



## Menthol confers neuroprotection through Nrf2/ARE pathway in diabetic encephalopathy

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### ABSTRACT

**Background:** Chronic, long-standing hyperglycemia in diabetes results in diabetic encephalopathy (DE). It is hallmarked by cognitive dysfunction accelerated by hyperglycemia-induced oxidative stress.

**Objective:** This study explored the neuroprotective potency of menthol in experimental diabetes.

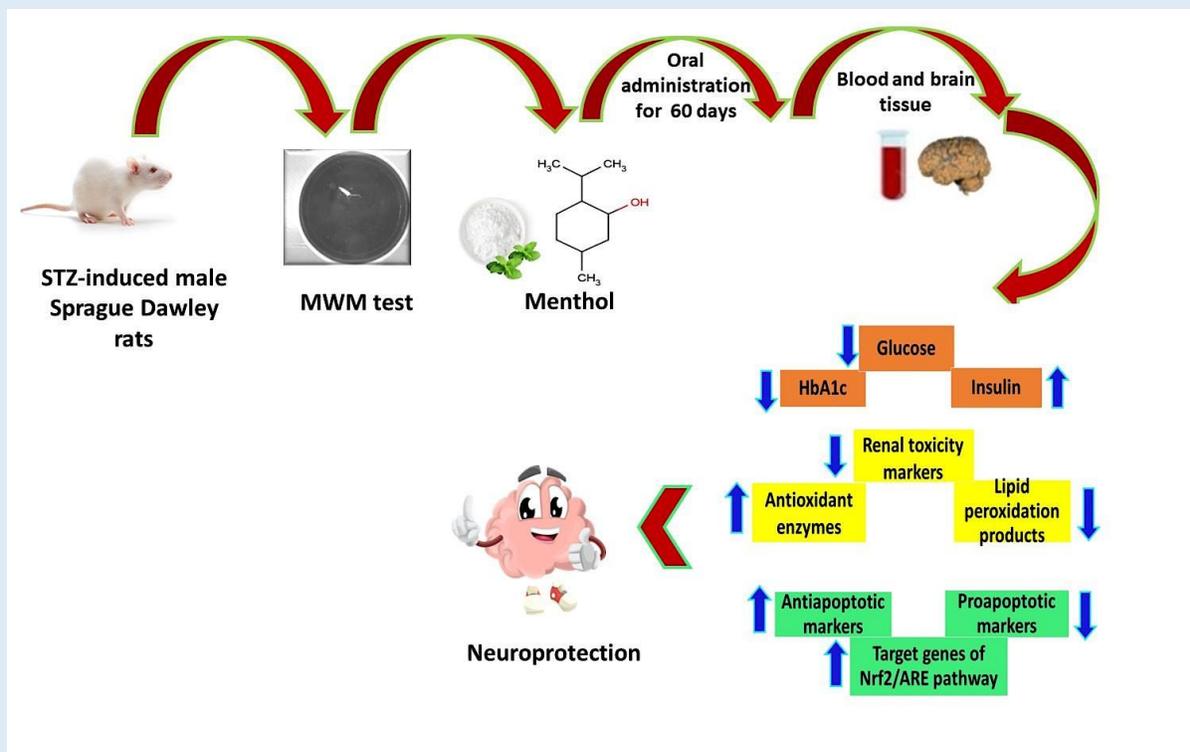
**Methodology:** Streptozotocin at a dose of 40 mg/kg body weight was injected into eighteen male Sprague- Dawley rats intraperitoneally to induce diabetes. The animals were kept without treatment for a period of 30 days for the development of DE. The cognitive deficit was confirmed by the Morris water maze test. Menthol (50 mg/kg body weight) was administered orally for 60 days. The behavioral test was conducted after 60 days of treatment. Results obtained were compared to diabetic rats fed with metformin (100 mg/kg body weight). Animals were then sacrificed to get blood and brain tissue for various biochemical examinations.

**Results:** Treatment with menthol improved cognitive performance in diabetic rats. In addition, menthol significantly decreased fasting blood glucose, HbA1c, renal toxicity markers, and lipid peroxidation products. Menthol enhances the

levels of plasma insulin and antioxidant enzymes. It also upregulated the mRNA expression of Bcl-2, Nrf2, Glo-1, and  $\gamma$ -GCS while diminishing the expression of Bax, cytochrome c, and caspase-3.

**Conclusion:** Menthol promotes neuroprotection by abating cognitive deficits, attenuating hyperglycemia, regulating oxidative stress, and curtailing apoptosis through Nrf2/ARE signaling.

**Keywords:** Diabetic encephalopathy, Menthol, Apoptosis, Nrf2/ARE pathway



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**INTRODUCTION**

Diabetes mellitus (DM) is a menacing health problem that affects around 415 million persons worldwide and is anticipated to rise to 642 million by 2040 [1]. It is caused by raised blood sugar levels brought on by flaws in insulin generation and/or action, giving rise to several macro and microvascular complications [2]. Diabetic encephalopathy (DE) is a dangerous microvascular consequence of diabetes that causes decreased cognitive

skills as well as electrophysiological, neurochemical, and structural abnormalities.

Hyperglycaemia raises the risk of DE, which can cause neuronal death and cognitive impairment. Free radicals produced by hyperglycemia cause impaired mitochondrial function, promoting the outflow of cytochrome c from the mitochondria and triggering death via a caspase-3-dependent mechanism[3].

Nrf2 (Nuclear factor erythroid 2–related factor 2) /

ARE (Antioxidant responsive element) pathway is a major antioxidant signaling pathway [4]. Under normal circumstances, Nrf2 is sequestered in the cytoplasm in association with Keap1 (kelch-like ECH-associated protein 1). During oxidative stress, the association between Nrf2 and Keap1 is broken. Therefore, Nrf2 is translocated, attaches to ARE (antioxidant response element) inside the nucleus, and activates endogenous antioxidant enzymes [5].

Methylglyoxal, a key intermediary in glucose metabolism, enhances the progression of diabetes-related cognitive deterioration [6]. It may lead to the formation of advanced glycation end products (AGEs) [7]. Glyoxylase 1 (Glo-1) is a vital enzyme that is involved in the elimination of alpha-carbonyl aldehyde, repressing methylglyoxal-induced glycosylation, and preventing AGE production by reduced glutathione (GSH) content [8]. Glo-1 activity is enhanced with GSH concentration, and a drop in GSH concentration reduces its cytoprotective potentiality in the face of oxidative stress. GSH is primarily generated with the aid of glutamylcysteine synthetase ( $\gamma$ -GCS), whereas  $\gamma$ -GCS is a renowned target gene under the control of the Nrf2/ARE signaling pathway [9]. It has been found that Glo-1

deficiency is associated with diabetes-related cognitive decline [6] as well as suppression of the Nrf2/ARE pathway [10].

There are currently no medications that are specifically made to prevent and reverse diabetes-related cognitive deterioration. Functional foods are vital for the management and prevention of diabetes mellitus, and complementary therapies can assist in managing some of the complications associated with diabetes[2]. The therapeutic efficacy of functional foods is due to the presence of bioactive compounds in them [11]. Menthol is a natural dietary monoterpene with the molecular formula  $C_{10}H_{20}O$ . It is a main component of peppermint oil and is found in mint plants. Figure 1 shows the chemical structure of menthol. Several studies revealed the antioxidant [12] antimicrobial[13], anticancer[14], and anti-inflammatory activities[15] of menthol. However, little information is available regarding the neuroprotective effect of menthol under diabetic conditions. Therefore, in the current work, we examined the neuroprotective role of menthol against hyperglycemia-mediated oxidative stress and apoptosis, unraveling the possible mechanisms associated with the activation of the Nrf2/ARE pathway.

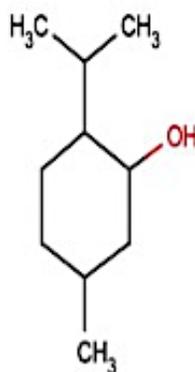


Figure 1. Chemical structure of menthol

## MATERIALS AND METHODS

**Chemicals:** The present investigation utilized analytical grade reagents from Sigma–Aldrich (St. Louis, MO) and Sisco Research Laboratories (Mumbai, India).

**Experimental design:** For the study, 30 male Sprague Dawley rats (body weight 150-250g) were procured from the animal house of the Department of Biochemistry, University of Kerala. Institutional Animal Ethics Committee of the Department of Biochemistry, University of Kerala approved the experimental procedure [IAEC 3-KU-04/2018-19-BCH-SM (44)]. Rats were categorized into 5 groups, each with 6 rats. Group I: normal control (N); Group II: normal rats administered with menthol, 50 mg/kg body weight (N+ M); Group III: Diabetic encephalopathic control (DE); Group IV: Diabetic encephalopathic rats administered with menthol, 50 mg/kg body weight (DE+M); Group V: Diabetic encephalopathic rats administered with metformin, 100 mg/kg body weight (DE+Met). 40 mg/kg of streptozotocin, delivered intraperitoneally in citrate buffer (0.1 M; pH 4.5), was employed to generate diabetes in groups III, IV, and V. The animals were provided with glucose solution (5%) overnight to counteract the hypoglycemia produced by STZ. 1 or 2 drops of blood were collected by tail nicking on the 3<sup>rd</sup> day following streptozotocin injection for blood glucose estimation using One touch horizon glucose strip. When the blood glucose levels exceeded 250 mg/dl, the animals were considered to have diabetes. Both group II and IV animals were supplemented daily with menthol (50 mg/kg body weight) in the vehicle corn oil intragastrically through oral gavage for 60 days. Based on the previous study [16], the dose and route of menthol supplementation were chosen. Group V was treated with metformin (100 mg/kg body weight) through oral gavage.

Following treatment for 60 days, the rats were fasted overnight, sacrificed, and blood and brain tissue were collected for various estimations.

**Morris water maze test (MWM test):** MWM test was performed according to the method of Morris, (1984) to determine the learning and memory of diabetic rats [17]. It was conducted in a water-filled, circular tank. The water was at a temperature of  $26 \pm 2^{\circ}\text{C}$ . In the tank, there were four equal quadrants, I, II, III, and IV. At a fixed point in one of the quadrants, 1 cm beneath the water surface, an escape platform was placed. Rats were then placed in the circular tank of water to localize the hidden escape platform. The rats underwent three trials for each stage over the course of four days. The time it takes to get to the platform (escape latency) was determined. Learning was deemed successful when the latency time from the first session was significantly reduced.

**Biochemical analysis:** Plasma insulin was measured using an ELISA kit from DRG Diagnostics, Marburg, Germany, and glycosylated hemoglobin was assessed using an HbA1c kit from Beacon Diagnostics Pvt Ltd. Commercially obtainable assay kits were employed to determine (Agappe Diagnostics Pvt. Ltd, India), the blood glucose, urea, uric acid and creatinine.

**Analysis of tissue antioxidant enzymes:** The antioxidant status of the brain tissue was assessed by analyzing the activities of superoxide dismutase (SOD)[18], catalase [19], glutathione peroxidase (GPx) [20], and glutathione reductase (GR) activity [21].

**Analysis of lipid peroxidation products in the brain:** Peroxidation of lipids was analyzed by determining

thiobarbituric acid reactive substances (TBARS) [22], conjugated dienes (CD), and hydroperoxides (HP) [23].

#### Isolation of RNA and reverse transcriptase polymerase chain reaction:

TRIzol, purchased from Sigma-Aldrich, St. Louis, MO, USA, was utilized to isolate total genomic RNA from the brain [24]. cDNA synthesis and PCR reactions were performed in a thermocycler (Eppendorf 5332),

using a kit purchased from Fermentas, Vilnius, Lithuania. Table 1 shows the primer sequences that were employed. The PCR products were electrophoresed on agarose gel (1.5%), accompanied by the densitometric scanning of the gels (Bio-Rad Gel Doc). The quantity one imaging software (Bio-Rad) quantified the mRNA level. The expression of  $\beta$  actin in the same sample was used to compare the relative expression.

**Table 1:** PCR primer sequences

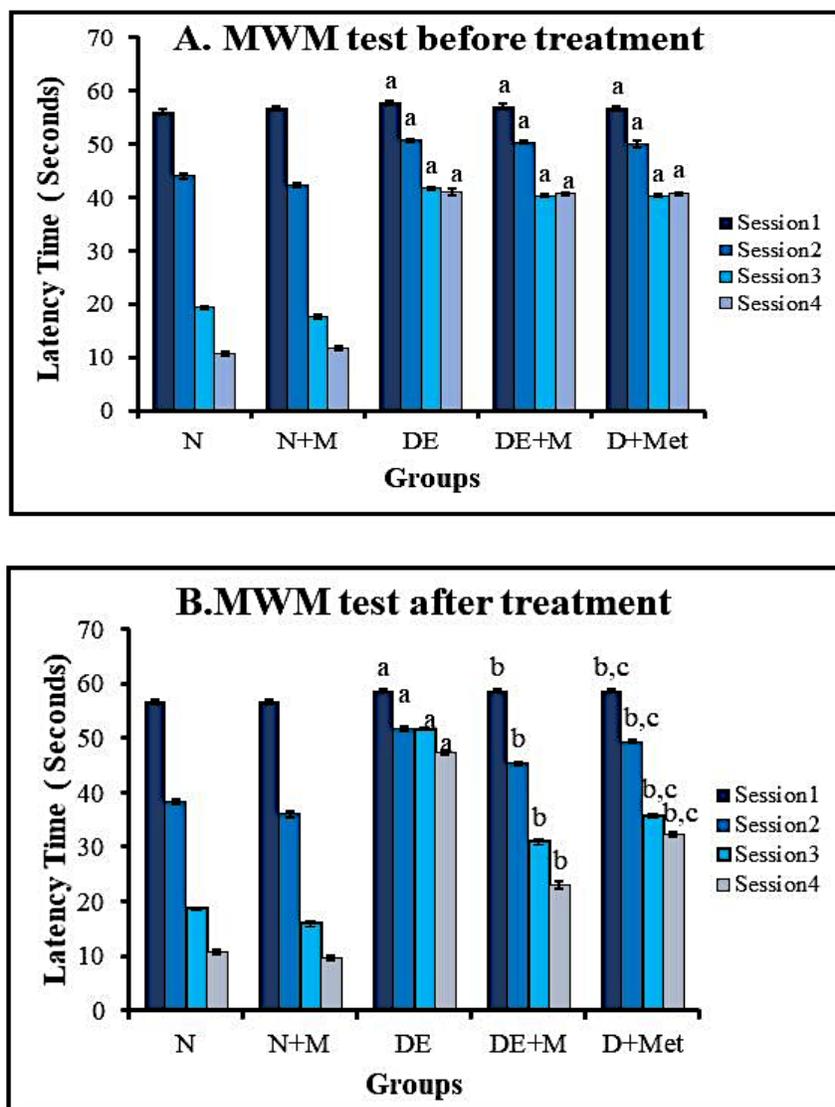
Gene	Forward primer (5'-3')	Reverse primer (3'-5')
$\beta$ actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTTC
Bax	CGGCGAATTGGAGATGAACTGG	CTAGCAAAGTAGAAGAGAGGGCA ACC
Bcl-2	TGTGGATGACTGACTACCTGAACC	CAGCCAGGAGAAATCAAACAGAG G
Caspase 3	GTGGAAGTACGATGATGATATG GC	CGCAAAGTACTGGATGAACC
Cytochrome c	CTTGGGCTAGAGAGCGGGACGTC	GTTATCAACAGTCAGAAGATAA
Nrf2	GAAATGATGTCCAAGGAGCAA	AAGACTTCAAGATACAAGGTGCTG
Glo1	GCCTCTAAGCCAGACCACAT	GCAGCACTCAAAGCCATAAC
$\gamma$ -GCS	GAAGATGACGAGACGCAGAGTTAC	CAGGATCTTGAACGAACGCCAGAC

**Statistical analysis:** The statistical software SPSS/PC+, Version 17 (SPSS Inc. Chicago, IL, USA), was utilized for the statistical analysis. One-way analysis of variance was used to analyze the data for the single group, and two-way analysis of variance (ANOVA) was employed to analyze the data for the groups. All the results were represented as mean value  $\pm$  standard error of the mean (SEM) (n = 6). p value less than 0.05 were regarded as significant.

## RESULTS

**In Learning and Memory:** The MWM test evaluated the role of menthol on learning and memory. Thirty days after diabetes induction, the untreated rats showed extended escape latencies during the training sessions

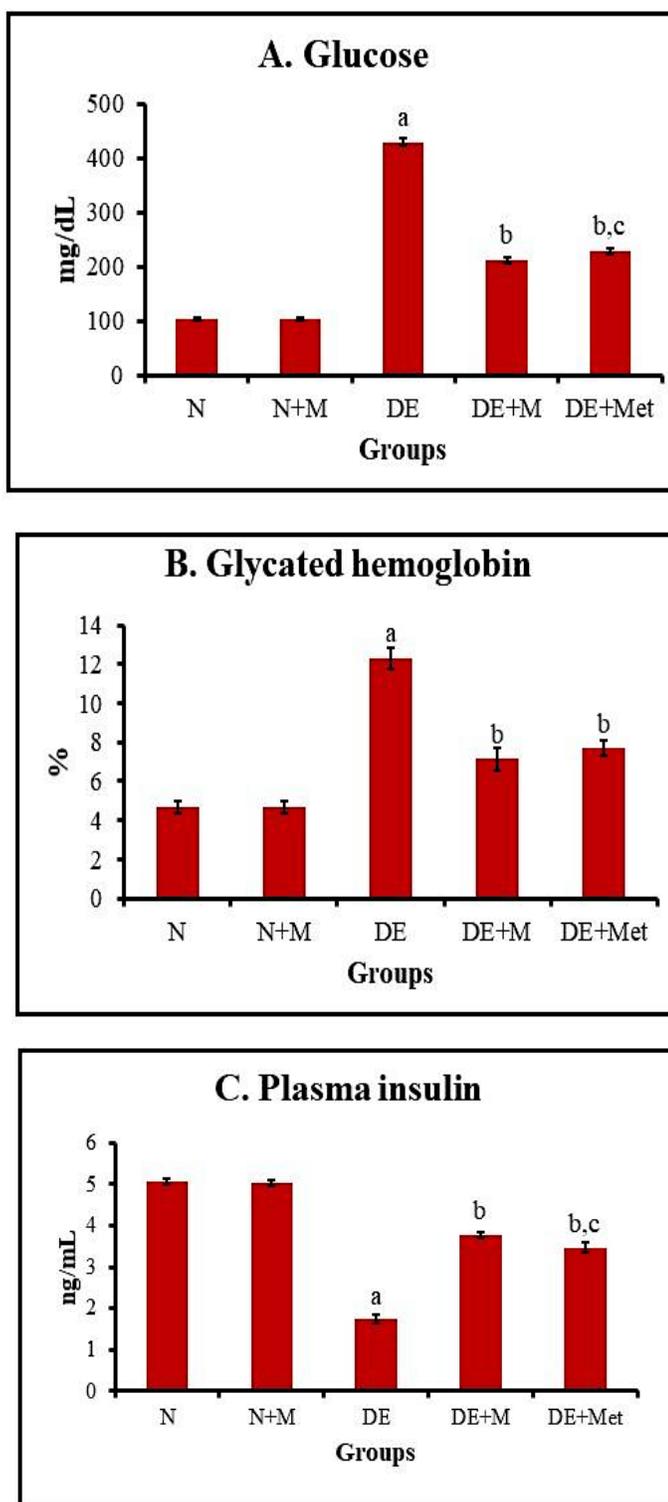
revealing cognitive impairment compared to normal rats (Figure 2A). For 60 days, diabetic rats were treated with menthol and metformin. Four consecutive trials of the MWM test displayed improved performance in normal control groups. Throughout the training sessions, STZ-injected untreated diabetic rats displayed extended escape latencies compared to normal control rats. Compared to the first session, menthol and metformin significantly ( $p < 0.05$ ) reduced the mean latency time in the third and fourth sessions. Learning and memory were improved in menthol treated diabetic group compared to the metformin-treated diabetic group (Figure 2B). The results indicated that menthol treatment improved cognitive impairment by reducing hyperglycemia-mediated oxidative stress.



**Figure 2.** Learning and memory. **2A.** MWM test before treatment, **2B.** MWM test after treatment. Mean ± SEM (n=6) is used to represent values. DE is compared with DE+M and DE+ Met. 'a' designates values that differed significantly from N, while 'b' designates values that differed significantly from DE. 'c' designates values that differed noticeably from DE+M. p < 0.05 is considered significant. N- Normal rats, N+M- Normal rats treated with Menthol, DE-Diabetic encephalopathic rats, DE+M-Diabetic encephalopathic rats treated with Menthol, DE+Met- Diabetic encephalopathic rats treated with Metformin.

**Blood glucose, Glycosylated hemoglobin, and plasma insulin:** In comparison to normal and normal control rats treated with menthol, blood glucose, and HbA1c were significantly higher in the DE control groups, and plasma insulin was lower. Supplementation of menthol and metformin diminished blood glucose and glycated hemoglobin in DE rats. Plasma insulin levels were higher

in DE+M and DE+Met groups than in the DE group (Figures 3A, 3B, and 3C). A significant positive effect was noticed in menthol-fed groups than metformin (p<0.05). In normal and normal rats fed with menthol, there was no significant alteration in blood glucose, HbA1c, or plasma insulin.



**Figure. 3 (A, B, C).** Serum glucose, HbA1c, and plasma insulin. Mean  $\pm$  SEM (n=6) is used to represent values. DE is compared with DE+M and DE+ Met. 'a' designates values that differed significantly from N, while 'b' designates values that differed significantly from DE. 'c' designates values that differed noticeably from DE+M.  $p < 0.05$  is considered significant. N-Normal rats, N+M- Normal rats treated with Menthol, DE-Diabetic encephalopathic rats, DE+M-Diabetic encephalopathic rats treated with Menthol. DE+Met- Diabetic encephalopathic rats treated with Metformin.

**Renal toxicity markers:** As shown in Table 2, the concentration of renal toxicity indicators viz, urea, uric acid, and creatinine were augmented in the DE rats compared to the control rats. With menthol supplementation, the levels of renal toxicity markers were significantly ( $p < 0.05$ ) declined compared to the DE

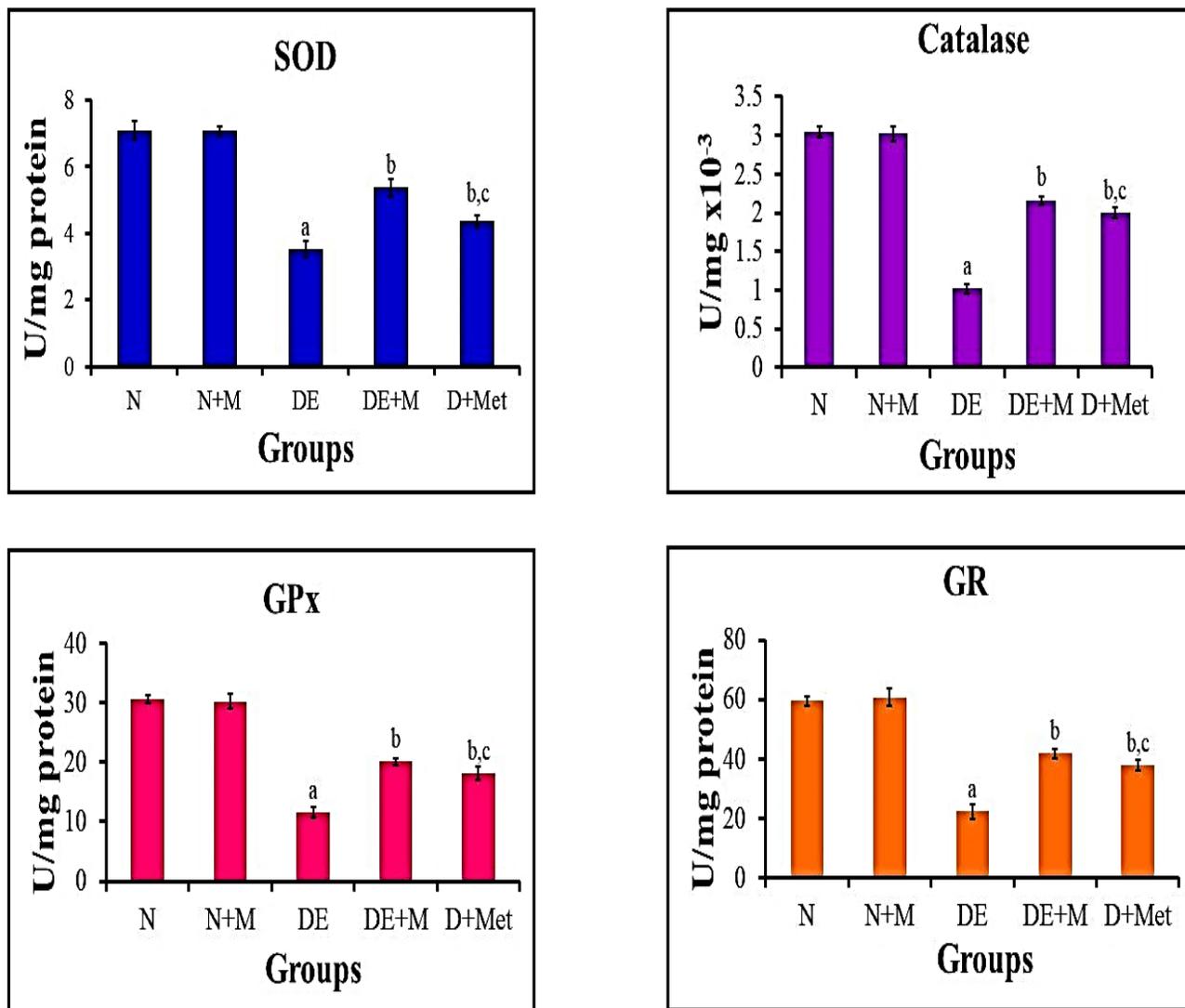
rats. Comparable results were obtained in rats treated with menthol and metformin. Renal toxicity markers in normal control rats and normal control rats fed with menthol were the same. These results suggest that menthol administration in DE rats could modulate the renal toxicity markers more than in the DE+Met group.

**Table 2:** Renal toxicity markers

Groups	Urea (mg/dL)	Uric acid (mg/dL)	Creatinine(mg/dL)
N	28.46 ± 0.60	4.53 ± 0.16	1.12 ± 0.02
N+M	28.80 ± 0.93	4.37 ± 0.14	1.12 ± 0.04
DE	57.20 ± 0.90 <sup>a</sup>	10.38 ± 0.23 <sup>a</sup>	2.90 ± 0.22 <sup>a</sup>
DE+M	42.45 ± 1.13 <sup>b</sup>	8.32 ± 0.20 <sup>b</sup>	2.00 ± 0.06 <sup>b</sup>
DE+Met	50.37 ± 1.36 <sup>b, c</sup>	8.91 ± 0.22 <sup>b, c</sup>	2.29 ± 0.01 <sup>b, c</sup>

Mean ± SEM (n=6) is used to represent values. DE is compared with DE+M and DE+ Met. 'a' designates values that differed significantly from N, while 'b' designates values that differed significantly from DE. 'c' designates values that differed noticeably from DE+M.  $p < 0.05$  is considered significant. N-Normal rats, N+M- Normal rats treated with Menthol, DE-Diabetic encephalopathic rats, DE+M-Diabetic encephalopathic rats treated with Menthol, DE+Met- Diabetic encephalopathic rats treated with Metformin.

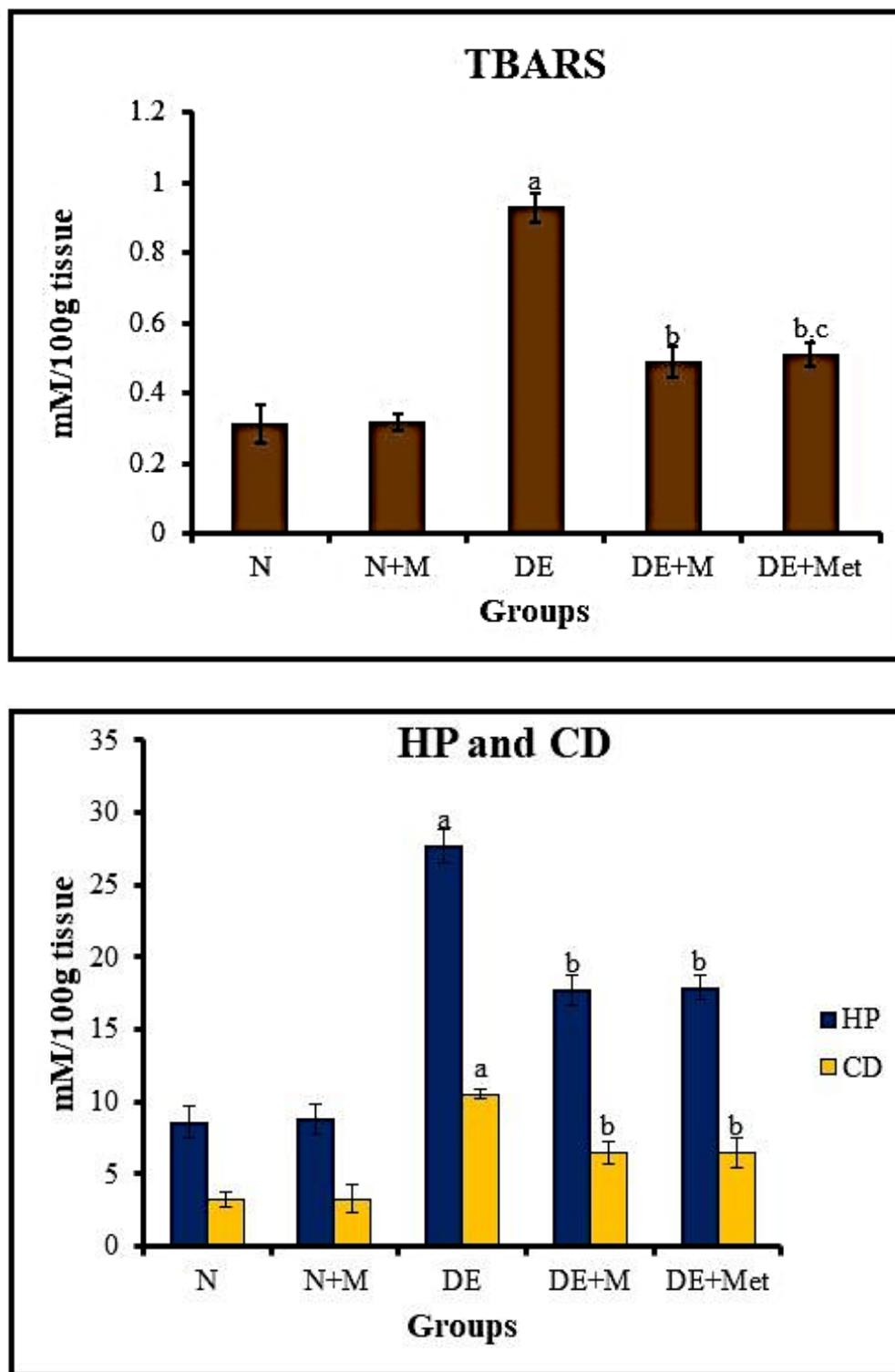
**Brain antioxidant enzymes:** When compared to the normal rats, the antioxidant enzymes SOD, GPx, GR, and catalase, were significantly ( $p < 0.05$ ) diminished in the brain of streptozotocin-induced DE rats. However, the antioxidant enzyme activities were significantly ( $p < 0.05$ ) higher in the diabetic groups with menthol and metformin treatment (Figure 4). These results suggest that menthol treatment affords protection and improves the antioxidant status in the brain under hyperglycemia-induced oxidative stress.



**Figure.4.** Antioxidant enzymes in the brain. Mean ± SEM (n=6) is used to represent values. DE is compared with DE+M and DE+ Met. 'a' designates values that differed significantly from N, while 'b' designates values that differed significantly from DE. 'c' designates values that differed noticeably from DE+M. p < 0.05 is considered significant. N-Normal rats, N+M- Normal rats treated with Menthol, DE-Diabetic encephalopathic rats, DE+M-Diabetic encephalopathic rats treated with Menthol, DE+Met- Diabetic encephalopathic rats treated with Metformin.

**Lipid peroxidation products:** In contrast to normal control, DE rats had significantly higher levels of a thiobarbituric acid reactive substance (TBARS), conjugated dienes (CD), and hydroperoxides (HP) (Figure 5). Nevertheless, supplementation with menthol and

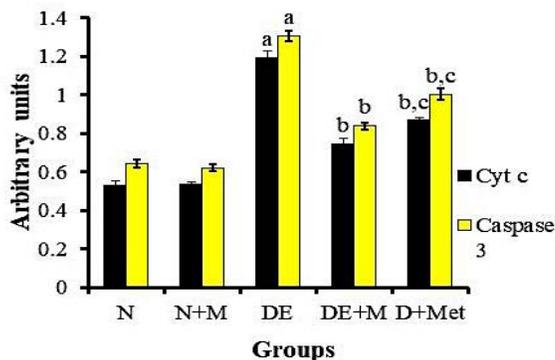
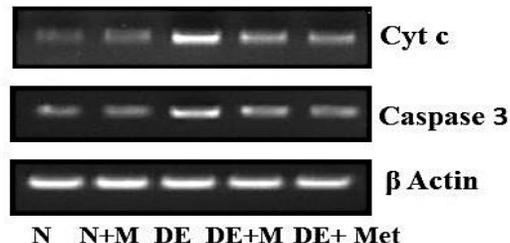
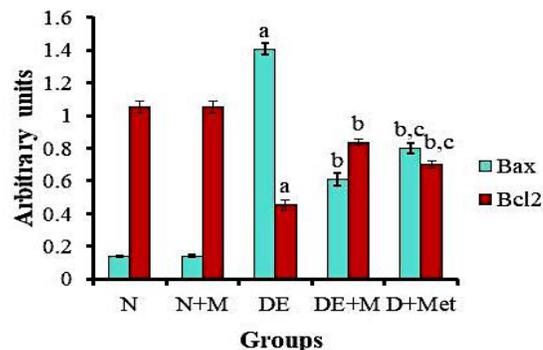
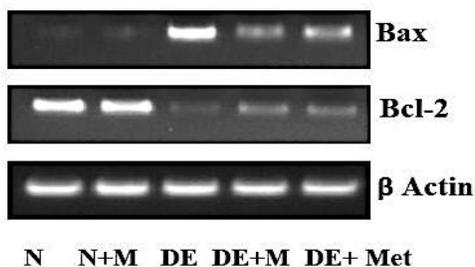
metformin led to a significantly (p < 0.05) reduced level of brain lipid peroxidation products. These findings suggested that treatment with menthol reduces lipid peroxidation products in the brain.



**Figure.5.** Lipid peroxidation products in the brain. Mean  $\pm$  SEM (n=6) is used to represent values. DE is compared with DE+M and DE+ Met. 'a' designates values that differed significantly from N, while 'b' designates values that differed significantly from DE.  $p < 0.05$  is considered significant. N-Normal rats, N+M- Normal rats treated with Menthol, DE-Diabetic encephalopathic rats, DE+M-Diabetic encephalopathic rats treated with Menthol, DE+Met- Diabetic encephalopathic rats treated with metformin.

**Apoptotic markers in the brain:** In diabetic rats, mRNA expression of the primary pro-apoptotic marker gene, Bax, was upregulated, and the antiapoptotic gene Bcl2 was significantly downregulated compared to normal control. However, the administration of menthol significantly ( $p < 0.05$ ) downregulated Bax expression and upregulated Bcl2 expression in DE rats. A superior effect was noticed in menthol treated group than in the

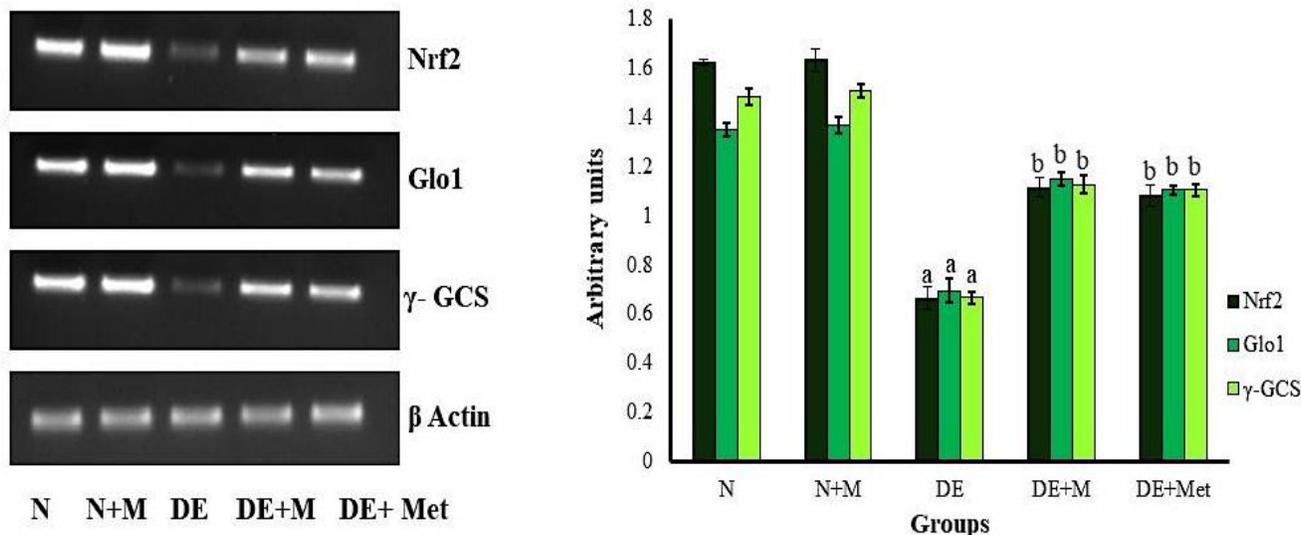
metformin-treated group (Figure 6A). Major apoptogenic factors, cytochrome c (Cyt c) and executioner caspase, caspase 3, were upregulated in DE rats in comparison with normal rats. However, treatment of menthol and metformin significantly ( $p < 0.05$ ) downregulated the mRNA expression of cytochrome c and caspase3 (Figure 6B). These results advocated that menthol might protect brain cells from apoptosis.



**Figure.6.** mRNA expression of apoptotic genes.6A. mRNA expression of Bax and Bcl2, 6B. mRNA expression of cytochrome c and caspase 3. Mean  $\pm$  SEM (n=6) is used to represent values. DE is compared with DE+M and DE+ Met. 'a' designates values that differed significantly from N, while 'b' designates values that differed significantly from DE. 'c' designates values that differed noticeably from DE+M.  $p < 0.05$  is considered significant. N- Normal rats, N+M- Normal rats treated with Menthol, DE-Diabetic encephalopathic rats, DE+M- Diabetic encephalopathic rats treated with Menthol, DE+Met- Diabetic encephalopathic rats treated with Metformin.

**Markers of Nrf2/ARE pathway:** To investigate whether menthol mediates neuroprotection via the Nrf2/ARE pathway, mRNA expression of Nrf2 and target genes Glo1 and  $\gamma$ -GCS were analyzed. Nrf2, Glo1, and  $\gamma$ -GCS mRNA expressions were downregulated in STZ-induced diabetic rats. Meanwhile, menthol markedly elevated the

expressions of Nrf2, Glo 1, and  $\gamma$ -GCS (Figure 7). These findings imply that menthol increased Glo-1 and  $\gamma$  GCS expression as with metformin treatment and this might be due to the triggering of Nrf2/ARE signaling pathway in STZ-induced DE rats.



**Figure 7.** mRNA expression of target genes of Nrf2/ARE pathway. Mean  $\pm$  SEM (n=6) is used to represent values. DE is compared with DE+M and DE+ Met. 'a' designates values that differed significantly from N, while 'b' designates values that differed significantly from DE. 'c' designates values that differed noticeably from DE+M.  $p < 0.05$  is considered significant. N- Normal rats, N+M- Normal rats treated with Menthol, DE-Diabetic encephalopathic rats, DE+M- Diabetic encephalopathic rats treated with Menthol, DE+Met- Diabetic encephalopathic rats treated with Metformin.

**DISCUSSION**

Diabetes is allied with progressive deficits in the central nervous system (CNS), termed diabetic encephalopathy. A major public health burden associated with diabetes mellitus is cognitive impairment[25]. Currently, there is no effective drug that can prevent diabetes-associated cognitive dysfunction [26]. At present, researchers are focusing on functional foods for managing diabetic complications effectively. Functional foods are natural or processed foods enriched with bioactive components to combat chronic diseases [27].

Bioactive compounds come under the category of secondary metabolites [28]. Menthol is one such secondary metabolite included under the category of terpenoids. A major clinical barrier to CNS disease treatment is the blood-brain barrier (BBB). Menthol is a robust candidate because of its capacity to cross the blood-brain barrier [29] and its hypoglycemic effects. The current investigation tested menthol for its potential

ameliorative effects against diabetes-mediated cognitive impairment and apoptosis.

The MWM test is an extensively employed behavioral test for assessing cognitive deficits in rodents. In the current study, DE, when compared to normal control, exhibited debilitated spatial learning and memory, as evidenced by the MWM test. It may be due to elevated oxidative stress and a decline in the number of viable neurons in the hippocampus. These findings are consistent with a prior study by Shyma and Mini, 2022 [30], who found that diabetic rats experienced cognitive impairment. Menthol supplementation significantly alleviated the impairment by abating escape latency in diabetic rats; similar results were also observed in the metformin treatment. The increased number of viable neurons in the hippocampus of diabetic rats may be responsible for the amelioration of cognitive impairment. According to reports, persistent hyperglycemia is the primary cause of the majority of diabetic problems,

including cognitive impairment [31]. In the present research, diabetic rats displayed augmented blood glucose levels compared to normal control, which is in accordance with the outcomes of Anjali et.al., 2022[32]. Blood glucose levels were substantially lowered in diabetic rats treated with menthol and metformin. An elevated glucose level is well identified by glycated hemoglobin (HbA1c). Observed increases in HbA1c in DE rats were caused by high blood glucose. In the menthol-treated DE group, HbA1c levels were markedly reduced; this could be due to improved insulin release from the prevailing or rejuvenated pancreatic  $\beta$ -cells[16]. Glucose and HbA1c levels in the normal and normal+ Menthol-supplemented groups did not differ considerably. According to a previous study, streptozotocin-induced diabetic rats displayed reduced insulin production and secretion [33]. The outcomes of our study corroborate this since plasma insulin levels were lower in DE groups when compared to normal control. Plasma insulin levels were markedly raised by menthol and metformin supplementation.

To evaluate the ability of menthol to prevent renal toxicity in STZ-induced diabetic conditions, renal toxicity markers were determined. The present study revealed elevated levels of renal toxicity markers viz, urea, uric acid, and creatinine in diabetic rats. Renal toxicity markers were reduced by menthol or metformin treatment. Menthol treatment showed better results, suggesting that it can regulate streptozotocin-induced renal damage. Additionally, menthol supplementation did not alter renal toxicity markers in normal control rats, indicating the nontoxic nature of menthol. Similar outcomes were observed in an earlier study where supplementation of syringic acid to diabetic rats decreased the activities of these toxicity markers [34].

Augmented generation of ROS resulting from oxidative stress performs a prominent role in neuronal

injury[35]. SOD, CAT, and GPx are the key enzymes crucial for the maintenance of antioxidant defense in living systems[36]. As oxidative stress rises the antioxidant defense activities were decreased. This is consistent with earlier research [37]. Treatment with menthol or metformin improved the level of antioxidant enzymes aiding neuroprotection. CNS is particularly susceptible to lipid peroxidation due to its rapid oxygen utilization, high concentration of polyunsaturated fatty acids, and relative deficiency of antioxidant enzymes[38]. Interaction of lipids with ROS results in the formation of lipid peroxidation products and advanced lipoxidation end products (ALEs), which have been associated with the pathogenesis of diabetic complications [39]. Malondialdehyde (MDA) has been acknowledged as a significant lipid peroxidation indicator. Lipid peroxidation products were remarkably increased in the DE group, whereas supplementation of menthol or metformin abated lipid peroxidation and thus reduced the underlying complications. Comparable outcomes were noticed in the previous study[40], where hibiscus anthocyanin extract exerts hepatoprotective potential by abating lipid peroxidation and regulating antioxidant status.

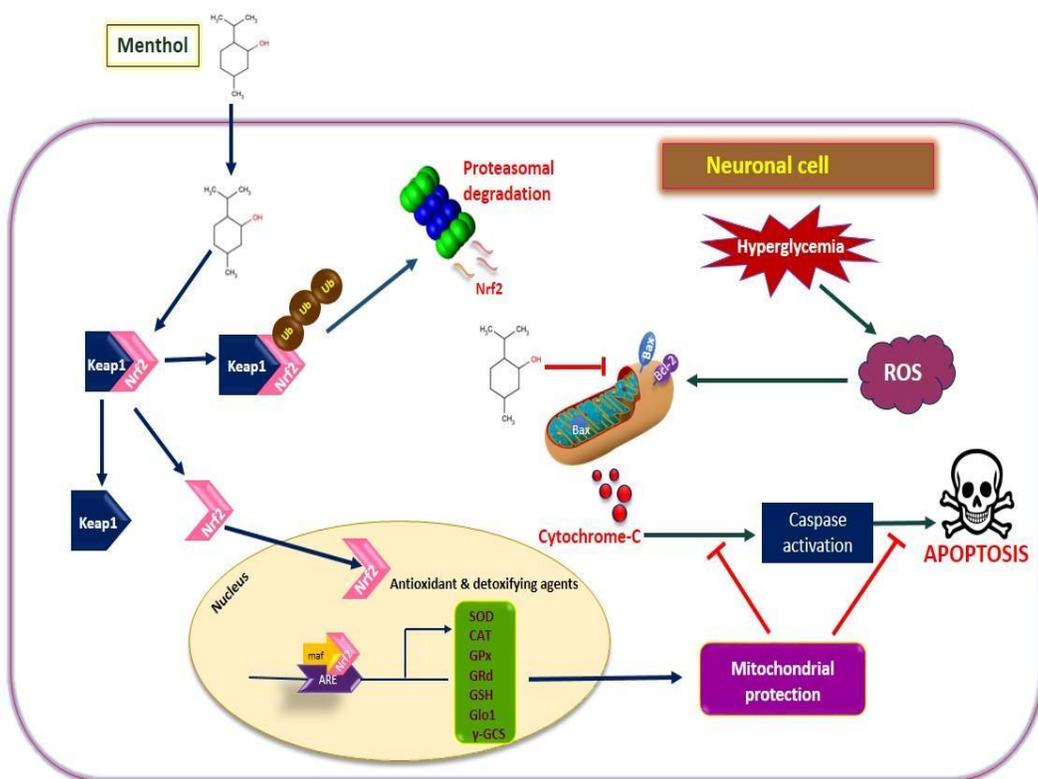
Diabetic encephalopathy is caused by hyperglycemia, which can cause neuronal death and cognitive impairments. Members of the Bcl-2 family are responsible for regulating the mitochondrial apoptotic pathway. Activation of the mitochondrial pathway is allied with the alteration of mitochondrial permeability, liberation of cytochrome c, and stimulation of caspase-9 [41],[42]. Bcl-2 is an anti-apoptotic factor, while Bax is pro-apoptotic. Under normal conditions, Bax and Bcl-2 are in equilibrium. Cell apoptosis is induced when the equilibrium is disturbed. One of the distinguishing characteristics of apoptosis is the release of cytochrome c from mitochondria. Typical morphological changes

connected with apoptosis are caused by the cleavage of cytoskeletal proteins by caspase-3, a central executioner molecule of apoptosis [43]. In DE rats, mRNA expression of Bcl-2 was down-regulated, whereas the expression of Bax, Cyt c, and caspase 3 were upregulated and contradicted the genes expressed in normal control rats. Bcl-2 expression was enhanced after menthol treatment. Nevertheless, Bax, Cytc, and caspase 3 expressions were decreased. Our findings are consistent with earlier research by Kamboj S.S. and Sandhir R., 2011 [44] and Chandirasegaran et al., 2018 [45].

The binding of Nrf2 to ARE is responsible for regulating phase II detoxification enzymes and several antioxidant enzymes [46]. Cells are shielded from oxidative stress by Nrf2 activation in response to oxidative damage. In the current investigation, the Nrf2/ARE pathway was upregulated by menthol in DE rats, as demonstrated by the raised mRNA expression of Glo1 and  $\gamma$ -GCS, the main targets of Nrf2 /ARE signaling. Previous research validated that the decline in the

expression of Glo-1 in rat primary cerebrocortical and hippocampal neurons grown in high glucose conditions was found to be associated with defective Nrf2/ARE signaling [10].

In addition to enhancing the expression of Bcl-2, Nrf2 also improves the expression of an array of additional cytoprotective genes elaborated in detoxification, antioxidant defense, and drug transport [47]. In light of this, it is rational to consider that the concerted stimulation of cytoprotective genes and anti-apoptotic Bcl-2 mediated by the menthol-stimulated Nrf2 activation played a central role in the decreased apoptosis and increased cell survival. Moreover, menthol repressed caspase-dependent apoptosis by reducing the expression of the Bax gene. Together, activation of Nrf2/ARE pathways accompanied by the production of antioxidant and detoxifying enzymes are essential for preventing and treating diabetic complications in the CNS (Figure 8).



**Figure 8.** The postulated molecular pathway for the neuroprotective role of menthol in experimental diabetes

## CONCLUSION

The current investigation demonstrates that menthol ameliorates hyperglycemia-induced cognitive deficit, reduces oxidative stress, and protects neuronal cells from apoptosis in experimental diabetes through upregulation of the Nrf2/ARE pathway. Thus, menthol may be a promising nutraceutical for relieving the neurological problems associated with diabetes mellitus.

**Abbreviations:** DM: Diabetes mellitus, DE: Diabetic encephalopathy, STZ: Streptozotocin, CNS: Central nervous system, ROS: Reactive oxygen species, HbA1c: Glycosylated hemoglobin, MWM: Morris Water Maze, SOD: Superoxide dismutase, GRd: Glutathione reductase, GPx: Glutathione peroxidase, TBARS: Thiobarbituric acid reactive substances, HP: Hydroperoxides, CD: Conjugated dienes, Nrf2: Nuclear factor erythroid 2-related factor 2, ARE: Antioxidant responsive element, Glo-1: Glyoxylase 1,  $\gamma$ -GCS:  $\gamma$  glutamylcysteine synthetase

**Authors' contribution:** The original concept was formulated by S. Soumya and S Mini and was discussed with N. P. Soumya and Sukanta Mondal. All authors ultimately concurred on the paper's primary theme and key concepts. S. Soumya carried out and analyzed the experiments, and S. Mini conceptualized the main ideas. The main text of the paper was written by S. Soumya and S. Mini. S Mini and N P Soumya revised and edited the manuscript, with Sukanta Mondal contributing to the editing and writing parts. All authors contributed to the writing and editing of the final draft.

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