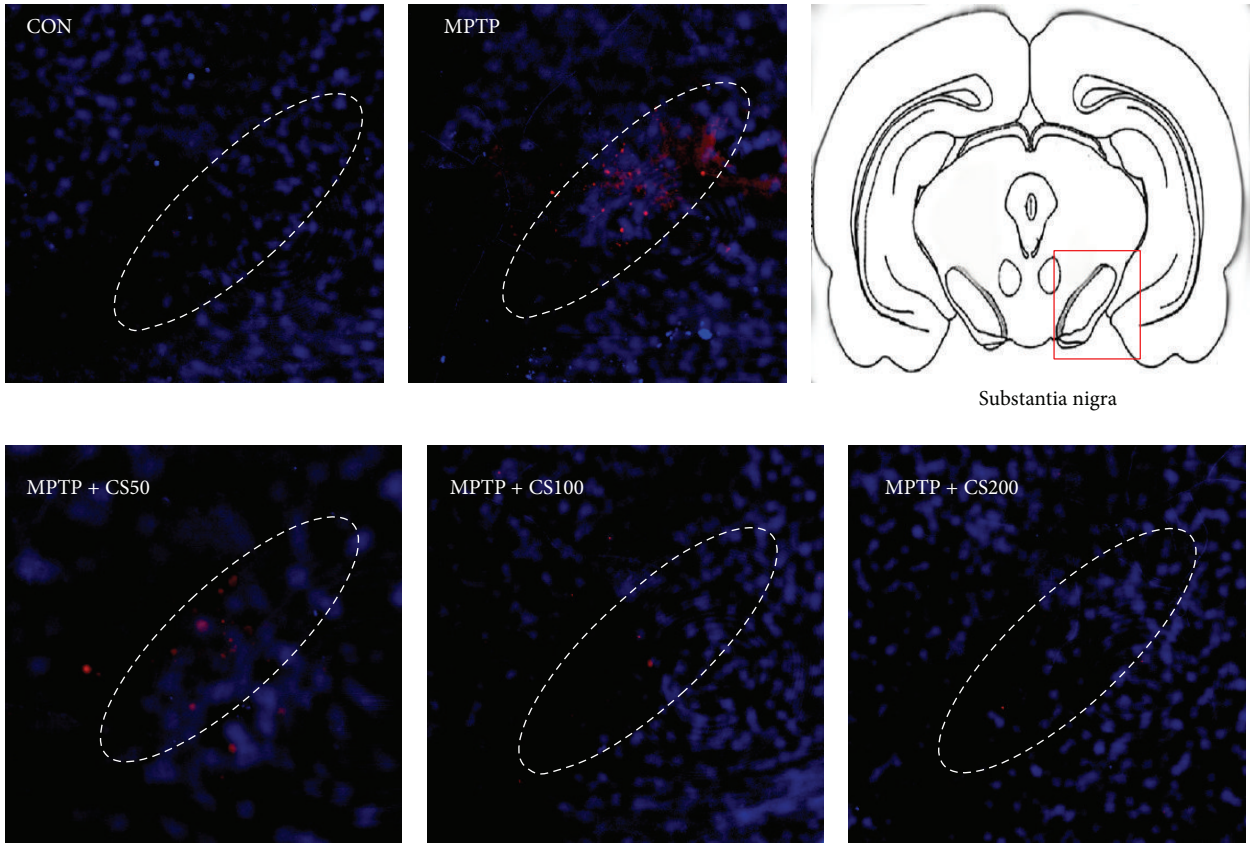

Figure 7: MPTP-induced ROS generation is inhibited by CS. CS inhibits MPTP-induced O_2^- and O_2^--derived oxidant production and protein oxidation in the SN. The animals received hydroethidine 48 h after the intranigral injection of MPTP in the absence or presence of CS. After 45 min, the brains were harvested, and sections of the SN were prepared for hydroethidine histochemistry to detect extracellular superoxide. Confocal microscopic observation revealed ethidium fluorescence (red) in the SN. The nuclei were counterstained with Hoechst 33258 (blue). These data are representative of five to six animals per group. Dotted lines indicate the SN. The schematic brain sections are from the atlas (Paxinos and Watson, 1986). The square represents SN in the mouse brain.

results in the generation of reactive oxygen species (ROS) by the mitochondria, including nitric oxide (NO), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) [33, 34]. ROS may result in oxidative stress to the DA neurons and induce and/or aggravate neurotoxicity. Some studies have implicated oxidative stress as a major cause of neuronal injury in neurological diseases such as PD [35–37]. As a pathogenic condition, oxidative damage may account for the degeneration of DA neurons in the SN of PD brains [38, 39] and in the brains of MPTP-treated mice [40]. The ROS responsible for these molecular modifications can be generated by microglial NADPH oxidase and play important roles in the development of oxidative stress in the MPTP model of PD. These data support the hypothesis that the observed neuroprotective effects of CS were mediated by reductions in oxidative damage.

To evaluate the toxicity of MPTP on cell viability, we used the toxicity of MPP+ instead of MPTP against differentiated PC12 cells. Upon nerve growth factor stimulation, PC12 cells not only reveal abundant neuritic growth but also choose a neurochemical dopaminergic phenotype [41]. MPP+-induced cellular toxicity is mediated through ROS generation [42]. In line with a previous study [43], PC12 cells produced a significant amount of intracellular ROS when exposed to 75 μM MPP+ for 24 h. However, pretreatment with CS suppressed the MPP+-induced accumulation of ROS. These findings show that CS exhibits broad-spectrum antioxidant activity. Oxidative stress results from a redox imbalance between the formation and deletion of ROS. Glutathione (GSH) is the most plentiful antioxidant in cells and plays an important role in cellular defense against oxidative stress [44]. In the present study, MPP+ induced a marked decline in the total GSH level in PC12 cells, whereas pretreatment with CS significantly restored the MPP+-induced decrease in total GSH.

CS has anti-inflammatory activities that reduce the production of cytokines and chemokines by mouse bone marrow-derived dendritic cells when these cells are stimulated with LPS [45]. However, CS was not found to be effective in reducing the microglial activation in the SN of MPTP-injected mice. MPTP directly damages DA neurons when it is taken up through the DA transporter; this uptake results in oxidative mitochondrial damage, which leads to neuronal death. MPP+-induced neuronal damage activates microglia

[16] to produce some potentially toxic substances that can damage neurons via an indirect mechanism. Our results imply that the neuroprotective effects of CS are associated with a blockade of oxidative stress in DA neurons and are not associated with the microglial activation that occurs in the MPTP-induced PD animal model.

5. Conclusion

In conclusion, CS protected dopaminergic cells from MPP+ toxicities in PC12 cells and against MPTP toxicities in C57BL/6 mice. *In vitro*, the neuroprotective effects of CS are due to its antioxidant activities, including the inhibition of ROS and an increase in GSH. *In vivo*, CS inhibited the production of ROS, prevented oxidative damage to neurons, and led to increased neuronal survival. These results suggest that CS may be beneficial as a novel therapeutic agent for neurodegenerative diseases that are related to oxidative damage, including PD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Cognitive-Enhancing Effect of Steamed and Fermented *Codonopsis lanceolata*: A Behavioral and Biochemical Study

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory impairment. *Codonopsis lanceolata* (*C. lanceolata*) has been employed clinically for lung inflammatory diseases such as asthma, tonsillitis, and pharyngitis. The present study was undertaken to evaluate the effect of fermented *C. lanceolata* (300, 500, and 800 mg/kg) on learning and memory impairment induced by scopolamine by using the Morris water maze and passive avoidance tests. To elucidate possible mechanism of cognitive-enhancing activity, we measured acetylcholinesterase (AchE) activity, brain-derived neurotrophic factor (BDNF), and cyclic AMP response element-binding protein (CREB) expression in the brain of mice. Administration of fermented *C. lanceolata* (800 mg/kg) led to reduced scopolamine-induced memory impairment in the Morris water maze and passive avoidance tests. Accordingly, the administration of fermented *C. lanceolata* inhibited AchE activity. Interestingly, the level of CREB phosphorylation and BDNF expression in hippocampal tissue of scopolamine-treated mice was significantly increased by the administration of fermented *C. lanceolata*. These results indicate that fermented *C. lanceolata* can ameliorate scopolamine-induced memory deficits in mouse and may be an alternative agent for the treatment of AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that results in impaired memory and cognition and is the most common cause of dementia among older people [1, 2]. There are multiple causes of AD and some causes have yet to be discovered. The pathogenesis of AD is defined by the presence of senile plaques, neurofibrillary tangles, and several biochemical factors such as inflammation and oxidative stress. Plaques are formed by the accumulation of β-amyloid, and inflammation around plaques in the brain can lead to cell death. Neurofibrillary tangles consist of the protein tau, a microtubule-associated protein. The presence of neurofibrillary tangles, together with the accumulation of β-amyloid, interferes with normal cellular functioning.

Acetylcholine (Ach), a neurotransmitter of the cholinergic system, plays an important role in memory and information processing. Decreased levels of Ach as well as the Ach synthesizing enzyme choline acetyltransferase (CHAT) in the cerebral cortex are another neuropathology associated with AD [3]. Acetylcholinesterase (AChE) inhibitors, including donepezil, galantamine, and tacrine, and an Ach receptor

agonist have been used in the treatment of AD [4, 5]. However, these medicines have several side effects, such as pain, nausea, and vomiting. Medicinal plants such as *Ginkgo biloba*, *Salvia officinalis* (sage), *Melissa officinalis* (balm), and *Papaver somniferum* (opium poppy) are reported to have beneficial effects in patients with AD and to have fewer side effects [6]. Scopolamine, a muscarinic cholinergic antagonist, has been used to develop animal models of AD and can be used to study the effects of antiamnesic drugs [7]. Scopolamine increases AchE activity in the cortex and hippocampus. In addition, it impairs mitochondrial function and reduces ATP levels [8].

Brain-derived neurotrophic factor (BDNF) plays a role in learning and memory formation and is regulated via transcription factor and cAMP-response element-binding protein (CREB). Downregulation of BDNF expression is associated with memory impairment [9–11].

Codonopsis lanceolata (Campanulaceae) is a traditional medicinal plant used for the treatment of hypertension and several lung inflammatory diseases, such as asthma, tonsillitis, and pharyngitis; it has been used in East Asia for thousands of years [12, 13]. *C. lanceolata* contains various compounds such as saponins, alkaloids, tannins, steroids, and polysaccharides [14]. Previous reports have shown that *C. lanceolata* inhibits the production of TNF-α and nitric oxide, the expression of interleukin (IL)-3 and IL-6, and LPS-mediated phagocytic uptake in RAW 264.7 cells (regulatory effects of *C. lanceolata* on macrophage-mediated immune responses) [15]. It also shows antilipogenic and anti-inflammatory effects in mice with alcohol-induced fatty liver [16]. Lancemaside A, major compounds in *C. lanceolata,* showed enhancing effect of memory, inhibiting AChE activity, and inducing BDNF and p-CREB expressions [17].

Recently, we verified that the fermented *C. lanceolata* ameliorated memory and learning impairment and the steamed and fermentation process significantly improved the cognitive recognition activity compared to original *C. lanceolata* [18–20]. In the present study, we modified the steamed and fermentation process for mass production of fermented *C. lanceolata* and confirmed the effect of fermented *C. lanceolata* on memory and learning impairment using scopolamine-induced memory deficits in the Morris water maze and passive avoidance tests. We investigated the possible mechanism of cognitive enhancement effect of fermented *C. lanceolata* and found that the administration of fermented *C. lanceolata* inhibited AchE activity. Intriguingly, the administration of fermented *C. lanceolata* also increased the expression of BDNF in accordance with increased CREB phosphorylation in hippocampus. Collectively, these results suggest that fermented and steamed *C. lanceolata* can ameliorate scopolamine-induced memory deficits in mouse and may be a possible agent for the treatment of AD.

2. Materials and Methods

2.1. Plant Materials. The roots of *C. lanceolata* were collected from Hoengseong, Gangwon, Korea. The *C. lanceolata* was washed several times with tap water to remove foreign material and dried in the shade at 20–30°C for 2 days. Dried *C. lanceolata* was steamed using a steam device (Dechang Stainless, Seoul, Korea) at 90°C for 8 h and this steaming process was repeated 5 times.

2.2. Fermentation and Extraction. *Bifidobacterium longum* (KACC 20587), *Lactobacillus acidophilus* (KACC 12419), and *Leuconostoc mesenteroides* (KACC 12312) were obtained from the Korean Agricultural Culture Collection (Suwon, Korea). The steamed *C. lanceolata* was mixed in distilled water 8 times the weight of the *C. lanceolata* and aseptically inoculated with approximately 10^6 CFU/g of *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Leuconostoc mesenteroides* (1 : 1 : 1). The inoculated *C. lanceolata* was fermented for 48 h at 30°C. Then, fermented *C. lanceolata* was extracted in 70% ethanol with 5 times the weight of the *C. lanceolata* at 80°C. This extract was concentrated using 24 h of reflux extraction at 70°C and harvested by centrifuging at 15,000 rpm. After evaporation, spray drying was conducted to obtain the fermented *C. lanceolata*.

2.3. Animals. ICR mice (3-week-old males; weight: 25–30 g) were obtained from the Dae Han Biolink Co. (Eumseong, Korea). The mice had access to commercial pellet feed and water *ad libitum*. Mice were housed 7 per cage and kept in a temperature-controlled room (20 ± 3°C) with a 12/12-h light-dark cycle. They were used after a 1-week adaptation period. All animal experiments in this study were carried out in accordance with the guidelines of the Kangwon National University IACUC (KIACUC).

2.4. Scopolamine Injection and Drug Administration. In Morris water maze test, the mice were administered 0.5% carboxymethylcellulose (CMC; control group), fermented *C. lanceolata* (300, 500, and 800 mg/kg, dissolved in CMC), and donepezil (used as a positive control; 1 mg/kg) orally 90 min before treatment with scopolamine. The control group received normal saline, subcutaneously (SC), and all other groups were given scopolamine (1 mg/kg SC, dissolved in saline) to induce amnesia. The first trial of the test was performed 30 min after scopolamine treatment.

In passive avoidance test, the mice were administered 0.5% CMC (control group), fermented *C. lanceolata* (300, 500, and 800 mg/kg), and donepezil (1 mg/kg) orally 90 min before treatment with scopolamine. Scopolamine (1 mg/kg SC, dissolved in saline) was administered 30 min prior to testing.

2.5. Morris Water Maze Test. The water maze test was performed as described in our previous study [18, 21]. The water maze consisted of a circular pool (90 cm diameter and 40 cm height) filled with water up to 30 cm and maintained at 20 ± 1°C and areas of the maze were defined as four equal quadrants. Starting points on the outside of the pool were changed each day. A white escape platform (10 cm diameter and 26 cm height) was placed in the center of one quadrant and submerged 1 cm below the surface of the water. All swimming behaviors of the mice were monitored and

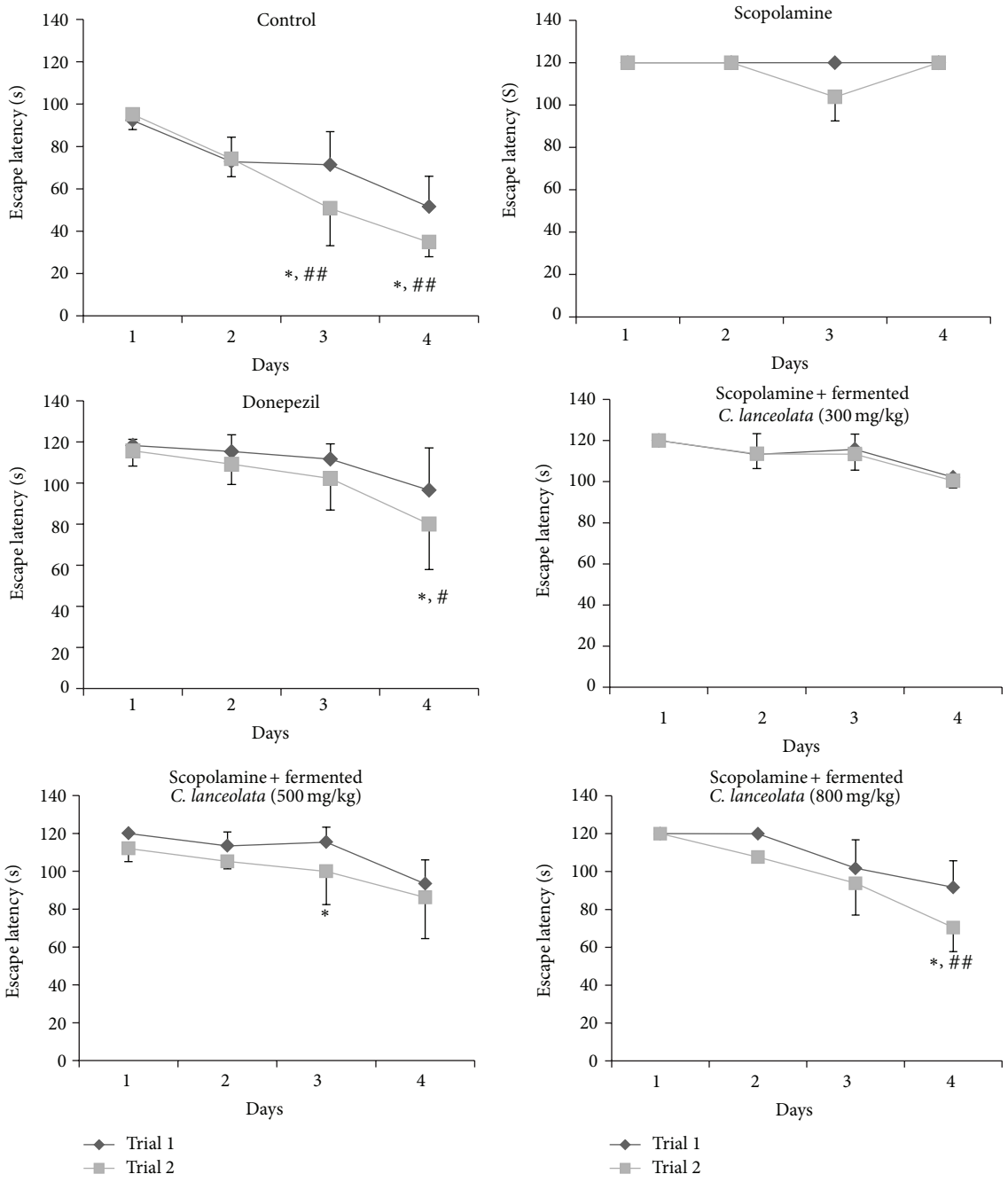


Figure 1: The effect of fermented *C. lanceolata* on escape latency in scopolamine-injected mice in the Morris water maze test. Control group (0.5% CMC (10 mL/kg body weight, P.O.), scopolamine group (1 mg/kg body weight, S.C.), donepezil group (1 mg/kg body weight, P.O.)) and fermented *C. lanceolata* group (300, 500, and 800 mg/kg body weight, P.O. treated 1 h before scopolamine administration). The values shown are the mean escape latency ± SD ($n = 7$). Escape latency of trial 2 significantly differed from that of trial 1: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the scopolamine group.

analyzed by Smart (ver. 2.5.21) video-tracking system. The escape latency and the time to locate the platform were used as measures for the development of spatial memory. Mice were given 60 s to swim in the absence of the platform on the day of the probe trial. The mice received two trial sessions per day for 4 consecutive days, with a 20 min intertrial interval. Location of the platform was unchanged between trial 1 and trial 2 during the test period. For the probe trial, the platform was removed for a period of 60 s on the last day. The time spent in the target quadrant was investigated to determine the memory of the mice.

2.6. Passive Avoidance Test. The passive avoidance test was carried out as previously described [18]. The mice were tested

in a passive avoidance apparatus (Gemini, San Francisco, USA), which consisted of two equally sized compartments (17 cm × 12 cm × 10 cm) with an electrifiable grid floor. The two compartments were divided by a guillotine door. On the first day, the mice performed a training trial in the avoidance apparatus. Twenty-four hours after the training trial, the mice were initially placed in the light compartment and after 20 s the door between the two compartments was opened. Movement of the mice in the dark compartment caused the guillotine door to close. An electric footshock (0.1 mA/10 g body weight, 2 s duration) was then delivered through the grid floor. During each trial, the time taken to move to the dark compartment was recorded as the latency time. The mice were again placed in the light compartment and the latency time was measured 24 h later. The maximum entry latency time to darkroom was 180 s.

2.7. Acetylcholinesterase Activity Determination. Acetylcholinesterase (AChE) activity test described by Ellman method was used with slight modification [22]. Mice were euthanized after behavioral test, and brain was removed. Hippocampus was dissected out from the brain and rapidly homogenized with sodium phosphate buffer. The mixture contained 33 μL of homogenate, 470 μL of sodium phosphate buffer, and 167 μL of DTNB. Then, 280 μL of acetylcholine iodide was added to the reaction mixture. After incubation, the reaction was measured at 412 nm using spectrophotometer. The AChE activity was calculated as the optical density value per mg protein.

2.8. Tissue Preparation and Western Blot Analysis. Mice were sacrificed 30 min after last behavior test. The brains were promptly collected and hippocampus was excised. Hippocampal tissues were homogenized in 200 μL of ice-cold RIPA buffer containing a protease inhibitor cocktail and centrifuged at 13,000 ×g for 20 min. The supernatants were stored at −80°C. The supernatant containing 20 μg of protein was subjected to 15% SDS-PAGE for 2-3 h at 100 V and transferred to PVDF membrane. The membrane was blocked in 5% skim milk for 1 h at room temperature and incubated overnight with a 1 : 2000 dilution of β-actin, 1 : 1000 dilution of CREB, 1 : 500 dilution of pCREB and 1 : 1000 dilution of BDNF antibody at 4°C. After incubation, the membrane was washed three times with 0.1% PBST.

Secondary antibodies (goat-anti-rabbit IgG HRP 1 : 2000 for BDNF, donkey-anti-goat IgG HRP 1 : 2000 for pCREB, and goat-anti-mouse IgG HRP 1 : 2000 for CREB and β-actin) were conjugated for 2 h at room temperature and then were washed three times with 0.1% PBST.

2.9. Statistical Analyses. Data from the Morris water maze test were analyzed by two-way ANOVA. Data from the probe trial test, passive avoidance test, and AChE activity values were analyzed by one-way ANOVA. All results were expressed as means ± SEM. If the results were significant, significant differences in direct comparisons were determined by Tukey's post hoc test. Statistical significance was set at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

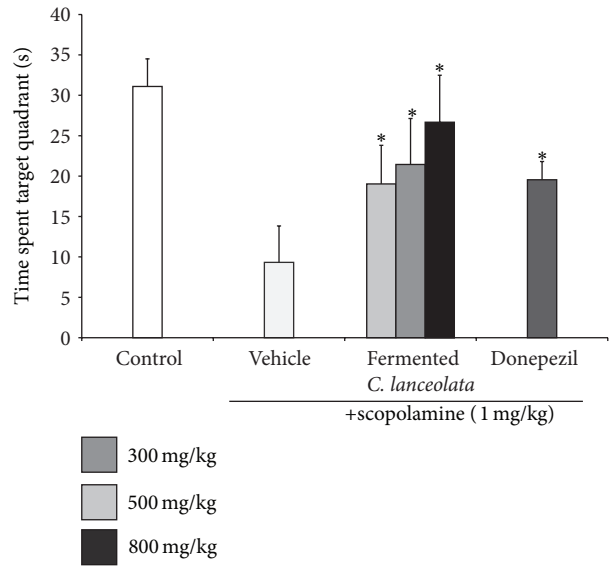
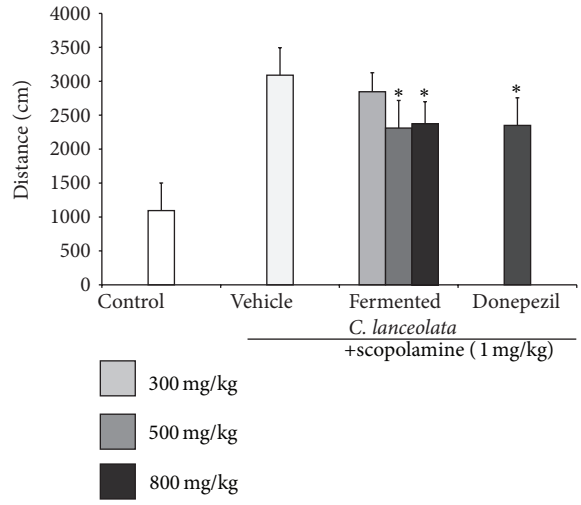


Figure 2: Mean escape latency of each group in the probe trial. The time spent in the quadrant where the platform was previously placed. Data represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the scopolamine group.

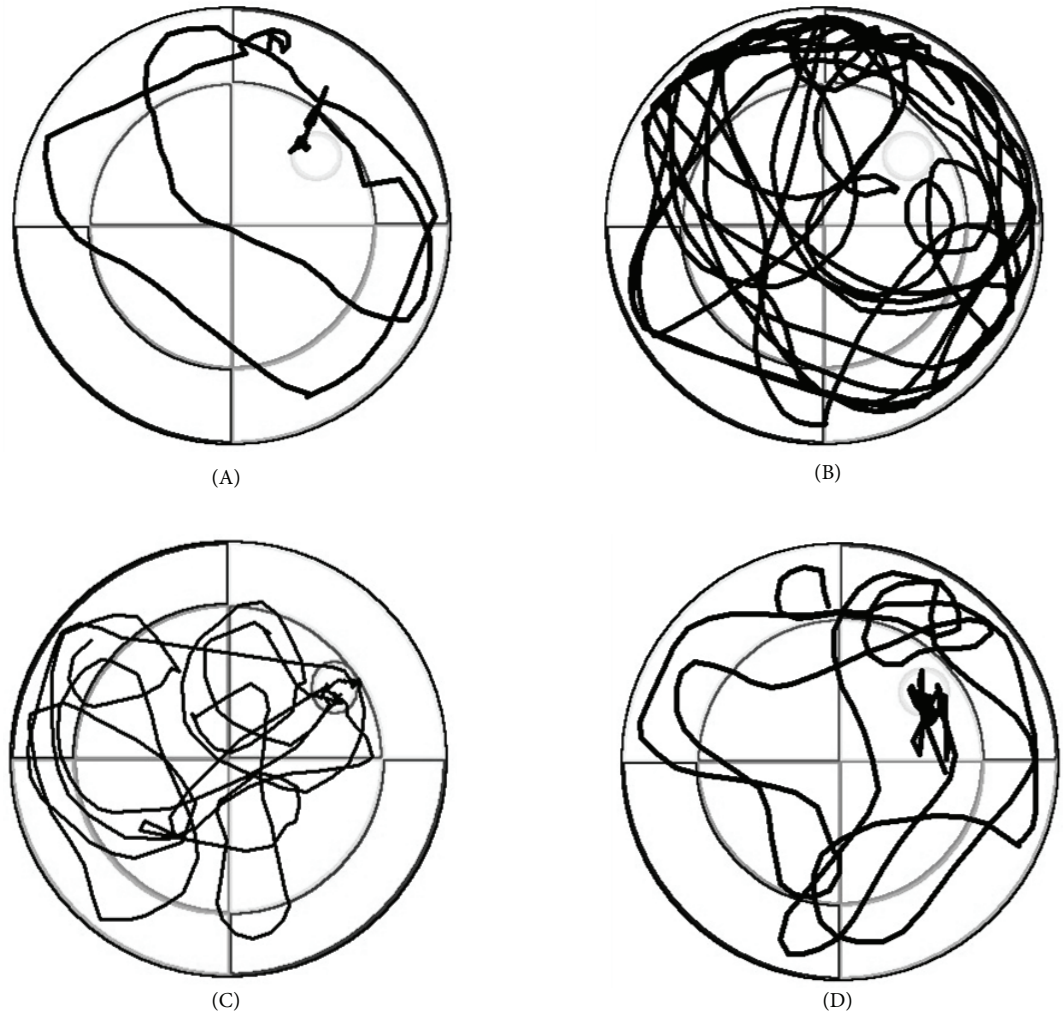
3. Results

3.1. Morris Water Maze Test. Based on our recent report, we found that the steamed and fermented *C. lanceolata* showed significantly increased cognitive enhancement activity. After modifying the steam and fermentation process for the mass production of steamed and fermented *C. lanceolata,* we investigated the effect of newly processed steamed and fermented *C. lanceolata* extract on scopolamine-induced spatial memory impairment by using the Morris water maze test (Figure 1).

The scopolamine-treated group did not show decreased escape latency time during the 4 trial days. The steamed and fermented *C. lanceolata* group showed shorter escape latency than the scopolamine-treated group from trial 1 and trial 2 after the 1st trial day. The donepezil-treated group also showed significantly reduced escape latency from trial 2 during the 3rd and 4th trial day. In the Morris water maze test, significant effects were observed for the fermented *C. lanceolata* group for treatment [$F(6, 98) = 2.21$, $P < 0.001$], for days [$F(3, 98) = 2.87$, $P < 0.001$], and for the interaction between treatment and day [$F(18, 98) = 1.82$, $P = 0.95$]. Compared to the control group, the scopolamine group spent less swimming time in the target quadrant after the platform was removed following the probe trial on the last day. The fermented *C. lanceolata* group showed significantly increased swimming time in the target quadrant than did the scopolamine group (Figure 2). Fermented *C. lanceolata* (800 mg/kg, P.O.) significantly decreased the scopolamine-induced increase in the average distance to the platform during the water maze test. The typical swimming routes of each group are shown in Figure 3.



(a)



(b)

Figure 3: (a) Mean distance travelled to the platform in the Morris water maze test. Results are expressed as mean ± SD ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with scopolamine group. (b) Typical swimming routes of each group in the Morris water maze test. (A) Control group; (B) scopolamine-treated group; (C) scopolamine + donepezil-treated group; (D) scopolamine + fermented *C. lanceolata* (800 mg/kg, P.O.).

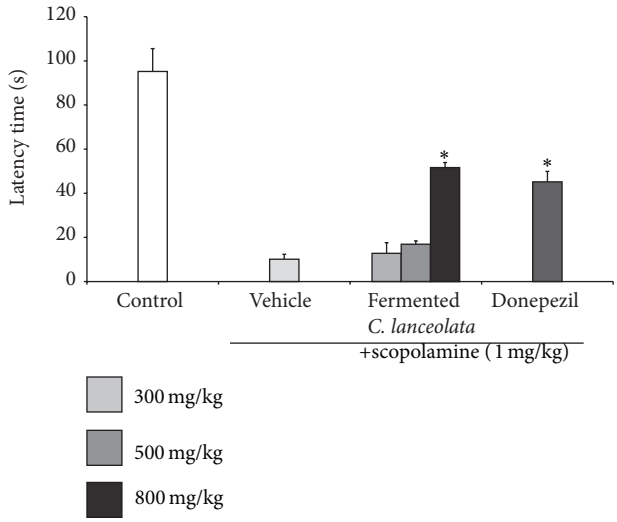


Figure 4: Effect of fermented *C. lanceolata* on scopolamine-induced memory impairment in the passive avoidance test. Mean latency time (s) ± SD ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the scopolamine group.

3.2. Passive Avoidance Test. We also investigated the effect of newly processed steamed and fermented *C. lanceolata* on scopolamine-induced memory deficit in the passive avoidance test. Passive avoidance test is generally used to assess long-term memory mice [23]. The latency time in the scopolamine group was significantly decreased as compared to that in the control group. Compared to the scopolamine-treated mice, the fermented *C. lanceolata*-treated mice (800 mg/kg, P.O.) that were cotreated with scopolamine exhibited significant reversal of latency time. This effect increased in a dose-dependent manner (Figure 4). Donepezil, an acetylcholinesterase inhibitor, was used as positive control. Treatment with donepezil also showed a significant increase in latency time. In this study, the steamed and fermented *C. lanceolata*-treated group (800 mg/kg, P.O.) showed similar latency time to donepezil-treated group.

3.3. Acetylcholinesterase Activity Determination. Since AchE activity was reported to be involved in memory impairment, the effect of fermented *C. lanceolata* on AchE activity in hippocampus of mice was evaluated, as shown in Table 1. Scopolamine treatment significantly increased AchE activity in hippocampus as compared with those of control group. Fermented *C. lanceolata* (800 mg/kg) treatment significantly decreased AchE activity by 35.63%. After treatment with donepezil, AchE activity of scopolamine-treated mice was decreased by 27.59%. This result demonstrated that the steamed and fermented *C. lanceolata* exerts cognitive-enhancing effect through the inhibition of AchE in hippocampus.

3.4. The Effect of Steamed and Fermented C. lanceolata on BDNF Expression and CREB Phosphorylation. The activation of CREB and BDNF plays a role in the enhancement of memory. Furthermore, increased CREB and BDNF activation improved long-term memory [24]. Further, scopolamine treatment was known to be involved in the inhibition of BDNF expression and CREB phosphorylation (pCREB). Thus, we decided to assess whether the effect of steamed and fermented *C. lanceolata* ameliorated memory impairment was linked to the increased BDNF and CREB activation in the hippocampus of mice. To determine the effect of steamed and fermented *C. lanceolata* on the BDNF and pCREB expression reduced by scopolamine in hippocampus, we conducted western blot analysis.

As shown in Figure 5, the BDNF expression in hippocampus was decreased following exposure of scopolamine to mice. The BDNF expression in mice with the steamed and fermented *C. lanceolata* (300, 500, and 800 mg/kg, P.O.) administration is significantly increased compared to scopolamine-treated mice. Scopolamine administration also resulted in a reduced pCREB expression compared to the control group. BDNF level was significantly elevated in mice treated with fermented *C. lanceolata* (800 mg/kg, P.O.) compared to scopolamine-treated mice. A correlation was observed between results in behavioral tests and levels of pCREB and BDNF.

Table 1: Acetylcholinesterase inhibitory effects of fermented *C. lanceolata* on memory deficit in mice.

Groups	AchE activity (U/mg protein)
Control	0.68 ± 0.038
Scopolamine	0.87 ± 0.008
Donepezil	0.63 ± 0.023
Fermented *C. lanceolata*	
300 mg/kg	0.74 ± 0.043
500 mg/kg	0.63 ± 0.048
800 mg/kg	0.56 ± 0.021***

Data represent the mean ± SD. $^{***}P < 0.001$ versus the scopolamine group.

4. Discussion

We established a new steamed and fermented method for large-scale production of fermented *C. lanceolata*. This method can be used with commercial application of fermented *C. lanceolata*.

The present study evaluated the cognitive-enhancing effect of fermented *C. lanceolata* extract by modified process on scopolamine-induced memory impairment in mice. The Morris water maze test is one of the most commonly used tests to investigate hippocampal-dependent spatial learning and memory in mice. The passive avoidance test is also used to assess memory retention based on the natural tendency of animals formed by an aversive stimulus. Scopolamine, a competitive muscarinic acetylcholine receptor antagonist, inhibits cholinergic activity. Cholinergic deficiency is associated with the cognitive impairments observed in AD [25, 26]. We found that fermented *C. lanceolata* extract improved memory and learning in mice with scopolamine-induced deficits in the Morris water maze and passive avoidance

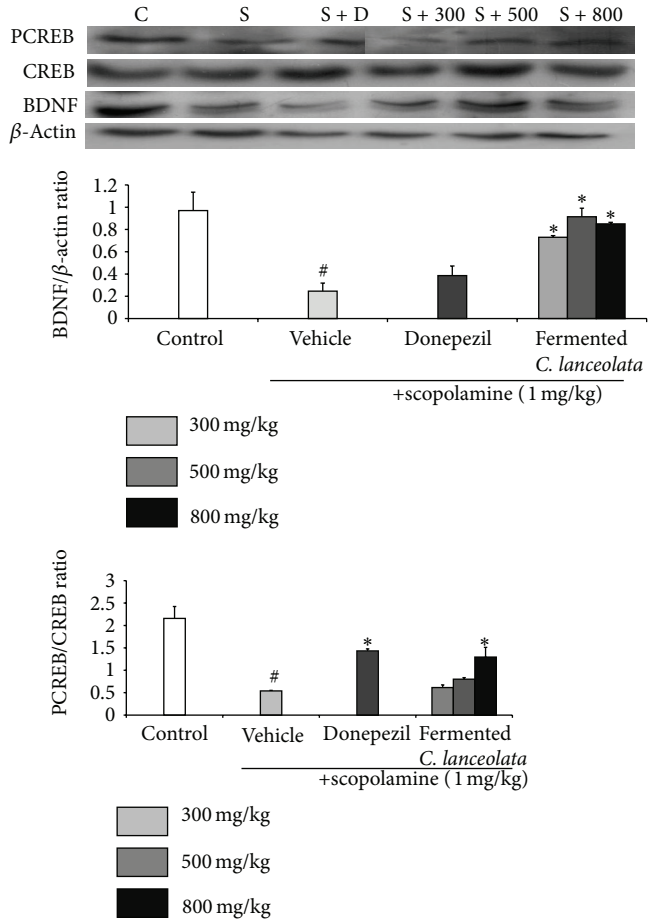


Figure 5: The proton levels of BDNF and pCREB in hippocampus. Control group (C; 0.5% CMC (10 mL/kg body weight, P.O.)), scopolamine group (S; 1 mg/kg body weight, S.C.), donepezil group (S + D; 1 mg/kg body weight, P.O.), and fermented *C. lanceolata* group (S + 300, S + 500, and S + 800; 300, 500, and 800 mg/kg body weight, P.O. treated 1 h before scopolamine administration) ($n = 5$ per group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the scopolamine group).

tests. In the Morris water maze test, the fermented *C. lanceolata*- (800 mg/kg) treated mice exhibited significantly shorter escape latency on day 4, between the 1st trial and 2nd trial, suggesting an improvement in short-term memory. The memory deficits induced by scopolamine were reversed significantly by treatment with fermented *C. lanceolata* extract at a dose of 800 mg/kg. These results implied that new large-scale process has stability of memory-enhancing activity of fermented *C. lanceolata*.

AchE is the enzyme involved in Ach hydrolysis at central cholinergic synapses. AchE activity in hippocampus was increased by administered scopolamine [27]. This study showed fermented *C. lanceolata* extract inhibited AchE activity in a doses-dependent manner. The hippocampal neurogenesis is important for learning and memory. Increased neurogenesis improved memory and new neurons increase memory capacity. Regulation of gene expression via BDNF plays a role in the long-term potentiation (LTP) and memory formation [28]. BDNF belongs to the neurotrophin family and activity is corrected with increased neurogenesis in the dentate gyrus of the hippocampus and enhanced memory [29]. Elevation of CREB activity leads to an upregulation of BDNF [30]. CREB promotes transcription of many specific target genes, including BDNF by phosphorylation at serine 133. CREB-mediated transcription interacts with coactivator CREB-binding protein [31, 32]. We tested the effect of fermented *C. lanceolata* on CREB phosphorylation (pCREB) and BDNF expression. Scopolamine reduced pCREB and BDNF levels in the hippocampus. We observed that fermented *C. lanceolata* treatment increased BDNF and pCREB levels.

Fermented *C. lanceolata* improved scopolamine-induced memory deficit during behavioral tests. In addition, fermented *C. lanceolata* inhibited AchE activity and increased pCREB and BDNF expression. These results of the present study suggest that the effect of fermented *C. lanceolata* may be associated with the muscarinic cholinergic receptor and may reverse cognitive impairment by affecting brain cholinergic activity. Moreover, the memory improving activity of fermented *C. lanceolata* is exerted via upregulation of

pCREB and BDNF in hippocampus. Our results support new mechanism of cognitive-enhancing effect of fermented *C. lanceolata* through BDNF and pCREB expression.

In summary, fermented *C. lanceolata* exhibited a cognitive-enhancing effect in the Morris water maze and passive avoidance test by inhibition of AchE activity and elevation of BDNF and pCREB expression. Further studies are needed to determine the role of fermented *C. lanceolata* in CREB and BDNF expression pathway.

Conflict of Interests

The authors have declared that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

The authors confirm that the paper has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. The authors further confirm that the order of authors listed in the paper has been approved by all of them.

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Research Article

Aqueous Extract of *Phyllanthus niruri* Leaves Displays *In Vitro* Antioxidant Activity and Prevents the Elevation of Oxidative Stress in the Kidney of Streptozotocin-Induced Diabetic Male Rats

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P. niruri has been reported to possess antidiabetic and kidney protective effects. In the present study, the phytochemical constituents and *in vitro* antioxidant activity of *P. niruri* leaf aqueous extract were investigated together with its effect on oxidative stress and antioxidant enzymes levels in diabetic rat kidney. *Results*. Treatment of diabetic male rats with *P. niruri* leaf aqueous extract (200 and 400 mg/kg) for 28 consecutive days prevents the increase in the amount of lipid peroxidation (LPO) product, malondialdehyde (MDA), and the diminution of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activity levels in the kidney of diabetic rats. The amount of LPO showed strong negative correlation with SOD, CAT, and GPx activity levels. *P. niruri* leaf aqueous extract exhibits *in vitro* antioxidant activity with IC_{50} slightly lower than ascorbic acid. Phytochemical screening of plant extract indicates the presence of polyphenols. *Conclusion*. *P. niruri* leaf extract protects the kidney from oxidative stress induced by diabetes.

1. Introduction

Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems with increased production of reactive oxygen species (ROS) or reduced activity of antioxidant defences or both [1]. In oxidative stress, oxidation of macromolecules such as proteins, lipids, carbohydrates, and DNA is elevated. Hyperglycaemia has been identified as a major cause for ROS generation [2]. Hyperglycaemia causes glucose autooxidation, protein glycation, and advanced glycation end products (AGE) formation which could lead to the development of diabetic complications including retinopathy, neuropathy, and macro- and microvascular damage [3]. Oxidative stress has been considered as a common pathogenetic factor for diabetic nephropathy (DN), which is often associated with morphological changes of the kidney leading to end-stage renal failure (ESRF) [4, 5].

Phyllanthus niruri which belongs to the Euphorbiaceae family is also known as kidney stone crusher [6]. Traditionally, *P. niruri* is used to treat problems related to the gastrointestinal and genitourinary tracts [7]. There are reports which indicate that *P. niruri* can block calcium oxalate crystals [8] and stone formation in the kidney, ureter, and urinary bladder [9]. *P. niruri* was also reported to display anticarcinogenic [10], hypolipidemic [11], hepatoprotective [12],

anti-inflammatory [13], and antiplasmodial [14] effects as well as an effective treatment for hemorrhagic cystitis [15]. Recent studies have also revealed antidiabetic [16] and antioxidant properties of this herbal extract [9] both *in vivo* and *in vitro*.

In the present study, we hypothesized that *P. niruri* helps to prevent oxidative stress in the kidney of diabetics. Our study therefore investigated this plant effect on oxidative stress parameters, that is, activity levels of antioxidant enzymes (SOD, CAT, and GPx) and the amount of LPO product in the kidney of diabetic rats. In addition, *in vitro* antioxidant activity and phytochemical constituent of this plant extract were also investigated.

2. Material and Methods

2.1. Chemicals. DPPH (1,1-diphenyl,2-picrylhydrazyl), NBT (nitro blue tetrazolium), NADH (nicotinamide adenine dinucleotide phosphate reduced), PMS (phenazine methosulphate), ferrous chloride ($FeCl_2$), streptozotocin, glibenclamide, epinephrine, thiobarbituric acid (TBA), reduced glutathione (GSH), and 5-5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Co. (St Louis, Mo, USA). All other chemicals were of analytical grade.

2.2. Collection and Preparation of Plant Material. Fresh leaves of *P. niruri* were collected from Tirupati and authenticated by Dr. K. Madhava Chetty, Botanist, Sri Venkateswara University, Tirupati, India. The plant was deposited in Herbarium of Department of Botany, Sri Venkateswara University, Tirupati, India, with the number: 856201. The leaves of *P. niruri* were air-dried at room temperature and ground into powder. About 800 g of the powdered leaves was extracted in 1 L of cold sterile distilled water maintained on a mechanical shaker. The aqueous extract was filtrated with No. 1 Whatman Millipore filter paper (0.45 μm Ref. HAWP04700, Bedford, MA, USA) and concentrated to dryness with a rotary evaporator (Rotavapor, R-210, Buchi Laborotechnik, AG, Flawil, Switzerland) at 50 ± 5C and lyophilized. A yielded freeze-dried material of approximately 36 g was obtained. The freeze-dried sample was stored in a cool dry place until ready for use.

2.3. Phytochemical Screening of P. niruri Leaves. The phytochemical components of *P. niruri* leaves were screened using standard method described by Harborne [17]. The leaf extract was screened for alkaloids, flavonoids, saponins, tannins, steroids, lignins, glycosides, terpenoids, polyphenols, coumarins, and resins. The colour intensity of the precipitate formed was used as analytical responses to these tests.

2.4. In Vitro Antioxidant Activities. Few different assays were performed to determine the antioxidative power of *P. niruri* leaf aqueous extract (20–300 μg/mL) as described below. In each of these assays, ascorbic acid (20–300 μg/mL) was used as a reference substrate. The ability of the extract to scavenge or inhibit free radicals was expressed as percentage inhibition and was calculated using the following formula:

$$\%\ \text{of inhibition} = \frac{(A_0 - A_t)}{A_0} \times 100, \quad (1)$$

where A_0 is absorbance of the control group (without plant extract) and A_t is absorbance of *P. niruri* leaf extract. All determinations were carried out in triplicate.

2.4.1. DPPH Radical Scavenging Activity. DPPH radical scavenging activity of *P. niruri* leaf aqueous extract was determined according to the method by Katalinic et al. [18]. In brief, 0.5 mL of 0.1 mM DPPH solution was prepared in methanol just before use. 1.0 mL of *P. niruri* leaf aqueous extract was added at different concentrations (20–300 μg/mL) to DPPH solution. Double distilled H_2O was used in the control group instead of samples, with the same procedures applied. The ability of the substrate to reduce the stable radical DPPH from deep purple to yellow-coloured diphenylpicrylhydrazine indicates its antioxidative potential. The mixture was shaken vigorously and left to stand for 30 min in the dark, and absorbance was measured at 517 nm using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Lower absorbance at 517 nm represents higher DPPH scavenging activity.

2.4.2. Superoxide Radical Scavenging Activity. Measurement of superoxide radical scavenging activity of *P. niruri* leaf extract followed the method by Xiang and Ning [19]. In brief, superoxide anions were generated in nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of NBT in the presence of different concentrations (20–300 μg/mL) of the extract. The reaction was initiated by adding 0.75 mL of PMS (120 μM) to the mixture. The absorbance was measured at 560 nm by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) following 5-minute incubation at room temperature.

2.4.3. Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity of *P. niruri* leaf extract was measured according to a modified method by Eswar Kumar et al. [20]. The reaction mixture contained 60 μL of 1.0 mM $FeCl_2$, 90 μL of 1 mM 1,10-phenanthroline, 2.4 mL of 0.2 M phosphate buffer (pH 7.8), 150 μL of 0.17 M hydrogen peroxide (H_2O_2), and 1.5 mL of different concentrations of the extract (20–300 μg/mL). H_2O_2 was added at the start of the reaction. After incubation at room temperature for 5 min, absorbance of the mixture was measured by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 560 nm.

2.4.4. Hydrogen Peroxide Scavenging Activity. The ability of *P. niruri* leaf extract to scavenge hydrogen peroxide was determined according to the method by Ruch et al. [21]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). *P. niruri* leaf extract at different concentrations (20–300 μg/mL) was added to hydrogen peroxide

solution (0.6 mL and 40 mM). Absorbance of the mixture was determined after 10 minutes against a blank solution containing phosphate buffer by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 230 nm.

2.5. Experimental Animals. Adult male Wistar rats weighing 180–210 g were obtained from Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. Five to six animals were housed together under a standard environmental condition of temperature 25 ± 2°C, relative humidity between 45 and 55%, and 12 hr light/dark cycle. Rats had free access to standard food pellet (Harlan diet, UK) and water *ad libitum*. The experimental protocol was in accordance with ARRIVE guidelines (Animals in Research: Reporting *In Vivo* Experiments) and European Community Guidelines (EEC Directive, 1986). This study was approved by the Faculty of Medicine, Animal Care and Use Committee, University of Malaya, with ethics number: 2013-07-15/FIS/R/NS. Acute toxicity study was conducted according to the Organization for Economic Cooperation and Development (OECD) revised up-and-down procedure for acute toxicity testing (OECD guideline 425) [22]. No signs of acute toxicity were observed in the tested animals.

2.6. Induction of Diabetes. Diabetes mellitus was artificially induced in overnight-fasted rats by a single intraperitoneal injection of STZ (55 mg/kg bw) dissolved in 0.1 M cold citrate buffer (pH 4.5) [23]. Rats were allowed to drink 5% glucose solution overnight to overcome drug-induced hypoglycemia. STZ was reported to selectively destroy the pancreatic β cells [23]. Diabetes was confirmed by the presence of polyuria, polydipsia, and weight loss and only animals exhibiting blood glucose levels above 300 mg/dL on the third day following STZ injection were considered as diabetics. Treatment was started three days after STZ injection which was considered to be day one. *P. niruri* leaf aqueous extract was administered orally by using a gavage tube daily for 28 consecutive days. The extract was administered in the form of suspension in 1% sodium carboxymethyl cellulose (Na-CMC) dissolved in distilled water.

2.7. Experimental Design. Animals were randomly divided into five groups with six rats in each group:

Group 1: normal and nondiabetic: received 1% Na-CMC vehicle only;

Group 2: diabetic: received 1% Na-CMC vehicle only;

Groups 3 and 4: diabetic: treated with *P. niruri* leaf aqueous extract at 200 mg/kg [24] and 400 mg/kg [25] body weight, respectively;

Group 5: diabetic: treated with standard antidiabetic agent, glibenclamide at 600 μg/kg body weight.

2.8. Collection of Tissue Samples. After 28-day treatment, rats were fasted overnight prior to sacrifice. Intraperitoneal injection of pentobarbitone sodium anesthesia (60 mg/kg) was given prior to cervical dislocation. Kidneys were immediately harvested and washed with ice-cold saline, immersed in liquid nitrogen, and stored at −80°C for biochemical analysis.

2.9. Preparation of Kidney Cytosolic Fraction. Kidney was weighed and 10% of tissue homogenate was prepared in phosphate buffer (0.1 m, pH 7.4) using a glass-Teflon homogenizer (Heidolph Silent Crusher M, Germany). Homogenates were then centrifuged for 10 min, 500 g at 4°C. Supernatant was collected and recentrifuged at 2000 g for 10 min. Supernatant was again collected and recentrifuged at 12000 g for 10 min at 4°C and pellet was resuspended in 200 mM mannitol, 50 mM sucrose, and 10 mmol/L Hepes-KOH (pH 7.4). The final supernatant was taken and centrifuged for 1 h at 40000 g [26]. The cytosolic fraction was frozen at −80°C until further used.

2.10. Estimation of LPO Product. LPO was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), where the latter was a product formed from membrane lipid peroxidation [27]. In brief, 2.5 mL homogenate, 0.5 mL of 0.9% NaCl, and 1.0 mL 20% w/v TCA were added into the mixture. The mixture was then centrifuged for 20 minutes at 4000 ×g at 4°C. 0.25 mL TBA reagent was added to 1.0 mL supernatant and the mixture was incubated at 95°C for 1 hr and cooled under running tap water prior to addition of 1 mL *n*-butanol. After a thorough mixing, the mixture was centrifuged for 15 minutes at 4000 ×g at 4°C. The organic layer was transferred into a clear tube and absorbance was measured at 532 nm with a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). The rate of lipid peroxidation was expressed as μ moles of MDA formed/gram wet weight of the tissue.

2.11. Estimation of SOD Activity. SOD activity was assayed according to the method by Misra and Fridovich [28]. The assay procedure involves inhibition of epinephrine autooxidation to adrenochrome in an alkaline medium (pH 10.2), which was markedly inhibited in the presence of SOD. 1.5 mL carbonate buffer (0.05 M, pH 10.2) and 0.5 mL ethylenediaminetetraacetic acid (EDTA) (0.49 M) were added to 0.5 mL supernatant. The reaction was initiated by the addition of 0.4 mL epinephrine (3 mM). Changes in absorbance were recorded at 480 nm for one min at 15 sec interval, 3 min each, by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). SOD activity levels were expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50%, which was equal to 1 U per milligram of protein.

2.12. Estimation of CAT Activity. CAT enzyme activity was determined on the basis of hydrogen peroxide decomposition [29]. The reaction solution contained 2.5 mL of 50 mmol phosphate buffer (pH 5.0) and 0.4 mL of 5.9 mmol H_2O_2. The reaction was initiated by adding 0.1 mL enzyme extract. Changes in the absorbance of the reaction solution were monitored every 30*s* and was read by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 240 nm. The enzyme

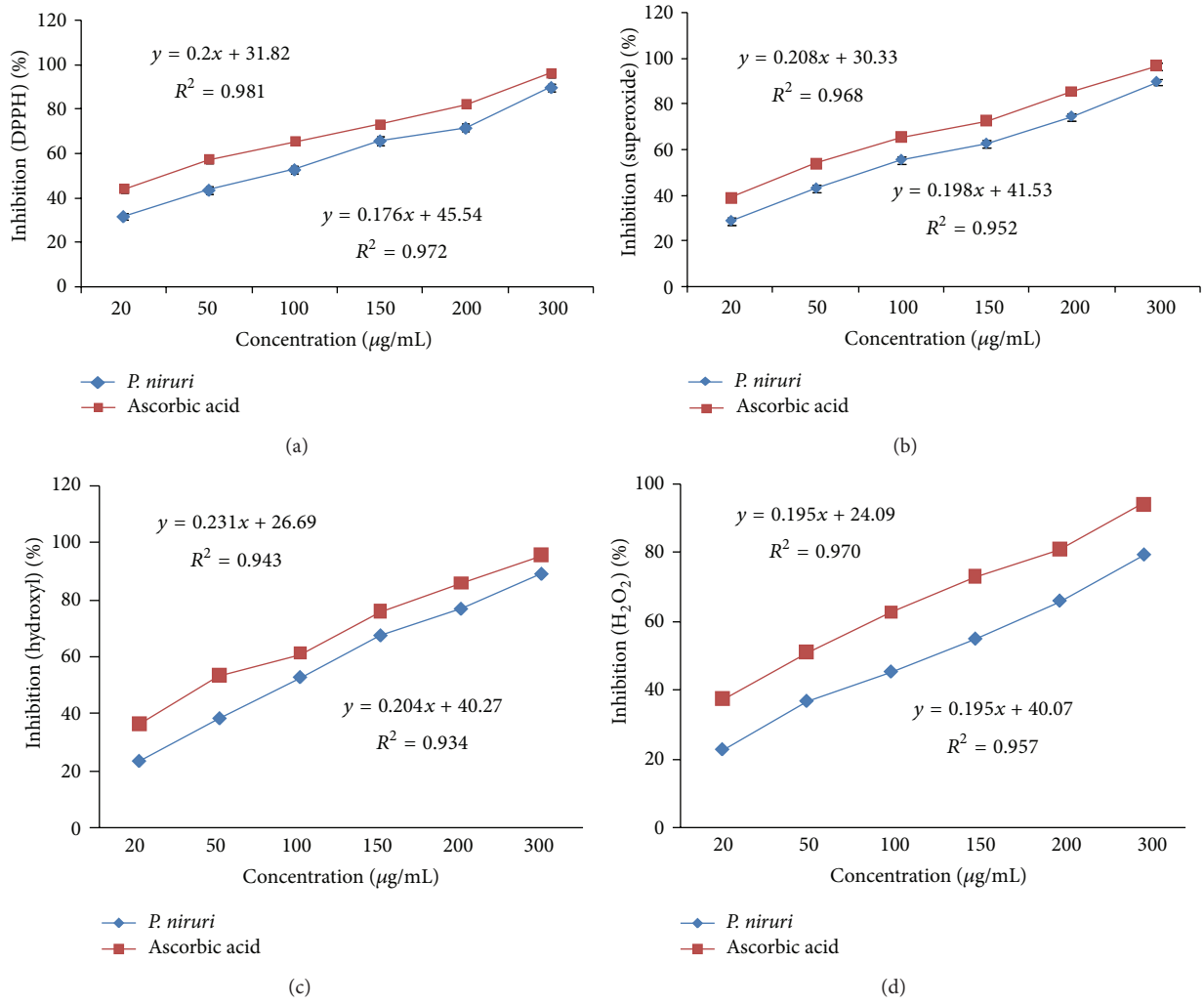

Figure 1: *In vitro* antioxidant assay of *P. niruri* leaf aqueous extract. The graphs show (a) DPPH radical, (b) superoxide radical, (c) hydroxyl radical, and (d) hydrogen peroxide scavenging activities of *P. niruri* leaf extract and ascorbic acid. Results represent means of triplicates of each concentration. For each assay, the IC_{50} for *P. niruri* leaf extract was slightly less than ascorbic acid.

activity levels were expressed as μmol of hydrogen peroxide (H_2O_2) metabolized/mg protein/min.

2.13. Estimation of GPx Activity. GPx activity was measured according to the method by Rotruck et al. [30]. The reaction mixture consists of 0.2 mL, 0.8 mM EDTA; 0.1 mL, 10 mM sodium azide; 0.1 mL, 2.5 mM H_2O_2; 0.2 mL GSH; 0.4 mL, 0.4 mM phosphate buffer (pH 7.0); and 0.2 mL homogenate which were incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 mL of 10% TCA and was centrifuged at 2000 rpm. 3.0 mL of 0.3 M disodium hydrogen phosphate and 1.0 mL of DTNB were added to the supernatant and the colour changes were read immediately by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 420 nm. GPx activity levels were expressed as μMol of GSH consumed/mg protein/min.

2.14. Statistical Analysis. Statistical differences were evaluated by analysis of variance (ANOVA) followed by Student's *t*-test. P value < 0.05 was considered to be significant. Post hoc statistical power analysis was performed and all values obtained were >0.8 which indicate adequate sample size. Shapiro-Wilk test results were >0.05 which indicate data normality.

3. Results

3.1. Phytochemical Screening. Preliminary phytochemical screening showed the presence of alkaloids, flavonoids, saponins, tannins, lignins, terpenoids, polyphenols, and coumarins; however, steroids, glycosides, and resins were not detected in the leaf extract of *P. niruri* (data not shown).

3.2. In Vitro Antioxidant Activities

3.2.1. DPPH Radical Scavenging Activity. DPPH radical scavenging activity of aqueous leaf extract of *P. niruri* is presented in Figure 1(a). Our findings indicate that 20 μg/mL of

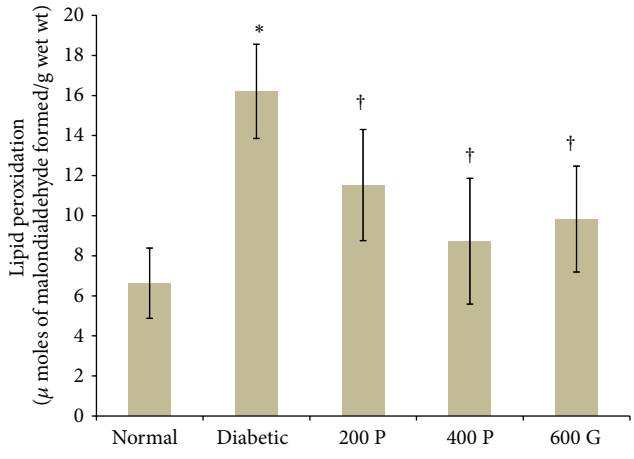

Figure 2: Estimation of LPO product, MDA, in the kidney in different experimental groups. Higher MDA levels were noted in diabetic rats as compared to normal, nondiabetic control rats. Administration of *P. niruri* leaf extract resulted in decreased MDA levels in the kidney in diabetes. 200P: 200 mg/kg/day *P. niruri* leaf extract; 400P: 400 mg/kg/day *P. niruri* leaf extract; 600G: 600 μg/kg/day glibenclamide. $n = 6$ per treatment group, $^{*}P < 0.05$ as compared to normal, nondiabetic control rats, and $^{\dagger}P < 0.05$ as compared to nontreated diabetic rats.

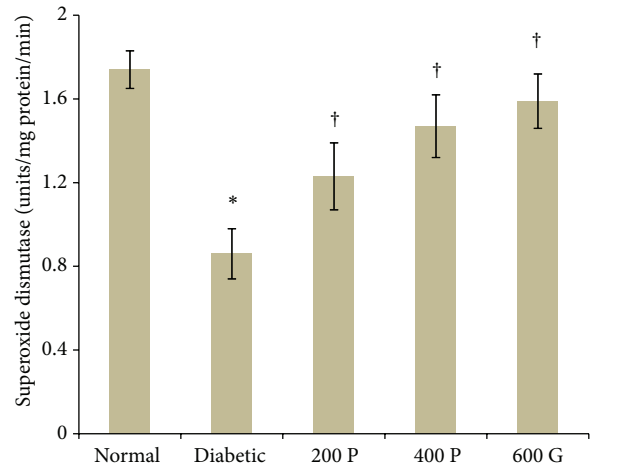

Figure 3: SOD activity levels in the kidney in different experimental groups. SOD activity levels were reduced in diabetic rats as compared to normal, nondiabetic rats. Administration of *P. niruri* leaf extract at 200 or 400 mg/kg/day and glibenclamide resulted in higher SOD activity in the kidney as compared to nontreated diabetic rats. 200P: 200 mg/kg/day *P. niruri* leaf extract; 400P: 400 mg/kg/day *P. niruri* leaf extract; 600G: 600 μg/kg/day glibenclamide. $n = 6$ per treatment group, $^{*}P < 0.05$ as compared to normal, nondiabetic control rats, and $^{\dagger}P < 0.05$ as compared to nontreated diabetic rats.

the leaf extract and ascorbic acid conferred 31.66% and 43.89% inhibition on DPPH radicals, respectively. The IC_{50} for *P. niruri* leaf extract and ascorbic acid was 90.86 μg/mL and 25.31 μg/mL, respectively. The IC_{50} of the leaf aqueous extract was 3.58 times lower than ascorbic acid. The extract exhibits gradual dose-dependent increase on DPPH inhibition.

3.2.2. Hydroxyl Radical Scavenging Activity. Hydroxyl radical scavenging activity of aqueous leaf extract of *P. niruri* is presented in Figure 1(b). At 20 μg/mL, the aqueous leaf extract and ascorbic acid inhibit hydroxyl radical by 23.66% and 36.56%, respectively. *P. niruri* leaf aqueous extract displays a dose-dependent inhibition on hydroxyl radicals with IC_{50} of 100.6 μg/mL. The IC_{50} for ascorbic acid was 47.44 μg/mL. The IC_{50} of *P. niruri* leaf was 2.12 times lower than ascorbic acid.

3.2.3. Superoxide Radical Scavenging Activity. Figure 1(c) shows dose-dependent scavenging activity of *P. niruri* leaf aqueous extract and ascorbic acid on superoxide radical. At 20 μg/mL, the percentage inhibition of *P. niruri* leaf aqueous extract and ascorbic acid was 28.46% and 38.67%, respectively. The IC_{50} for *P. niruri* leaf extract was 94.34 μg/mL, while ascorbic acid was 42.64 μg/mL. The IC_{50} of *P. niruri* leaf extract was 2.21-fold lower than ascorbic acid.

3.2.4. Hydrogen Peroxide Scavenging Activity. The ability of *P. niruri* leaf aqueous extract and ascorbic acid to scavenge hydrogen peroxide free radical is shown in Figure 1(d). H_2O_2 inhibition by *P. niruri* leaf extract and ascorbic acid at 20 μg/mL was 22.54% and 37.57%, respectively. The IC_{50} for *P. niruri* leaf extract and ascorbic acid was 132.57 μg/mL and 50.81 μg/mL, respectively. The IC_{50} of *P. niruri* leaf aqueous extract was 2.61-fold lower than ascorbic acid.

3.3. Effect of P. niruri Leaf Aqueous Extract on Renal MDA Levels. The levels of renal LPO product, MDA, were significantly higher in nontreated diabetic rats (144.49%) as compared to normal, nondiabetic rats (Figure 2). Administration of 200 mg/kg *P. niruri* leaf extract to diabetic rats caused the levels of MDA to decrease by 28.87% as compared to the nontreated diabetic rats. Meanwhile, the levels of MDA were 46.2% lower in diabetic rats treated with 400 mg/kg *P. niruri* leaf aqueous extract as compared to the nontreated diabetic rats (Figure 2). In glibenclamide-treated diabetic rats, the levels of MDA were 39.35% lower than the nontreated diabetic rats. 400 mg/kg *P. niruni* leaf extract was 0.88-fold less potent than glibenclamide in preventing the increase in LPO product in the kidney of diabetic rats (Figure 2).

3.4. Effect of P. niruri Leaf Aqueous Extract on Renal SOD Levels. Figure 3 shows the effect of *P. niruri* leaf aqueous extract and glibenclamide on SOD activity levels in diabetic rat kidney. In the nontreated diabetic rats, SOD activity level was 50.57% lower than normal, nondiabetic rats. However, renal SOD activity level was 43.02% higher in diabetic rats treated with 200 mg/kg *P. niruri* leaf extract as compared to the nontreated diabetic rats. Following treatment with 400 mg/kg *P. niruri* leaf extract, the SOD activity levels were 79.93% higher than the nontreated diabetic rats. Glibenclamide-treated diabetic rats have 84.88% higher renal SOD activity level as compared to the nontreated diabetic rats. Treatment with 400 mg/kg *P. niruri* leaf aqueous extract was less potent

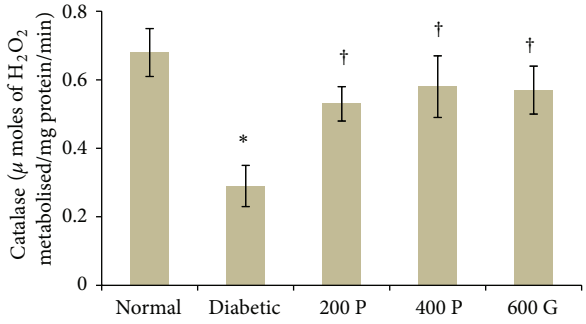

Figure 4: CAT activity levels in the kidney in different experimental groups. Administration of *P. niruri* leaf extract at 200 and 400 mg/kg/day or glibenclamide to diabetic rats prevented decrease in CAT activity in the kidney in diabetes. 200P: 200 mg/kg/day *P. niruri* leaf extract; 400P: 400 mg/kg/day *P. niruri* leaf extract; 600G: 600 μg/kg/day glibenclamide. $n = 6$ per treatment group, $^{*}P < 0.05$ as compared to normal, nondiabetic control rats, and $^{\dagger}P < 0.05$ as compared to nontreated diabetic rats.

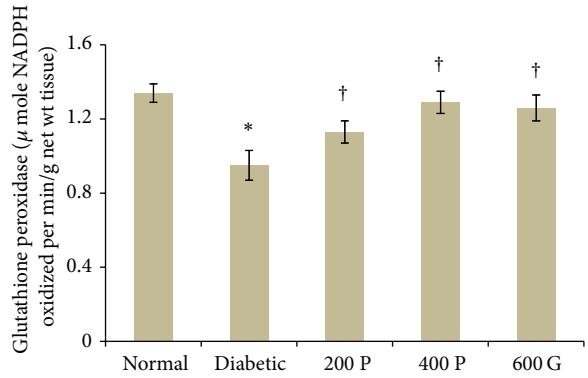

Figure 5: GPx activity levels in the kidney in different experimental groups. Administration of *P. niruri* leaf extract at 200 and 400 mg/kg/day or glibenclamide prevented deterioration of GPx activity levels in the kidney in diabetes. 200P: 200 mg/kg/day *P. niruri* leaf extract; 400P: 400 mg/kg/day *P. niruri* leaf extract; 600G: 600 μg/kg/day glibenclamide. $n = 6$ per treatment group, $^{*}P < 0.05$ as compared to normal, nondiabetic control rats, and $^{\dagger}P < 0.05$ as compared to nontreated diabetic rats.

than glibenclamide in preventing the decrease in SOD activity levels in diabetic rat kidney.

3.5. Effect of P. niruri Leaf Aqueous Extract on Renal CAT Levels. The activity levels of CAT in the kidney of normal, STZ-induced diabetic rats and diabetic rats which received *P. niruri* leaf aqueous extract or glibenclamide are presented in Figure 4. Our findings indicate that CAT activity levels were 57.35% lower in STZ-induced diabetic rat kidney as compared to normal, nondiabetic rats. 28-day treatment with 200 mg/kg/day *P. niruri* leaf aqueous extract resulted in higher CAT activity level (82.75%) as compared to the nontreated diabetic rats. Treatment with 400 mg/kg/day *P. niruri* leaf aqueous extract resulted in 100% higher CAT activity levels as compared to the nontreated diabetic rats. Meanwhile, glibenclamide treatment resulted in 96.55% higher CAT activity levels as compared to the nontreated diabetic rats. No significant difference in CAT activity levels was noted between treatment with 400 mg/kg/day *P. niruri* leaf extract and glibenclamide.

3.6. Effect of P. niruri Leaf Aqueous Extract on Renal GPx Levels. Figure 5 shows the effect of *P. niruri* leaf aqueous extract or glibenclamide on renal GPx levels in diabetic rats. Our findings indicate that GPx activity levels were lower in diabetic rats (29.1%) as compared to normal, nondiabetic rats. Treatment with 200 mg/kg/day and 400 mg/kg/day aqueous leaf extract of *P. niruri* resulted in a significantly higher GPx activity levels (18.94% and 35.78%, resp.) as compared to the nontreated diabetic rats. Glibenclamide treatment resulted in 32.63% higher GPx activity levels in diabetic rats as compared to the nontreated diabetic rats. No significant difference in GPx activity levels was noted between treatment with *P. niruri* leaf aqueous extract and glibenclamide.

3.7. Correlation between Levels of MDA and Antioxidant Enzymes in the Kidney. Negative correlations were observed between the levels of kidney LPO products as reflected by MDA amount (Figures 6(a), 6(b), and 6(c)) and SOD, CAT, and GPx activity levels. A strong negative correlation was observed between renal MDA content and SOD activity levels ($r = -0.9675, P = 0.007$), MDA content and CAT activity levels ($r = -0.9856, P = 0.0021$), and renal MDA content and GPx activity levels (Figure 6(c)), with correlation coefficient $= -0.9872, P = 0.0017$.

4. Discussion

Hyperglycaemia increases the production of free radicals and decreases the tissue antioxidative capacity in diabetes. These imbalances lead to tissue oxidative stress. The tissue antioxidative potential needs to be raised in order to overcome oxidative damage. The kidney is involved in maintaining body homeostasis as well as regulating electrolytes and acid-based balances and blood pressure. Diabetes-related kidney oxidative stress was reported to cause glomerular hypertrophy, basement membrane thickening, mesangial expansion, tubular atrophy, interstitial fibrosis, and arteriolar thickening [31]. A number of medicinal plants have been reported to possess antioxidative capacity which could help reduce free radical formation and promote endogenous antioxidant enzyme activity in the kidney [18]. One of the plants which has been reported to display kidney protective effect is *P. niruri*. The aqueous extract of *P. niruri* has been shown to reduce the crystals aggregation in rat urine which could help to inhibit the formation of urinary stones [8]. In the present study, aqueous leaf extract of *P. niruri* was found to display *in vitro* free radical scavenging activity, reduce endogenous LPO product formation, and increase activity levels of endogenous antioxidant enzymes in the kidney, which makes this herb a potential treatment for diabetic nephropathy.

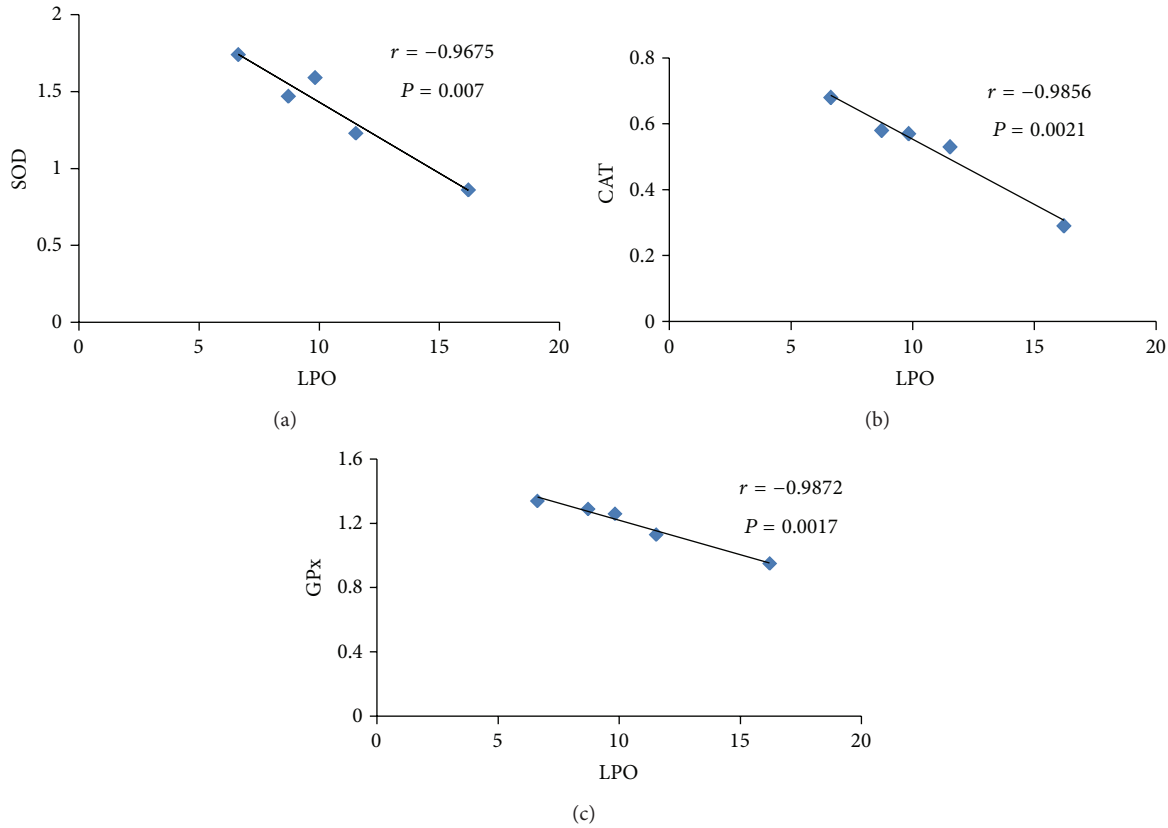

Figure 6: (a–c) Correlation between MDA and activity levels of SOD, CAT, and GPx in the kidney. Strong negative correlations were noted between the levels of MDA and activity of antioxidant enzymes indicating that the extent of tissue damage due to oxidative stress was directly related to the levels of endogenous antioxidant enzymes.

We have shown that the aqueous leaf extract of *P. niruri* displays *in vitro* antioxidant activity from the dose-dependent inhibition on DPPH, superoxide and hydroxyl radicals formation. Additionally, this extract also possesses hydrogen peroxide scavenging activity. Together with superoxide inhibition, *P. niruri* leaf aqueous extract could inhibit formation of peroxides and breaks autooxidative chain reaction. Our findings were consistent with the previous reports on *in vitro* radical scavenging activity of *P. niruri* [32, 33]. The free radical scavenging activity of this herb could be due to the presence of various bioactive compounds such as alkaloids, flavonoids, coumarins, and polyphenols which were reported to possess antioxidant capabilities [34].

Many studies have shown that hyperglycaemia was able to increase tissue oxidative stress via activating the polyol pathway, nonenzymatic protein glycosylation, and autooxidation of glucose leading to increased production of reactive oxygen species (ROS) which include superoxide radical ($O^{2\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). Additionally, hyperglycemia could also reduce antioxidant defence systems of the body [35, 36]. Experimentally induced diabetic rats were known to have high levels of tissue oxidative stress as characterized by high amount of LPO products, MDA, which indirectly reflects intensified free radical production [37]. The end product of lipid peroxidation was found to cause damage to the proteins, lipids, and DNAs [38]. In the present study, MDA levels in diabetic rat kidneys were higher than nondiabetic rats. Recently, Naik et al. [39] and others [40, 41] have reported that tissue oxidative damage as reflected by high amount of lipid peroxidation products occurs in the kidney of diabetic rats. Our findings which indicate a significantly lower LPO product accumulation in the kidney of diabetic rats following *P. niruri* leaf aqueous extract treatment provide evidence that consumption of this extract could prevent elevation of oxidative stress in the kidney in diabetes. Other *Phyllanthus* species such as *Phyllanthus amarus* have been reported to protect the kidney against the increase in LPO product in diabetes [42].

The physiological levels of antioxidant enzymes such as SOD, CAT, and GPx are important to reduce the formation of H_2O_2 by dismutating oxygen radicals, eliminating organic peroxides, and reducing hydroperoxides generations in hyperglycaemic condition [43]. SOD is involved in scavenging superoxide radicals ($O^{2\bullet-}$) and therefore prevents its conversion into H_2O_2 and molecular oxygen [35]. In our study, SOD activity levels were reduced in diabetic rat kidney which could be due to oxidative inactivation by H_2O_2 or glycosylation [44]. Fujita et al. [45] reported that downregulation of renal CuZn-SOD (SOD1) and CuZn-SOD (SOD3) activities could result in diabetic nephropathy. Kitada et al. [46] reported that resveratrol, a plant bioactive compound,

prevents nephropathy development by preventing Mn-SOD dysfunction in the kidney of diabetic mice. In view of this, the effect of the leaf extract of *P. niruri* in maintaining renal SOD activity levels near normal could help to preserve the kidney function and prevent nephropathy development in diabetes. Our previous study has shown that the aqueous leaf extract of *P. niruri* prevents the decrease in SOD activity in cardiac tissue following doxorubicin-induced myocardial toxicity in rats [47].

CAT and GPx are involved in the elimination of H_2O_2 [48]. CAT was regarded as a major renal antioxidant which helps to reduce H_2O_2 and protects the tissues from highly reactive hydroxyl radicals [49]. In our study, decreased CAT activity in the kidney was observed in diabetic rats. Hwang et al. [50] reported that CAT deficiency could accelerate kidney injury in diabetes through peroxisomal dysfunction. Administration of *P. niruri* leaf aqueous extract to diabetic rats could prevent the decrease in renal CAT activity most probably via preventing dysfunction of CAT enzyme by free radicals. GPx, a selenium containing enzyme, plays a role in minimizing tissue oxidative damage [41]. Reduced GPx activity in diabetes could be due to inactivation by free radicals [51]. In our study, GPx activity levels were maintained near normal in diabetic rat kidneys following *P. niruri* leaf aqueous extract treatment which suggest that this herb protects this tissue against oxidative damage by preventing enzyme dysfunction. A strong negative correlation between LPO and SOD and CAT and GPx activity levels indicated that kidney oxidative damage was dependent on activity levels of these enzymes. Additionally, the decreased levels of LPO product following *P. niruri* leaf aqueous extract treatment could also be due to reduced level of free radicals as observed from *in vitro* radical scavenging effect of the leaf extract.

In conclusion, this study has provided scientific evidence, whereby administration of *P. niruri* leaf aqueous extract to diabetic rats could reduce oxidative stress in the kidney via preventing the decrease in activity levels of endogenous antioxidant enzymes. Our findings therefore support the claims that this herb is beneficial in treating kidney disease due to diabetes.

Conflict of Interests

The authors reported no conflict of interests regarding the publication of the paper.

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Review Article

Neurological Effects of Honey: Current and Future Prospects

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Honey is the only insect-derived natural product with therapeutic, traditional, spiritual, nutritional, cosmetic, and industrial value. In addition to having excellent nutritional value, honey is a good source of physiologically active natural compounds, such as polyphenols. Unfortunately, there are very few current research projects investigating the nootropic and neuropharmacological effects of honey, and these are still in their early stages. Raw honey possesses nootropic effects, such as memory-enhancing effects, as well as neuropharmacological activities, such as anxiolytic, antinociceptive, anticonvulsant, and antidepressant activities. Research suggests that the polyphenol constituents of honey can quench biological reactive oxygen species and counter oxidative stress while restoring the cellular antioxidant defense system. Honey polyphenols are also directly involved in apoptotic activities while attenuating microglia-induced neuroinflammation. Honey polyphenols are useful in improving memory deficits and can act at the molecular level. Therefore, the ultimate biochemical impact of honey on specific neurodegenerative diseases, apoptosis, necrosis, neuroinflammation, synaptic plasticity, and behavior-modulating neural circuitry should be evaluated with appropriate mechanistic approaches using biochemical and molecular tools.

1. Introduction

Honey, a natural food product, is a sweet, viscous substance that is formed from the nectar of flowers by honeybees (*Apis mellifera*; Family: Apidae). The conversion of nectar to honey is an impressively complex process. Nectar is first collected from flowers and undergoes ripening by partial enzymatic digestion in the honey stomach of the honeybee. The ripened nectar is then matured by moisture evaporation through fanning by the bees, which leaves a moisture content of only approximately 13 to 18% in the honey [1]. Honey has been utilized by humans since prehistoric times, before civilization appeared approximately 5,500 years ago. Most ancient civilizations, such as the Egyptians, Greeks, Chinese, Mayans, Romans, and Babylonians, used honey both for nutritional purposes and for its medicinal properties [2]. Honey is the only insect-derived natural product, and it has therapeutic, religious, nutritional, cosmetic, industrial, and traditional value.

The global production of honey increased by 10%, from 1,419,072 to 1,555,980 tons, between 2005 and 2010 [3]. In addition to the consumption of raw honey, the use of honey in beverages is also increasing in popularity. Although modern science has reported its medical benefits, honey has historically been utilized in various food products as a sweetening agent and in medicine as a therapeutic agent for wound healing and for the treatment of cataracts [2, 4]. Raw honey has been used for centuries by traditional medical practitioners worldwide in numerous medical treatments, such as treatments for eye diseases in India, cough and sore throat in Bangladesh, leg ulcers in Ghana, and measles in Nigeria [5].

The traditional knowledge of honey and modern science are merged in "apitherapy," which denotes the medical use of honey and bee products. Apitherapy has become a major focus of research involving alternative medicine because a wide variety of well-known preventive or curative methods from folk medicine use honey to treat different ailments, and the therapeutic properties of honey have been increasingly documented in the modern scientific literature [6–8]. Recently, the oral ingestion of raw honey has been indicated for insomnia, anorexia, stomach and intestinal

Table 1: An overview of composition of raw honey [22].

Nutrient	Value in 100 g
Moisture	17.10 g
Carbohydrate	82.40 g
Glucose	35.75 g
Fructose	40.94 g
Sucrose	0.89 g
Maltose	1.44 g
Galactose	3.10 g
Total dietary fiber	0.20 g
Protein	0.30 g
Total lipid (fat)	0.00 g
Ash	0.20 g
Energy	304 kcal

ulcers, constipation, osteoporosis, and laryngitis. Externally applied honey is used to treat athlete's foot, eczema, lip sores, and both sterile and infected wounds caused by accidents, surgery, bedsores, or burns. In many countries, including France and Germany, physicians recommend using honey as a first-line treatment for burns, superficial wounds, and in some cases, even deep lesions such as abscesses [9].

2. Nutritional Facts about Honey

To date, approximately 300 varieties of honey have been identified [5]. These varieties exist due to the variable types of nectar that are collected by the honeybees. Although there have been many nutritional studies of honey, only a few are representative. Carbohydrates are the main constituents of honey and contribute 95 to 97% of its dry weight. In addition to carbohydrates, honey contains numerous compounds, such as organic acids, proteins, amino acids, minerals, and vitamins [10, 11] (Table 1). Pure honeys were also reported to contain polyphenols, alkaloids, anthraquinone glycosides, cardiac glycosides, flavonoids, reducing compounds, and volatile compounds [12–14].

Monosaccharides, such as fructose and glucose, are the predominant sugars present in honey, and they are said to be responsible for most of the physical and nutritional characteristics of honey [15]. Smaller quantities of other types of sugars, such as disaccharides, trisaccharides, and oligosaccharides, are also present in honey. The disaccharides primarily include sucrose, galactose, alpha,beta-trehalose, gentiobiose, and laminaribiose, whereas the trisaccharides primarily include melezitose, maltotriose, 1-ketose, panose, isomaltose glucose, erlose, isomaltotriose, theanderose, centose, isopanose, and maltopentaose [15–17]. Approximately 5 to 10% of total carbohydrates are oligosaccharides, and approximately 25 different oligosaccharides have been identified [18, 19]. Many of these sugars are not found in the nectar but are formed during the honey ripening and maturation phases.

Gluconic acid, which is a product of glucose oxidation by glucose oxidase, is the major organic acid that is found in honey; in addition, minor amounts of formic, acetic, citric, lactic, maleic, malic, oxalic, pyroglutamic, and succinic acids have also been detected [20]. These organic acids contribute to the acidic (pH between 3.2 and 4.5) characteristic of honey [21]. However, honey can also behave as a buffer.

Honey also contains several physiologically important amino acids, including all nine essential amino acids and all nonessential amino acids except for glutamine and asparagine. Among the amino acids present, proline is predominant, followed by aspartate, glutamate, and some other types of amino acids [22]. However, in another study, proline was reported as the primary amino acid in honey, followed by lysine [23]. Enzymes that are either secreted from the hypopharyngeal glands of the bee or originate from the botanical nectars constitute the main protein component of honey. These enzymes include the bee hypopharyngeal gland-derived diastase (an amylase that digests starch to maltose), invertases (e.g., saccharase and α-glucosidase that catalyzes the conversion of sucrose to glucose and fructose), glucose oxidase (which produces hydrogen peroxide and gluconic acid from glucose), and plant-derived catalase (which regulates the production of hydrogen peroxide), along with acid phosphatase [24].

The vitamin content in honey is generally low and does not meet the recommended daily intake (RDI). Usually, all of the water-soluble vitamins are present in honey, with vitamin C being the most abundant. Approximately 31 different minerals have been detected in honey, including all of the major minerals, such as calcium, phosphorus, potassium, sulfur, sodium, chlorine, and magnesium (Table 2). Some essential trace minerals are also reported to be present in honey, such as rubidium (RB), silicon (Si), zirconium (Zr), vanadium (V), lithium (Li), and strontium (Sr), as well as some trace elements, such as lead (Pb), cadmium (Cd), and arsenic (As), which could be present due to contaminants from surrounding environments [25]. Interestingly, the amounts of these minerals follow a geographical variation; the mineral compositions of honeys that are collected from similar regions are similar. However, several previous reports claimed that honey is a poor source of minerals [8, 22], whereas several other recent reports suggest that honey is rich in minerals [26, 27]. Essential trace elements are important, particularly among growing children because of their rapid growth and development. Nevertheless, a comparison with the RDI clearly indicates that honey contains a substantial amount of several essential trace elements that would partially meet the RDI for children (Table 2). For adults, honey is a good source of potassium.

3. Other Nonnutritional Components of Honey

Previous studies have reported the presence of approximately 600 different volatile compounds in honey, and these compounds can be used to characterize its botanical source [28]. In addition, volatile compounds can also impart aromatic characteristics to honey and contribute to its potential biomedical activity [28]. The volatile composition of honey is generally low but includes hydrocarbons, aldehydes, alcohols,

Table 2: A comparison of the minerals found in honey (major and essential trace minerals) with RDI as reported from several studies [25–27].

Major minerals	RDI	One tablespoon (21 g)	Essential trace minerals	RDI	One tablespoon (21 g)
Calcium	1000 mg	5.0 mg [27]	Copper	2 mg	0.4 mg [27]
Chloride	3400 mg	11.5 mg [25]	Fluoride	150 μg	280.0 μg [25]
Magnesium	350–400 mg	1.4 mg [26]	Iron	15–18 mg	4.6 mg [27]
Phosphorus	1000 mg	0.5 mg [26]	Molybdenum	75 μg	4.0 μg [25]
Potassium	3500 mg	21.0 mg [27]	Selenium	70 μg	104.0 μg [27]
Sodium	2400 mg	2.5 mg [26]	Zinc	15 mg	1.3 mg [27]

The values are daily reference values (DRVs) of RDI. The DRVs for major minerals are based on a caloric intake of 2,000 calories for adults and children (of four or more years of age). For trace elements, the RDIs that are given are the maximums for all sex and age groups [130, 131].

ketones, acid esters, benzene and its derivatives, furan and pyran, norisoprenoids, terpene and its derivatives, and sulfur, as well as cyclic compounds [29, 30].

Polyphenols and flavonoids, which act as antioxidants, are two important bioactive molecules that are present in honey. Emerging evidence from recent studies has confirmed the presence of approximately 30 different polyphenols in honey [31, 32]. The total polyphenol content of honey varies from 50 to 850 mg/kg, whereas the flavonoid content varies from 36 mg/kg to 150 mg/kg [12, 33, 34]. The presence and concentrations of these polyphenols in honeys can vary depending upon the floral source and the geographical and climatic conditions. Some bioactive compounds, such as Galangin, kaempferol, quercetin, isorhamnetin, and luteolin, are present in all types of honey, whereas others, such as hesperetin and naringenin, are reported only in specific varieties [35]. Overall, the most commonly reported phenolic and flavonoid compounds in honey include ellagic acid, gallic acid, syringic acid, benzoic acid, cinnamic acid, ferulic acids, chlorogenic acid, caffeic acid, coumaric acid, myricetin, chrysin, hesperetin, isorhamnetin, quercetin, galangin, apigenin, catechin, kaempferol, naringenin, and luteolin [7, 31, 32].

4. Effects of Honey on Brain Structures and Functions

4.1. Current Experimental Evidence of the Nootropic and Neuropharmacological Effects of Honey. Research from the past two decades has explored honey as an enigmatic gel that has gastroprotective, hepatoprotective, reproductive, hypoglycemic, antioxidant, antihypertensive, antibacterial, antifungal, anti-inflammatory, immunomodulatory, wound healing, cardio-protective and antitumor effects [6, 26, 36, 37]. Unfortunately, research on the nootropic and neuropharmacological effects of honey is scarce. Nevertheless, the belief that honey is a memory-boosting food supplement is actually ethnotraditional as well as ancient in nature. For instance, honey is reported to be an important component of Brahma rasayan, an Ayurvedic formulation that is prescribed to extend the lifespan and improve memory, intellect, concentration, and physical strength [38].

One established nootropic property about honey is that it assists the building and development of the entire central nervous system, particularly among newborn babies and preschool age children, which leads to the improvement of memory and growth, a reduction of anxiety, and the enhancement of intellectual performance later in life [26]. Additionally, the human brain is known to undergo postnatal development with the obvious maturation and reorganization of several structures, such as the hippocampus and cerebral cortex. It has been reported that this postnatal development occurs through neurogenesis, which occurs predominantly during childhood, and this development can also extend into adolescence and even through adulthood [39]. Empirical, but striking, evidence supporting this concept was provided by an experiment that was conducted on postmenopausal women; those who received honey showed improvements in their immediate memory but not in immediate memory after interference or in delayed recall [40]. In another study, the normal diet of two-month-old rats was supplemented with honey, and their brain function was assessed over a one-year period. Honey-fed rats showed significantly less anxiety and better spatial memory throughout all stages compared with the control group of rats. More importantly, the spatial memory of honey-fed rats, as assessed by object recognition tasks, was significantly greater during later months (i.e., 9 and 12) [41].

In agreement with the previous study, both short- and long-term supplementations with honey at a dose of 250 mg/kg body weight significantly decreased the lipid peroxidation in brain tissue with a concomitant augmentation of superoxide dismutase (SOD) and glutathione reductase activity. Thus, honey consumption ameliorates the defense mechanism against oxidative stress and attenuated free radical-mediated molecular destruction [39]. Furthermore, honey decreased the number of degenerated neuronal cells in the hippocampal CA1 region, a region that is known to be highly susceptible to oxidative insult [42]. Theoretically, the cumulative macromolecular destruction by free radicals due to an imbalance between the prooxidant and antioxidant defense systems is implicated in aging [43]. Many studies have focused on the evidence of oxidative stress in neurodegenerative diseases, such as Alzheimer's disease (AD), mild cognitive impairment, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) [44].

Emerging research has documented the neuropharmacological effect of honey as a nutraceutical. Oyekunle et al. [45] conducted the first such study, in which rats were fed with different concentrations of honey (10, 20 and 40%) at a dose of 0.5 mL/100 g. Significant dose-dependent

increases in exploratory activities in a hole board test and in locomotor, rearing and grooming activities in an open-field test were found in the honey-fed test groups rats compared with the control group rats. These findings indicate that the consumption of honey mitigates anxiety and exerts an excitatory effect on the central nervous system, especially at the highest nonsedative dose [45]. In another study, the neurological effects of honey were investigated by assessing spatial working memory in mice using (1) the Y-maze test and (2) pentobarbital-induced hypnosis and assessing, (3) its anxiolytic activities using hole-board and elevated plus-maze tests, (4) its anticonvulsant activity in an picrotoxin seizure model, (5) its antinociceptive activity in hot-plate and tail-flick tests, and (6) its antidepressant effects using the forced swimming test. The authors of that study concluded that honey is a functional food that possesses anxiolytic, antinociceptive, anticonvulsant, and antidepressant effects [46].

Indeed, the neuropharmacological impact of honey reflects the preliminary modulatory ability of the neural circuit and associated neurochemical systems that underlie the behavioral and molecular changes associated with the experimental paradigm. These insights into the neuropharmacological effects of honey highlight the neurological factors that are influenced by treatment with honey. Exploratory behaviors often involve the excitatory neural systems, such as the cholinergic and dopaminergic systems, whereas anxious behavior often involves the inhibitory neural system, specifically γ-aminobutyric acid (GABA) [47–49]. Several lines of experimental evidence support the hypothesis that the neuropharmacological effects of honey are mediated via dopaminergic and nonopioid central mechanisms, such as the voltage-gated sodium channel blocking hypothesis, the activation of the noradrenergic inhibitory system and/or serotonergic systems, and the GABAergic system [45, 50].

In addition to neural effects, glial cells may also respond to honey therapy because honey shows a neuroprotective effect in the cerebral focal-induced ischemia model in rats [51]. Moreover, honey attenuated ischemia-induced neuroinflammation by activating microglia, and neuroinflammatory processes in the brain are believed to play a crucial role in the development of neurodegenerative diseases as well as in neuronal injury associated with stroke [52, 53]. Interestingly, ischemia-induced cognitive impairments that result from microglia- and/or astrocyte-mediated neuroinflammation were also significantly attenuated by honey therapy [52, 54].

5. The Effects of Physiologically Active Moieties in Honey on Brain Function

Oxidative stress is a common manifestation of all types of biochemical insults to the structural and functional integrity of neural cells, such as aging, neuroinflammation, and neurotoxins. The brain is highly susceptible to oxidative damage due to its high oxygen demand as well as to the high amount of polyunsaturated fatty acids (PUFAs) in the neuronal membranes [55]. Different phytochemical compounds have been shown to have scavenging activities and can activate key antioxidant enzymes in the brain, thus breaking the vicious cycle of oxidative stress and tissue damage [56, 57]. Several supplementary research reports have suggested that the neuroprotective effect of the polyphenols present in honey involves several important activities within the brain. These effects include protection against oxidative challenge; the attenuation of neuroinflammation; the promotion of memory, learning, and cognitive function; and protection against neurotoxin-induced neuronal injury. We describe several important constituents in honey that may play this protective role.

Apigenin is a common flavonoid that is frequently identified in honey. In addition to its radical scavenging activity, apigenin protects neurons against oxygen-glucose deprivation/reperfusion-induced injury in cultured primary hippocampal neurons by improving sodium/potassium-ATPase (Na^+/K^+-ATPase) activities [58]. Apigenin also inhibits the kainic acid-induced excitotoxicity of hippocampal cells in a dose-dependent manner by quenching reactive oxygen species and by inhibiting the depletion of reduced glutathione (GSH) levels [59]. Apigenin suppresses the interferon gamma (IFN-γ)-induced expression of CD40, whereas the signaling of CD40 is critically involved in microglia-related immune responses in the brain. Rezai-Zadeh et al. suggested that apigenin may have neuroprotective and disease-modifying properties in several types of neurodegenerative disorders [60]. Moreover, apigenin stimulates the adult neurogenesis that underlies learning and memory [39].

Caffeic acid, another important antioxidant, is a type of phenolic acid that is present in honey, as well as in coffee, fruits and vegetables. An *in vitro* study has demonstrated the neuroprotective effects of caffeic acid on neuronal cells [61]. The neuroinflammatory suppression activity of caffeic acid can be inferred from the observation that caffeic acid reverses the aluminum-induced overexpression of 5-lipoxygenase (5-LOX) in brain tissues [62]. Caffeic acid also prevents the aluminum-induced damage of the cerebrum that is associated with neuronal death in the hippocampus and with learning and memory deficits [62]. *In vitro* treatment with caffeic acid at several different concentrations has been reported to increase the acetylcholinesterase activity in the cerebral cortex, cerebellum, and hypothalamus. A similar scenario is also observed in the cerebellum, hippocampus, hypothalamus, and pons when caffeic acid is administered *in vivo*. All of these findings strongly support the proposition that caffeic acid improves memory by interfering with cholinergic signaling, in addition to its neuroprotective effects [63].

Catechin is a flavonoid that contributes to the antioxidant activities of honey. Several studies have repeatedly demonstrated the neuroprotective effects of catechin on neuronal death in a wide array of cellular and animal models of neurological diseases [64, 65]. Although catechin possesses potent iron-chelating, radical-scavenging, and anti-inflammatory activities, current studies have indicated that the modulation of signal transduction pathways, cell survival, or death genes and mitochondrial function significantly contribute to the induction of cell viability [66]. For instance, according to Unno et al., the daily consumption of green tea, which contains high levels of catechin, can delay the memory regression

that is associated with age-related brain atrophy and cognitive dysfunction [67]. Animal studies have indicated that the long-term administration of green tea may prevent age-related learning and memory decline by modulating the transcription factor cAMP-response element binding protein (CREB) and by upregulating synaptic plasticity-related proteins in the hippocampus [68, 69]. Similar memory-ameliorating effects were also shown in the context of neurodegenerative diseases, such as PD, AD, and multiple sclerosis [64].

Chlorogenic acid is a derivative of caffeic acid and is another common phenolic acid that is found in honey. A dose-dependent protective effect of chlorogenic acid against apoptosis was observed in pheochromocytoma-12 (PC12) cell lines that were exposed to methyl mercury-induced apoptotic damage. The protective activity of chlorogenic acid was associated with a reduction in the generation of reactive oxygen species (ROS) and the attenuation of apoptosis by the activation of caspase-3 [70]. In a study by Kwon et al. [71], the neuroprotective effects of chlorogenic acid on scopolamine-induced learning and memory impairment were investigated using several behavioral tests, such as the Y-maze, passive avoidance, and Morris water maze tests. Chlorogenic acid was found to significantly improve memory-related performance in all of the tests. It was concluded that chlorogenic acid may exert antiamnesic activity via the inhibition of acetylcholinesterase and malondialdehyde in the hippocampus and frontal cortex because chlorogenic acid inhibited the acetylcholinesterase activity of the hippocampus and frontal cortex in both *ex vivo* and *in vitro* model systems [71]. Chlorogenic acid inhibits the synthesis and release of inflammatory mediators, such as tumor necrosis alpha and nitric oxide (NO), thus contributing to anti-inflammatory and analgesic activities against carrageenan-induced inflammation [72]. Therefore, the chlorogenic acid in honey might have the capacity to attenuate neuroinflammation.

Chrysin (5,7-dihydroxyflavone) is another important flavonoid antioxidant that is present in honey. A behavioral experimental model revealed that chrysin is an anxiolytic that acts as a central receptor for benzodiazepine in instances where anxiety was reported to hamper cognitive function and learning capacity [73]. A study conducted by He et al. [74] showed that the therapeutic potential of chrysin in neurodegeneration-associated dementia resulted from cerebral hypoperfusion. The effects of chrysin were further investigated in a rat model of cognitive deficits and brain damage generated by the permanent occlusion of the bilateral common carotid arteries [74]. Such surgically induced hypoperfusion leads to a significant increase in the escape latency in the Morris water maze, with biochemical features of neural damage, such as increases in glial fibrillary acidic protein expression and apoptosis. Interestingly, chronic treatment with chrysin significantly alleviated neuronal damage and spatial memory deficits, with a reduction in lipid peroxidation and glutathione peroxidase activity but a decrease in SOD activity [74], indicating the neuroprotective role of honey.

p-Coumaric acid is the most abundant of the three hydroxy derivatives of cinnamic acid. A previous study demonstrated the oxidative stress reduction capacity and antigenotoxic capacity of p-coumaric acid [75]. In doxorubicin-induced cardiotoxicity, p-coumaric acid was able to increase the levels of GSH, SOD, and catalase activities with a concomitant reduction of lipid peroxidation [76]. p-Coumaric acid exhibited neuroprotective effects against 5-S-cysteinyl-dopamine-induced neurotoxicity. The extent to which p-coumaric acid confers neuroprotection was reported to be equal to or greater than that observed for the flavonoids (+)-catechin, (−)-epicatechin, and quercetin [77].

Ellagic acid is a phenolic acid that is found not only in fruits and vegetables but also in honey. In addition to its antioxidant activity, ellagic acid exerts chemopreventive effects, as indicated by its antiproliferative activity [78]. Interestingly, the chemopreventive effects of ellagic acid are executed through the reduction of oxidative stress at the cellular level [30]; moreover, oxidative stress is involved in neurodegeneration and age-related memory deficits. Hence, the probable neuroprotective effect of ellagic acid is promising. Treatment with ellagic acid also restores the levels of lipid peroxides and NO (nitric oxide), the activities of catalase and paraoxonase, and the total antioxidant status of the brain to normal levels [79]. Other experiments also support the hypothesis that ellagic acid reduces oxidative stress in the brain, which is reflected by improvements in cognitive function. Ferulic acid, another polyphenol that is found in honey, is a phenolic acid. Ferulic acid can provide neuroprotection against cerebral ischemia/reperfusion injury-associated apoptosis in rats. Ferulic acid treatment resulted in a decrease of the extent of apoptosis, with decreased levels of ICAM-1 mRNA and reduced numbers of microglia and macrophages. This phenomenon ultimately results in the downregulation of inflammation-induced oxidative stress and oxidative stress-related apoptosis [80]. In another study [81], the ameliorating effects of ferulic acid on apoptosis caused by cerebral ischemia or reperfusion were investigated. Ferulic acid was found to show neuroprotective effects against p38 mitogen-activated protein (MAP) kinase-mediated NO-induced apoptosis. It was also reported that ferulic acid inhibits Bax translocation, the release of cytochrome c, and p38 MAP kinase phosphorylation and enhances the expression of the GABAB1 receptor [81]. Ferulic acid could also alleviate learning and memory deficits through the concomitant inhibition of acetylcholinesterase activity and the augmentation of SOD activity while lowering the concentration of glutamic acid and malondialdehyde in the hippocampus of rats. These results suggested that the antioxidant activities of the honey may contribute to the improvement of the cholinergic system in the brain or to the inhibition of nerve injury by excitatory amino acids [82]. Ferulic acid may be useful for preventing trimethyltin-induced cognitive dysfunction as well as for boosting the activation of choline acetyltransferase (ChAT) in dementia [83].

Gallic Acid. Gallic acid prevents the apoptotic death of cortical neurons *in vitro* by inhibiting amyloid beta (25–35)-induced glutamate release and the generation of ROS [84]. Gallic acid possesses an antianxiolytic activity, which provided the primary evidence in support of

the memory-ameliorating effect of gallic acid because anxiety is associated with memory disturbance [85]. The memory-ameliorating effects of gallic acid were further confirmed by Al Mansouri et al. [86], who revealed its neuroprotective effect on 6-hydroxydopamine-induced and cerebral oxidative stress-induced memory deficits. Gallic acid improved memory concomitant with increases in the total thiol pool and glutathione peroxide activity and decreased lipid peroxidation in the hippocampus and striatum [87]. However, we cannot claim that these biochemical findings are entirely responsible for the improvements in memory.

Kaempferol is a plant flavonoid that is also frequently found in honey. The toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is a neurotoxin, leads to behavioral deficits, a depletion of dopamine, reductions in SOD and glutathione peroxidase activities, and an elevation of lipid peroxidation in the substantia nigra. The administration of kaempferol has been reported to reverse all of these behavioral and biochemical alterations and to prevent the loss of TH-positive neurons that is induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [88]. In another study, kaempferol demonstrated the ability to protect primary neurons from rotenone-induced apoptotic challenge. Specifically, kaempferol-ameliorated antioxidant defenses and antiapoptotic effects involve the enhancement of mitochondrial turnover, which is mediated by autophagy [89]. Furthermore, kaempferol may be an optimal treatment for improving cognitive function due to its positive effects on depression, mood, and cognitive functions [90].

Luteolin is a flavonoid from the flavone class that has been reported to be found in honey. As is the case for most flavonoids, luteolin has antioxidant, anti-inflammatory, and antitumor properties [91]. Luteolin also has neuroprotective effects against microglia-induced neuronal cell death. The consumption of luteolin has been found to improve the spatial working memory of aged rats by mitigating microglia-associated inflammation in the hippocampus [92]. The impairment of learning acquisition induced by cholinergic neurotoxins and muscarinic and nicotinic receptor antagonists were reported to be attenuated by luteolin. This phenomenon, however, was not observed for dopaminergic neurotoxin- and serotonergic neurotoxin-induced memory impairments, thus confirming the involvement of the central cholinergic system in the memory-restoring function of luteolin [93].

Interestingly, Tsai et al. showed that the augmenting effect of luteolin on Mn-SOD and (Cu/Zn)-SOD activity as well as on the GSH levels in the cortex and hippocampus was associated with the amelioration of amyloid beta (1–40)-induced oxidative stress and cognitive deficits [94]. Luteolin is believed to enhance basal synaptic transmission and facilitate the induction of long-term potentiation (LTP) by high-frequency stimulation in the dental gyrus of the hippocampus. At the molecular level, the LTP inductive effect of luteolin involves the activation of cAMP response element-binding protein (CREB) [95].

Myricetin is another well-known flavonoid that has also been reported to be found in honey. Yasuo et al. (1994) demonstrated that myricetin can reduce the calcium-induced increase in oxidative metabolism in rat brain neurons when administered at a concentration of 3 nM or greater [96]. In the case of the retinoid-induced apoptosis of human neuroblastoma cells, myricetin induced neuroprotection through a protective effect against retinoid-induced oxidative stress. The neuroprotective effect of myricetin was reported to be associated with a reduction in lipid peroxidation, retinoid-induced hydrogen peroxide generation, and superoxide radical generation (O^{2-}), as well as an elevation of the glutathione redox status [97]. In another study, myricetin was also reported to significantly prevent D-galactose-induced cognitive impairment. The results of this study also indicated that cognitive impairment was most likely mediated by the extracellular signal-regulated kinase- (ERK-) cyclic AMP response element binding protein (CREB) signaling pathway in the hippocampus [98].

Naringenin can confer a neuroprotective effect against quinolinic acid-induced excitotoxicity mediated by elevated intracellular calcium levels, NO-mediated oxidative stress, and, consequently, cell death [99]. Amyloid beta-protein-induced free radical-mediated neurotoxicity is also attenuated by naringenin [100]. Interestingly, free radical-mediated oxidative stress is a common manifestation of both amyloid beta- and quinolinic acid-induced neurotoxicity, and it has repeatedly been implicated in neurodegeneration and cognitive deficits. In a rat model, the administration of naringenin reversed the learning, memory, and cognitive impairments caused by the intracerebroventricular administration of streptozotocin [101]. Treatment with naringenin also increases the pool of GSH and the activities of glutathione peroxidase, glutathione reductase, glutathione-S-transferase, SOD, and choline acetyltransferase in the hippocampus in a rat model of Alzheimer's disease- (AD-) type neurodegeneration with cognitive impairment (AD-TNDCI), with a concomitant decrease in the loss of ChAT-positive neurons and impairments in spatial learning and memory [102].

Quercetin is another flavonoid with antioxidant activity that is commonly found in honey. An *in vitro* study demonstrates that quercetin can inhibit oxidative insults as well as oxidative stress-dependent and independent apoptosis in a neural cell model [103, 104]. Quercetin improves memory and hippocampal synaptic plasticity in models of memory impairment that is caused by chronic lead exposure [105]. Quercetin also exhibited neuroprotective effects against colchicine-induced cognitive impairments [106]. Another neuroprotective role confirmed for quercetin is the alleviation of neuroinflammation. According to Sharma et al., quercetin modulates an interleukin-1 beta-mediated inflammatory response in human astrocytes [107]. Quercetin also decreases the extent of ischemic injury in a lesion-repeated cerebral ischemic rat model and restores spatial memory through the suppression of hippocampal neuronal death [108, 109]. Interestingly, quercetin also showed ameliorating effects on the peripheral nervous system and the central nervous system (CNS). In another study, quercetin promoted the

functional recovery of the spinal cord following acute injury [110].

6. Honey as a Neuroprotective Nutraceutical

Generally, neurodamaging insults are categorized as either endogenous or exogenous in nature. Because the neurons of the mature nervous system are postmitotic, they cannot be easily replaced by cell renewal; therefore, neuronal cell death is the most widely studied neuronal pathologies. Neurodegeneration describes the progressive loss of neural structure and function that culminates in neuronal cell death. Acute neurodegeneration is usually caused by a specific or traumatic event, such as cardiac arrest, trauma, or subarachnoid hemorrhage, whereas chronic neurodegeneration occurs within the context of a chronic disease state with a multifactorial origin, such as AD, PD, HD, or amyloid lateral sclerosis [111]. The biochemical events underlying neurodegeneration include oxidative stress, mitochondrial dysfunction, excitotoxicity, neuroinflammation, misfolded protein aggregation, and a loss of functionality [112]. The ultimate fate of such a neurodamaging insult is neuronal cell death through apoptosis, necrosis, or autophagy [113]. Therefore, oxidative stress, mitochondrial dysfunction, and inflammation are prime candidates for neuroprotection [114].

Much research over the last few decades has established nutraceuticals as neuroprotective agents. In addition to the acute modulation of the antioxidant defense system, several nutraceuticals can also modulate gene expression to confer long-term protection [115, 116]. Phytochemicals can also modify cellular behaviors by influencing receptor function as well as by modulating intracellular events, such as cell-signaling cascades [117, 118]. Honey and its constituents can ameliorate oxidative stress and oxidative stress-related effects. The neuroprotective effects of honey are exerted at different stages of neurodegeneration and play prominent roles in early events (Figure 1).

7. Honey as a Nootropic Nutraceutical

Learning and memory are the most exclusive and basic functions of the brain. Synaptic plasticity is thought to be crucial for information processing in the brain and underlies the processes of learning and memory [119]. Synaptic plasticity describes the capacity of neurons to change their efficiency in neuronal transmission in response to environmental stimuli and plays an essential role in memory formation. Long-term synaptic plasticity, or long-term potentiation (LTP), is the molecular analog of long-term memory and is the cellular model that underlies the processes of learning and memory [120, 121]. The induction, expression, and maintenance of LTP involve a series of biochemical events [122]. LTP is induced by the influx of calcium into postsynaptic neurons through a set of receptors and/or channels and is usually followed by the amplification of calcium levels due to the release of calcium from the Ca^{2+}/$InsP_3$-sensitive intracellular store [123, 124].

The expression of LTP involves the activation of several calcium-sensitive enzymes, which include calcium/calmodulin-regulated protein kinases (CaMKII and CaMKIV), the cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), and MAPK/ERKs [125, 126]. Signaling events downstream and enzyme activation ultimately cause the initial expression and maintenance of LTP. However, the long-term expression and maintenance of LTP requires efficient gene expression. PKA may induce changes in the expression of genes via the phosphorylation of the transcription factor CREB. Phosphorylated CREB activates the transcription of genes with an upstream cAMP response element (CRE) [127]. The activation of CREB via MAPK/ERKs is thought to be connected to PKA and PKC signaling. Furthermore, CaMKII and CaMKIV may play a role in the maintenance of LTP through its effects on CREB phosphorylation [128]. Ultimately, CREB mediates the transcription and expression of at least two sets of genes, which include genes that regulate the transcription of other genes, such as *c-fos*, *c-jun*, *zif268*, and *Egr-3*, and effector genes, such as *Arc*, *Narp*, *Homer*, *Cox-2*, and *Rheb*, that directly act on cells to evoke different effects, including plastic changes [129].

Current research has clarified only a portion of the involvement of honey polyphenols in memory-related signaling pathways. However, the overall body of knowledge clearly suggests the neuroprotective roles of honey and several supplementary experimental studies support its memory-improving effects. Overall, honey or its bioactive constituents might influence multiple signaling pathways to exert its memory-improving effects (Figure 2).

8. Concluding Remarks and Future Prospects

The brain is the supervisory organ with critical functions, such as body homeostasis maintenance, learning, and memory. Any neurodamaging insult leads to either the death or the functional aberration of neural cells, which results in neurodegeneration and the loss of motor function and the executive functions of the brain, such as memory. There is strong scientific support for the development of nutraceutical agents as novel neuroprotective therapies, and honey is one such promising nutraceutical antioxidant. However, past research paradigms did not evaluate the neuropharmacological and nootropic effects of honey using appropriately in-depth mechanistic approaches concerning biochemical and molecular interventions.

Honey has an appreciable nutritional value. Raw honey possesses anxiolytic, antinociceptive, anticonvulsant, and antidepressant effects and improves the oxidative status of the brain. Several honey supplementation studies suggest that honey polyphenols have neuroprotective and nootropic effects. Polyphenol constituents of honey quench biological reactive oxygen species that cause neurotoxicity and aging as well as the pathological deposition of misfolded proteins, such as amyloid beta. Polyphenol constituents of honey counter oxidative stress by excitotoxins, such as kainic acid and quinolinic acid, and neurotoxins, such as 5-S-cysteinyl-dopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Honey polyphenols also counter direct

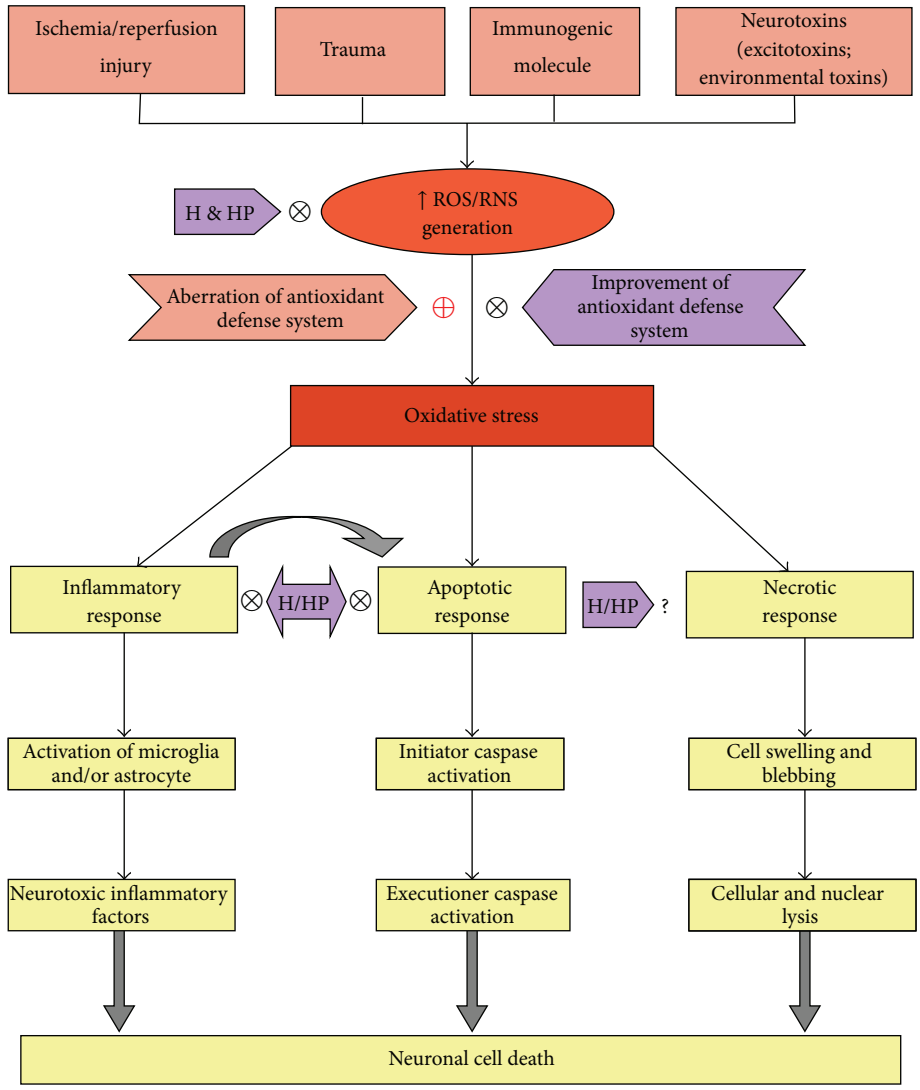

Figure 1: The putative neuroprotective mechanism of honey and its polyphenols. The generation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) increases irrespective of neurodamaging insults that lead to oxidative stress. The dysfunction of the antioxidant defense system synergistically causes reactive species accumulation, leading to oxidative stress. The ultimate outcome of such oxidative stress is neuronal cell death through an inflammatory, apoptotic, or necrotic response [111–114, 116, 119]. Honey (H) and its polyphenol constituents (HP) can counter oxidative stress by limiting the generation of reactive species as well as by strengthening the cellular antioxidant defense system. Honey and several honey polyphenols (apigenin, ferulic acid, and catechin) prevent neuronal cell death by attenuating neuroinflammation and apoptosis. However, the neuroinflammatory responses overlap with apoptosis, and the role of honey in necrotic cell death remains unclear. X = stop or prevent and + = improve or intensify.

apoptotic challenges by amyloid beta, methyl mercury-induced, and retinoid. Raw honey and honey polyphenol attenuate the microglia-induced neuroinflammation that is induced by ischemia-reperfusion injury or immunogenic neurotoxins. Most importantly, honey polyphenols counter neuroinflammation in the hippocampus, a brain structure that is involved in spatial memory. Honey polyphenols also counter memory deficits and induce memory formation at the molecular level. Several studies suggest that the modulation of specific neural circuitry underlies the memory-ameliorating and neuropharmacological effects of honey polyphenols.

Our information demands the evaluation of the benefits of raw honey and its individual constituents in specific neurodegenerative diseases, such as AD, PD, and HD. The ultimate biochemical impact of honey on mitochondrial dysfunction, apoptosis, necrosis, excitotoxicity, and neuroinflammation should also be explored. Furthermore, exploration of the actual cell signaling cascades that are associated with synaptic plasticity may provide more specific therapeutic interventions using honey. The effect of honey on synaptic plasticity under normal and disease conditions should also be determined. The neural circuits and receptors that are involved in the neuropharmacological effects of

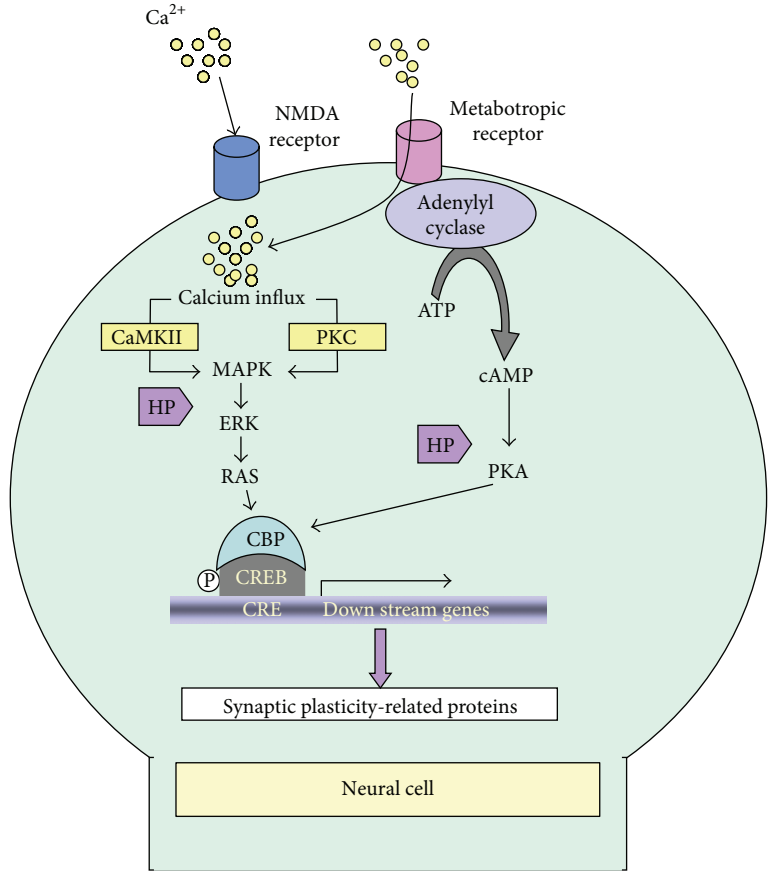

Figure 2: Putative nootropic mechanisms of honey and its polyphenols. Calcium influx via the N-methyl-D-aspartate receptor (NMDAR) occurs during the initial phase of NMDAR-dependent LTP. The inductive phase follows CREB phosphorylation through MAPK/ERKs signaling, which ultimately leads to the transcriptional regulation of synaptic plasticity-related proteins. Metabotropic receptors include ligand-gated ion channels that promote calcium influx (AMPA receptor) and enzyme-coupled receptors (such as cholinergic, glutamate, and dopamine receptors) that can trigger a second messenger (cAMP/cGMP) to activate downstream effector enzymes. The effector enzymes finally modulate the activation of CREB [123–128]. Honey polyphenols (HP: luteolin, myricetin, catechin) modulate synaptic plasticity through the activation of CREB by MAPK/ERKs and/or PKA-involved cellular signaling.

honey, such as anxiolytic, antinociceptive, anticonvulsant, and antidepressant activities, should be examined in further detail.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Recent Updates in the Treatment of Neurodegenerative Disorders Using Natural Compounds

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Neurodegenerative diseases are characterized by protein aggregates and inflammation as well as oxidative stress in the central nervous system (CNS). Multiple biological processes are linked to neurodegenerative diseases such as depletion or insufficient synthesis of neurotransmitters, oxidative stress, abnormal ubiquitination. Furthermore, damaging of blood brain barrier (BBB) in the CNS also leads to various CNS-related diseases. Even though synthetic drugs are used for the management of Alzheimer's disease, Parkinson's disease, autism, and many other chronic illnesses, they are not without side effects. The attentions of researchers have been inclined towards the phytochemicals, many of which have minimal side effects. Phytochemicals are promising therapeutic agents because many phytochemicals have anti-inflammatory, antioxidative as well as anticholinesterase activities. Various drugs of either synthetic or natural origin applied in the treatment of brain disorders need to cross the BBB before they can be used. This paper covers various researches related to phytochemicals used in the management of neurodegenerative disorders.

1. Introduction

Various neurodegenerative (progressive loss of structure and/or function of neurons) disorders share many common features at both cellular and subcellular levels. Intracellular and extracellular changes could be observed in Alzheimer's, Parkinson's, Huntington's, and other neurodegenerative diseases. As far as cellular and subcellular biological events are concerned, the cytosol and endoplasmic reticulum are responsible for the synthesis of new structural and functional protein molecules. Mechanisms of translational as well as posttranslational modifications are highly complex and sophisticated in nature. Any polypeptide that fails to fold properly is directed to its degradation processes or known as autophagy and ubiquitin proteasome system [1, 2].

Neurodegenerative disorders are usually characterized by accumulation of abnormal protein aggregation that leads to inflammation as well as oxidative stress in the central nervous

system (CNS). Parkinson's disease (PD) and Alzheimer's disease (AD) are the most common disorders of nervous system caused by environmental and genetic influences [3–5]. It has been observed that various types of biological mechanisms are associated with neurodegenerative disorders such as oxidative stress, aggregates of proteins in neurons, depletion or in sufficient synthesis of neurotransmitters, degradation of neurotransmitters in the synaptic cleft due to the higher activity of enzymes, abnormal ubiquitination, mitochondrial dysfunction, and excitotoxicity of neurons as well as disarrangement or damage of the blood brain barrier (BBB) (Figure 1).

AD is characterized by cognitive decline, neuronal loss, neuronal inflammation, and neuronal death, which is also known as apoptosis and/or necroptosis. Moreover, aggregation of β-amyloid (Aβ) is one of the main features of AD. The formation of hyperphosphorylated Tau (microtubule-associated protein) in the neurons is also linked with AD. PD is a movement disorder which is characterized by abnormal aggregation of α-synuclein protein in the neurons [33]. Similarly, abnormal long polyglutamine (PolyQ) may lead to Huntington's disease [34].

Another important brain disorder related to CNS inflammation and characterized by learning and social disabilities with no definite pathogenesis is known as autism spectrum disorder (ASD). Multiple biochemical and molecular features could be observed for the neurodegeneration in the brain of ASD [35, 36] including oxidative stress [37, 38], activated astrocytes and microglia [39, 40], neuronal loss [35, 40], elevated levels of 8-oxo-guanosine [41], and development of proinflammatory cytokines [40, 42].

Children with ASD tend to behave differently under stress or when exposed to certain foods, showing skin allergies [43]. Neurotensin with release of corticotrophin-releasing hormone under stressful conditions stimulates the microglia and mast cells leading to neurotoxicity and focal brain inflammation. In case of ASD, various pathological states could be observed but not in all ASD children including allergic symptoms, increased anti-brain protein autoantibodies, high anxiety, increased oxidative stress, and increased food intolerance while decreasing the levels of reduced glutathione, sulfation, and methylation [43]. Luteolin (a flavonoid) showed inhibitory effects on human mast cells that release tumor necrosis factor (TNF) [44]. Luteolin such as epigallocatechin gallate inhibits [45] mammalian target of rapamycin (mTOR) which stimulates the mast cells and microglia proliferation [46, 47] leading to the retardation of the release of TNF which could initiate apoptosis, necroptosis, and/or inflammation in the biological system. Various important biological actions of luteolin are illustrated (Figure 2) which may be helpful in children of ASD.

2. Blood Brain Barrier (BBB)

The blood brain barrier (BBB) is responsible for the regulation of small molecules (solutes) between the CNS and the blood circulation. Three different kinds of barriers could be observed where the central nervous system and blood interact; arachnoid barrier, blood-cerebrospinal fluid (CSF) barrier and the BBB. The neurons in the CNS signal by sending action potentials through which neurons interact in the biological system. The BBB had tight junctions between cells responsible for the reduction of flux mechanism through the paracellular pathway and intercellular cleft (physical barrier) and mediation of solute flux mechanisms (transport barrier) as well as enzyme metabolizing molecules (metabolic barrier). Moreover, the functions of barriers are equally operated in physiological and pathological states of BBB [48]. The tight junctions present between the astrocytes (part of the BBB) are composed of claudin and occludin proteins. Damage in these proteins or tight junctions can lead to the loss of BBB integrity with functional barrier loss [49, 50]. Various drugs, either synthetic or natural, may have their own mode of action but drugs used in the treatment of brain disorders have to cross the BBB to gain entry into the CNS, since structural and/or functional dysfunction in the BBB leads to inflammatory changes in the tissue such as movement of immune mediators in the brain, further contributing to the neurodegenerative process [51].

3. Inhibition of Cholinesterase Activity

Stimulating acetylcholine release in the brain region is one of the ways used in the treatment of neurodegenerative disorder such as AD that can further contribute to dementia and decline in higher cognitive function [52]. The pathological state of CNS particularly related to AD is characterized by neurofibrillary tangles, derangement of neurotransmitters in the neurons and synaptic cleft, and β-amyloids plaques all of which are related inflammatory mechanisms [53–55]. Both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are responsible for the breakdown of acetylcholine in the synaptic region and low levels of acetylcholine has been found to be related to age-related disorders that leads to loss of cognitive ability [18, 56].

Reactive oxygen species (ROS) developed as a result of oxidative stress in the biological system can contribute to damage of biological macromolecules and as a result pathological state at cellular level can become more evident. Such pathogenic state plays a crucial role in the aging process [57]. Cholinesterase inhibitors are not commonly used in allopathic and current treatments do not lead to sufficient production of acetylcholine to help in the management of AD [18]. The research in the field of phytochemicals has developed into investigation of natural compounds responsible for antioxidative (Table 2) and antiaging properties that can also be useful for neurodegenerative disorders [18, 58]. It is important to stimulate the cholinergic receptors in the CNS or enhance the prolonged production of acetylcholine in the synaptic cleft with the help of such active constituents that could retard the activities of AChE and BChE in the neuronal system. When the inhibition of enzyme activities is 60% or more by the plants extracts, compounds

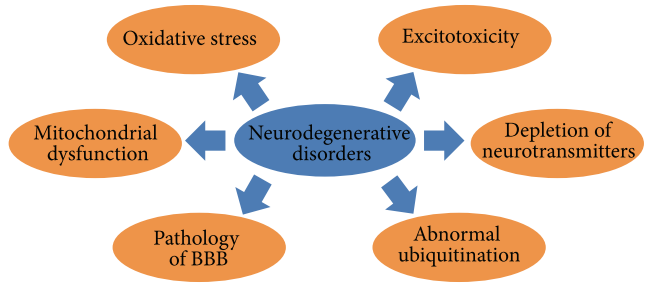

Figure 1: Various biological mechanisms contributing to neurodegenerative disorders.

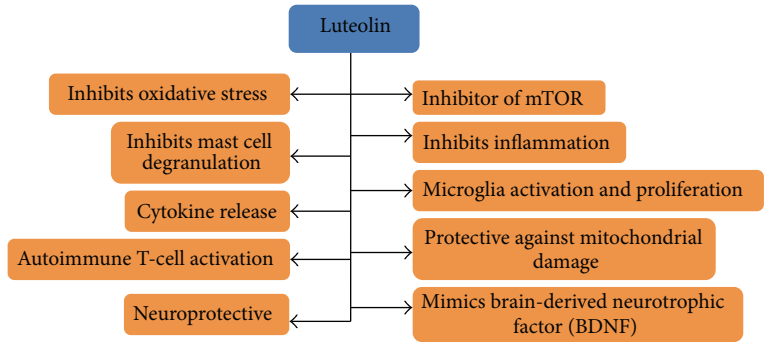

Figure 2: Luteolin (flavonoid) responsible for multiple biological functions.

are generally considered as strong inhibitors (Table 3) [59].

4. Anti-Inflammatory and Antioxidative Activites

Various medicinal plants have anti-inflammatory activities by inhibiting cyclooxygenase-1 (COX-1) that surrounds amyloids plaque in microglia. The accumulation of COX-1 enzyme in microglia in AD patients may be responsible for the local increase in oxidative stress and prostaglandin synthesis [10]. *Ferula assafoetida*, *Syzygium aromaticum*, and *Zingiber officinalis* have previously been reported to have activity against COX-1 enzyme [10]. *F. assafoetida* has previously been used as memory enhancer, antibacterial, antispasmodic, and antihelminthic in traditional medicines. *Z. officinalis* showed not only anti-COX-1 activity but also free radical scavenging activity that may be contributed to the presence of important phytochemicals such as gingerols and shogaols [10].

Sinapic acid (Brassicaceae) shows anti-inflammatory activity and can act as a neuroprotective agent by decreasing the levels of Aβ and by protecting neuronal cell death [15]. On the other hand, *Emblica officinalis* may be used in the treatment of mental disorders and as anti-inflammatory agent [60]. Several natural polyphenols such as vitamins, flavonoids, phenolic acids, and other polyphenols including thymol, ellagic acid, and eugenol have antioxidant properties and may be used for neurodegenerative diseases as promising therapeutic agents (Tables 1 and 3).

5. Computational Approaches towards Neurodegenerative Disorders

With the advancements in computational fields, particularly in the field of bioinformatics, the understanding of biological system at molecular level has improved drastically. The action of enzymes with their substrates, the synthesis of proteins, degradation of various biological macromolecules, ubiquitination, and many other processes could be observed with various computational programs including *in silico* molecular docking strategies. The normal homeostasis including metabolic equilibrium associated with many complex biological mechanisms under the supervision of autonomic nervous system as well as prediction for pathological state and possible therapeutic suggestions.

Jeyam et al. [7] used the *in silico* techniques for the understanding of molecular behavior of some traditional medicines for the management of PD. The loss of dopamine is considered as prominent feature of PD. Currently, levodopa (L-Dopa) is given in the form of supplementation for the management of PD. Catecholamine-O-methyltransferase (COMT), an enzyme, is responsible for the metabolism and conversion of L-Dopa into 3-O-methyl dopa. Hence, the inhibition of COMT may be one of the important

Table 1: Role of various plants and their active constituents in brain disorders.

Plant	Active Compounds	Disorder	References
Adhatoda vasica	Vasicine, vasicol, vasicinol, arachidic, cerotic, linoleic and oleic acids	AD, PD	[6]
Ginkgo biloba	Amentoflavone	PD	[7]
Mandukparni	Asiaticoside	Schizophrenia	[8]
Panax ginseng	Ginsenoside	PD	[7]
Rauvolfia serpentina	Reserpine	Schizophrenia	[8]
Withania somnifera	Withaferin A, sitoindoside IX, physagulin D, withanoside IV, viscosalactone B	Schizophrenia	[8]

Table 2: Plants with antioxidant properties which could be applied in the therapy used in neurodegenerative diseases.

Plants	Active Compounds	References
Abrus precatorius	Glycyrrhizin, precol, abrol, gallic acid, abrine	[9]
Acorus calamus	α-asarone, β-asrone, eugenol	[6]
Adhatoda vasica	Vasicine, vasicol, vasicinol, arachidic, cerotic, linoleic, oleic acids	[6]
Anogeissus leiocarpus	Castalagin, flavogallonic acid	[9]
Emblica officinalis	Emblicanins A, B, punigluconin, pedunculagin, punicafolin, ellagic acid, gallic acid	[10]
Entandrophragma angolense	7α-obacunyl acetate, cycloartane	[9]
Khaya senegalensis	Khayseneganin, luteolin, catechin	[9]
Medicago sativa	Soysaponin I, azukisaponin V	[6]
Mentha spicata	Spearmint oil, α, β-pinene, carvone, linalool, limonene	[6]
Myrtus communis	α-pinene, 1, 8-cineole, limonene	[6]
Pavetta crassipes	Quercetin	[9]
Piper nigrum	Piperine	[11]
Salvia triloba	Rosmarinic acid, ferulic acid, luteolin, quercetin	[11]
Sonchus eruca	Alkaloids, flavonoids, tannins, saponins	[12]
Terminalia arjuna	Arjunic acid, arjunolic acid, gallic acid, ellagic acid, proanthocyanidins	[10]
Terminalia chebula	Arjungenin, chebulosides, gallic acid, ellagic acid, luteolin, tannic acid, luteic acid, chebulic acid	[10]
Tribulus terrestris	Neohecogenin, β-D-galactopyranside	[6]
Withania coagulaus	Coagulin, withanolide, withaferin A	[6]
Withania somnifera	Withaferin A, sitoindoside IX, physagulin D, withanoside IV, viscosalactone B	[6]

ways to treat the disorder. Considering this way of treatment, the neuroprotective phytocompounds were evaluated using *in silico* studies [7]. Phytochemicals such as baicalin, stigmasterol, emodin, curcumin, wogonin, and eriodictyol were found to be having binding energies of approximately −7 kcal/mol which was similar to talcapone (synthetic drug to enhance the levodopa treatment) indicating that amentoflavone from *Ginkgo biloba* and ginsenoside from *Panax ginseng* are perceived as very good inhibitors for COMT as well as good adjuvants for L-dopa management. Kuhn and Kollman [61] studied and calculated the free energy activation of COMT considering the molecular dynamics of this enzyme. Moreover, Lee and Kim [62] investigated human COMT for designing anti-PD drug by using the ligand docking and comparative homology modeling.

Ayurveda medication has been evaluated for schizophrenia using *in silico* techniques [8]. Schizophrenia is associated with misbalancing of various chemicals of the brain involving the glutamate and dopamine. Studies on schizophrenia indicated that patients have abnormalities in brain structure such as decreased size of certain brain regions, enlargement of fluid-filled cavities, and less metabolic activities. Moreover, patients have delusions and hallucinations. From the Indian medication, three plants (*Rauvolfia serpentina*, *Withania somnifera,* and Mandukparni) were selected for the investigation of their role in the management of schizophrenia by using the tools of bioinformatics. The active molecules from these plants were docked with RGS-4 protein (regulator for G protein signaling-4) considered to be responsible for schizophrenia. The docking of RGS-4 protein with the combinations of reserpine, withanolide, and asiaticoside from *Rauvolfia serpentina*, *Withania somnifera*, and Mandukparni, respectively, showed that such combination therapy could be helpful in the management of schizophrenia [8].

6. Conclusion

In future, phytochemicals could be used as promising therapeutic agents for neurodegenerative disorders due to their anti-inflammatory and antioxidative as well as anticholinesterase activities. The neurodegenerative disorders

Table 3: List of plants having antioxidative and anticholinesterase activity.

Plant	Active compounds	Properties	References
Acorus calamus	α-asarone, β-asrone, eugenol	Antioxidative, anticholinesterase	[13]
Adhatoda vasica	Vasicine, vasicol, vasicinol, arachidic, cerotic, linoleic, oleic acids	Anticholinesterase	[10]
Bacopa monnieri	Bacoside, brahmin, herpestine, d-mannitol, luteolin, apigenin	Anticholinesterase	[14]
Brassica species	Brassicasterol, sinapic acid, sinapine	Anti-inflammatory, neuroprotective, anticholinesterase	[15–17]
Buddleja salviifolia	Phenols, flavonoids, proanthocyanidins	Antioxidative, anticholinesterase	[18]
Chamaecrista mimosoides	Phenols, flavonoids, proanthocyanidins	Antioxidative, anticholinesterase	[18]
Corydalis species		AChE inhibition	[19]
Corydalis ternate	Protopine	Anticholinesterase, antiamnesic	[20]
Cymbopogon schoenanthus	Piperitone, 2-carene	Antioxidative, anticholinesterase, antimicrobial	[21]
Ferula assafoetida	Cadinene, eremophilene	Anti-COX-1	[10]
Ginkgo biloba	Ginkgetin, ginkoglides-A, B	Anticholinesterase	[14]
Myricaria elegans	Crude extract	Anticholinesterase, antilipooxygenase	[22]
Nardostachys jatamansi	Angelicin, β-eudesmol, calarene, calarenol, elemol, nardol, oroselol	Antioxidative, anticholinesterase	[13]
Origanum ehrenbergii	Carvacrol, thymol	Antioxidative, anti-inflammatory	[23]
Origanum syriacum	Carvacrol, thymol	Antioxidative, anti-inflammatory, anticholinesterase	[23]
Peganum harmala	Norharmane, harmine, harmalol	Anticholinesterase	[10]
Piper nigrum	Piperine	Antioxidative, anticholinesterase	[11]
Ptychopetalum olacoides	Lupeol, α, β-pinene	Anticholinesterase	[24]
Salvia lavandulaefolia	Essential oil, terpenes	Anticholinesterase	[25]
Salvia miltiorrhiza	Diterpenoid	Anticholinesterase	[26]
Salvia miltiorrhiza	Terpenes, tanshinones	Anticholinesterase	[26, 27]
Salvia officinalis	Polyphenols	Antioxidative, anticholinesterase	[28, 29]
Salvia plebeian	Essential oil	Antioxidative	[30]
Salvia tiliifolia	Phenols, flavonoids, proanthocyanidins	Antioxidative, inhibition of cholinesterase	[18]
Salvia triloba	Rosmarinic acid, ferulic acid, luteolin, quercetin	Antioxidative, anticholinesterase	[11]
Schotia brachypetala (root)	Phenols, flavonoids, proanthocyanidins	Antioxidative, anticholinesterase	[18]
Schotia brachypetala (bark)	Phenols, flavonoids, proanthocyanidins	Antioxidative, anticholinesterase	[18]
Syzygium aromaticum	Eugenol, trans-β-caryophyllene, α-humulene	Anti-COX-1	[10]
Tabernaemontana divaricata	Voafinidine, lupeol, α-amyrin, β-sitosterol	Anticholinesterase	[31]
Terminalia chebula	Penta-O-galloyl-β-D-glucose	Anticholinesterase	[32]
Zingiber officinale	Gingerol, shogaol, zingerone	Anti-COX-1	[10]

such as AD, PD, Huntington's, and others share common features at cellular and subcellular levels as well as sharing mostly common molecular signaling pathways that may lead to apoptosis, necroptosis, and inflammation. Overall phytochemicals provide promising alternatives to current therapies for neurodegenerative disorders.

Conflict of Interests

The authors declare that they have no conflict of interests.

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