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Menthol Attenuates Cholinergic Dysfunction and Neurotransmitter Imbalance in Experimental Diabetes

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Abstract

One of the most predominant enduring consequences of Diabetes Mellitus (DM) is Diabetic Encephalopathy (DE), which has neither a reliable treatment nor an effective preventive strategy. Cognitive dysfunction is the primary problem allied with DE. The current inquiry aims to determine the potency of menthol in reducing the risk of brain complications induced by Streptozotocin (STZ) in diabetic rats. A single STZ intraperitoneal injection (40 mg/kg body weight) was employed to induce DM in Sprague-Dawley male rats and animals were held without treatment for 30 days to develop DE. The Morris water maze test, followed by the supplementation of menthol and metformin for 60 days at 50 and 100 mg/kg body weight dosages, verified the cognitive deficit in diabetic rats. After 60 days of therapy, rats were sacrificed to obtain blood and brain tissues for biochemical investigation. Oral delivery of menthol enhanced cognitive function in DE rats. Furthermore, menthol markedly reduced fasting blood sugar, glycosylated Hemoglobin (HbA1c), and elevated plasma insulin levels. In the brain, menthol increases neurotransmitter levels and choline acetyltransferase activity while decreasing AChE activity. Menthol also downregulated the expressions of monoamine oxidase A and B. Thus, the study indicates that menthol was effective in attenuating the neurodegenerative alterations in DE rats. It had a therapeutic potential and could be effectively utilized as a dietary supplement for regulating complications associated with encephalopathy.

Keywords: Cholinergic Dysfunction, Diabetic Encephalopathy (DE), Menthol, Neurotransmitters, Streptozotocin

1. Introduction

DM is characterized by hyperglycemia caused by defects in insulin secretion, insulin action, or both. A wide range of long-term problems are connected to chronic hyperglycemia. One such long-term complication of DM is DE, hallmarked by alterations in nervous system anatomy and cognitive function[1]. The pathophysiology of DE has been linked to several factors, including diminished secretion or action of insulin, imbalance in glucose homeostasis, raised plasma leptin levels, elevated glucocorticoid levels, neuronal inflammation, impairment in nerve impulse transmission, oxidative stress, apoptosis, and mitochondrial malfunction as well as variations in the morphology and functions of the hippocampus[2].

The cerebral cortex and hippocampus, specialized brain areas that control learning and memory, exhibit highly pronounced cholinergic neuroactivities[3]. Acetylcholine (ACh), the crucial neurotransmitter in the cholinergic system, is produced by choline acetyltransferase (ChAT) and cleaved by acetylcholinesterase (AChE) and mediates a significant function in learning and memory[4]. High levels of AChE are present in the brain, nerves, and RBCs. The cholinergic function is significantly influenced by the constant presence of acetylcholine, either through prolonged synthesis or decreased acetylcholinesterase activity; nevertheless, an upsurge in AChE could impact the degradation of neuronal processes[5].

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Evidence suggests that neurotransmitter systems are a potential early pathophysiological factor in cognitive impairment[6] and that many have been used to diagnose degenerative encephalopathy[7]. Acetylcholine and monoamine neurotransmitters are crucial for a variety of physiological processes. An upsurge or drop in the quantities of neurotransmitters has been connected to several central nervous system ailments. The progression of various diseases can be accelerated by balancing the levels of these neurotransmitters. Neurotransmitters like acetylcholine and monoamines are regulated by monoamine oxidase and cholinesterase enzymes[8].

Monoamine oxidases are flavoenzymes facilitating the oxidative deamination of neurotransmitters, viz, serotonin, norepinephrine, tyramine, dopamine, and certain other amines[9]. A set of central nervous system diseases, including memory loss, cognitive decline, and mental disorders like depression, can be treated symptomatically with monoamine oxidase and cholinesterase inhibitors to prevent further development of the condition[8]. Due to their fewer adverse effects and better efficacy, medicinal herbs and natural phytocompounds are increasingly used to treat diabetes. Terpenoids are a broad class of naturally occurring secondary metabolites having a variety of pharmacological effects. Menthol, a naturally occurring dietary monoterpene found in mint having the chemical formula $C_{10}H_{20}O$, is a principal component of peppermint oil, an herbal remedy adopted in conventional medicine. Figure 1 shows the chemical structure of menthol.

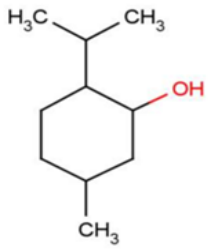

Figure 1. Chemical structure of menthol.

Various studies have demonstrated that menthol has antibacterial[10], anticancer[11], antimicrobial[12] and anti-inflammatory properties[13]. In our study, we aimed to figure out if menthol ameliorates cognitive deficits caused by hyperglycemia-induced cholinergic dysfunction and neurotransmitter imbalances in experimental diabetes induced by streptozotocin.

2. Materials and Methods

2.1 Chemicals

The analytical grade reagents employed in the current investigation were purchased from Sisco Research Laboratories (Mumbai, India) and Sigma–Aldrich (St. Louis, Missouri, United States).

2.2 Experimental Animals

Male Sprague Dawley rats weighing 150-250 g were employed in the investigation. The animals kept in propylene cages under standard experimental conditions (25°C, 12 h light/dark cycles) were provided water and a standard laboratory pellet diet. The experimental procedure obtained approval from the Institutional Animal Ethics Committee [IAEC 3-KU-04/2018-19-BCH-SM (44)].

2.3 Induction of Diabetes

STZ (40 mg/kg body weight) is soluble in 0.1 M citrate buffer with pH 4.5 was administered intraperitoneally into rats to develop diabetes. To prevent drug-induced hypoglycemia, a 5% glucose solution was provided to the animals overnight. When the blood glucose level exceeded 250 mg/dL on the third day following the STZ injection, the rats were deemed diabetic.

2.4 Experimental Design

Five groups of six rats each were used for the study.
Group I: Normal control (N)
Group II: Normal rats administered with Menthol (N+M)
Group III: Diabetic Encephalopathic control rats (DE)
Group IV: Diabetic Encephalopathic rats administered with Menthol (DE+ M)
Group V: Diabetic Encephalopathic rats administered with Metformin (DE+ Met)

The animals in Groups II and IV were intragastrically supplemented with menthol (50 mg/kg body weight) dissolved in corn oil for 60 days. The dosage and method of administering menthol were chosen in accordance with the outcomes of the prior investigation conducted by Muruganathan *et al*[14]. Metformin, 100 mg/kg body weight, was supplemented in Group V. After 60 days of therapy, animals fasted for 12 hrs were sacrificed, and blood and brain were taken for various analyses.

2.5 Morris Water Maze Test (MWM Test)

The MWM test was employed to gauge the cognitive abilities of diabetic rats[15]. The experiment was performed in a spherical, water-filled pool maintained at a temperature of 26 ± 2 ^{0}C. Four equal quadrants were marked (I, II, III and IV) in the pool. An escape platform (10 cm diameter) was set up permanently in one of the quadrants, 1 cm below the water surface. For the experiment, rats were placed into the water from four quadrants to identify a hidden platform to escape. For four days, the rats were exposed to three trials per stage. Escaping latency (time taken to arrive escape platform) was calculated. The average latency time for all three trials was determined. A substantial decrease in latency time was considered successful learning.

2.6 Biochemical Studies

2.6.1 Serum Glucose and Hepatic Toxicity Markers

The blood glucose, Aspartate Transaminase (AST), Alanine Transaminase (ALT) and Alkaline Phosphatase (ALP) were assessed by kits obtained from Agappe Diagnostics Pvt. Ltd, India.

2.6.2 Glycated Hemoglobin (HbA1c) and Plasma Insulin by ELISA

Glycated hemoglobin was measured by means of an HbA1c kit from Beacon Diagnostics Pvt Ltd., and plasma insulin using an ELISA kit from DRG Diagnostics, Marburg, Germany.

2.6.3 Acetylcholinesterase and Choline Acetyltransferase (ChAT) Activity

AChE activity and ChAT activity were determined according to the protocol of Ellman *et al.*,[16] and Liu *et al.*,[17] respectively.

2.6.4 Neurotransmitters

The brain Norepinephrine (NE) was assayed by the protocol of Weil-Malherbe and Bigelow[18], Dopamine (DA) by the technique of Atack[19] and serotonin (5-HT) by means of the procedure of Curzon and Green[20].

2.6.5 Determination of Protein

The protein concentration was measured according to Lowry *et al*[21].

2.6.6 RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction

TRI Reagent was adopted for the isolation of total genomic RNA from the brain (Sigma-Aldrich, St. Louis, MO, USA)[22]. cDNA synthesis and PCR reactions were carried out in a thermocycler (Eppendorf 5332) in accordance with the manufacturer's directions employing a kit acquired from Fermentas, Vilnius, Lithuania. The primer sequences utilized for the investigation are displayed in Table 1. The PCR products were electrophoresed on 1.5% agarose gels, and the gels were then scanned densitometrically using Bio-Rad Gel Doc to visualize the products. The mRNA level was quantified using the quantity one imaging software (Bio-Rad). In the same sample, the relative expression was compared to the expression of β actin.

2.6.7 Statistical Analysis

SPSS/PC+, Version 17 (SPSS Inc. Chicago, IL, USA), the statistical package, was employed for statistical analysis. Comparison among the groups was accomplished by one-way Analysis of Variance (ANOVA) and, subsequently,

Table 1. PCR primer sequences

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
β actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC
MAO A	AAGACACGCTCAGGAATGGG	ATGGCTACGTACATGGCTGG
MAO B	TTTGGCAGCCAGAACCAGAA	AGCTTGTGTTCCAGTCACCC

Duncan's posthoc multiple comparison tests. The results were denoted as mean value ± SE (n = 6). '*p*' values less than 0.05 were considered significant.

3. Results

3.1 Morris Water Maze Test

Spatial learning and memory in laboratory rats were investigated by adopting the MWM test. After thirty days following STZ induction, animals in untreated diabetic groups displayed greater escape latencies during training sessions than the healthy control rats (Figure 2A). It demonstrated cognitive impairment. Menthol and metformin were then administered to the diabetic rats for 60 days. In four successive trials, the normal healthy groups performed better on the MWM test. Throughout training sessions, the escape latencies of STZ-induced rats were longer than those of the normal rats. In comparison to diabetic controls, menthol, and metformin supplementation significantly ($p < 0.05$) diminished mean escape delay time (Figure 2B). These findings demonstrated that the diabetic group treated with menthol and metformin significantly improved their spatial learning abilities compared to the diabetic encephalopathic group.

3.2 Fasting Blood Glucose, HbA1c and Plasma Insulin Levels

Blood glucose and HbA1c levels were significantly higher in the DE control groups than in normal rats, but plasma insulin levels were lower. Administration of menthol and metformin reduced blood glucose and HbA1c and augmented plasma insulin levels in DE rats (Figure 3). A significant effect was noticed for blood glucose and plasma insulin in menthol supplemented group than metformin ($p < 0.05$). Nevertheless, for HbA1c, the results were comparable. In normal and normal rats treated with menthol, there was no substantial change in blood glucose, HbA1c and plasma insulin. These results indicate that menthol offers protection to the pancreas in diabetic rats and enhances insulin release from pancreatic β cells.

3.3 Liver Toxicity Markers

Several markers, including AST, ALT and ALP, are principal parameters representing the health of the fundamental liver function, and variations in these parameters can reveal the degree of hepatocytic damage[23]. As shown in Table 2, the liver enzymes AST, ALT, and ALP were elevated in DE rats. In comparison to diabetic control, menthol, and metformin supplementation significantly ($p < 0.05$) diminished AST, ALT and ALP activities (Table 2). Between the N and N+M groups, there were no significant alterations in the activities of hepatic toxicity markers. A superior effect was observed for menthol than metformin. Therefore, menthol promotes protection by averting the outflow of hepatic enzymes into the bloodstream without any toxic effects.

Values are represented as mean ± SE (n = 6). 'a' depicts values that differed significantly from N, DE is contrasted to DE + M and DE + Met ('b' depicts values that varied significantly from DE and DE + M is a contrasted with

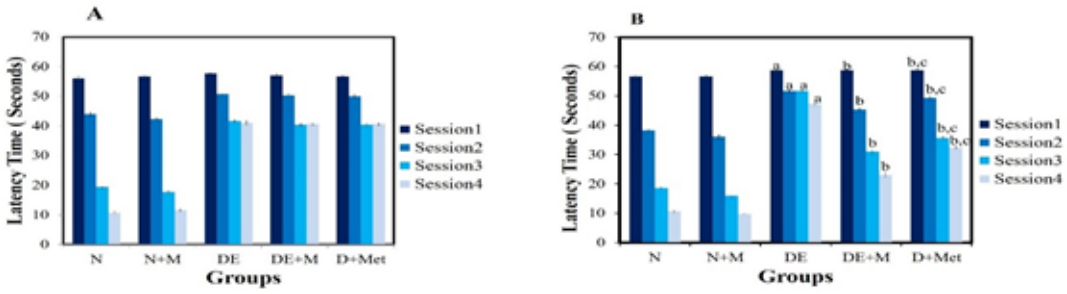

Figure 2. Learning and memory. **(A)** MWM test before treatment, **(B)** MWM test after treatment. Values are represented as mean ± SE (n = 6). 'a' depicts values that differed significantly from N, DE is contrasted to DE+M and DE+Met ('b' depicts values that differed significantly from DE and DE+M is a contrasted with DE+met ('c' depicts values that differed significantly from DE+M. Significance accepted at $p < 0.05$.

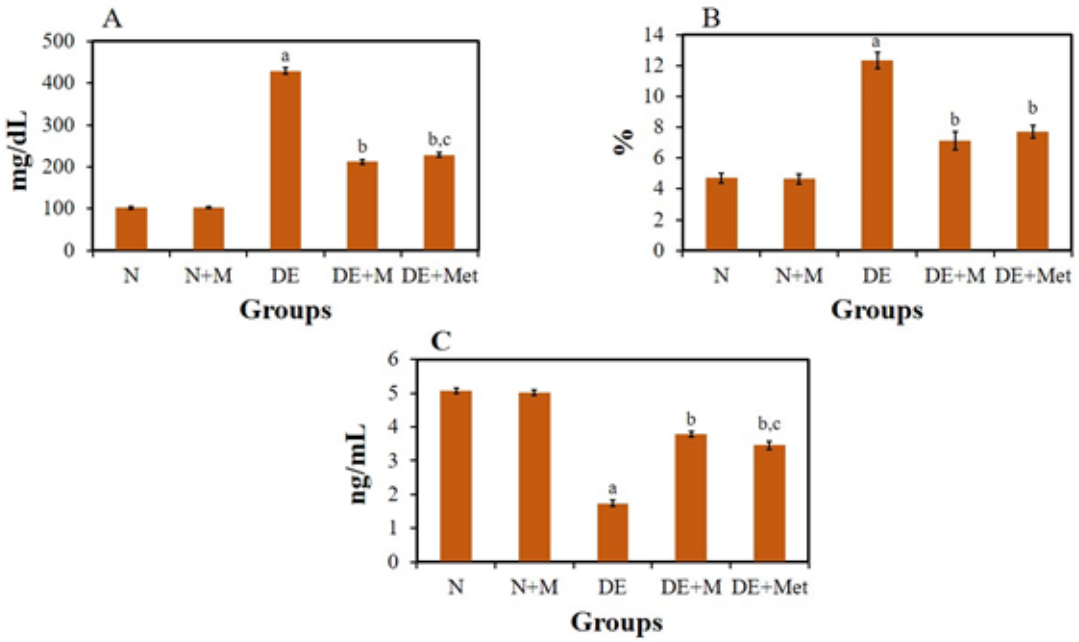

Figure 3. **(A)** Serum glucose, **(B)** Glycated hemoglobin, **(C)** Plasma insulin. Values are represented as mean ± SE (n = 6). 'a' depicts values that differed significantly from N, DE is contrasted to DE + M and DE + Met 'b' depicts values that differed significantly from DE and DE + M is a contrasted with DE + met 'c' depicts values that differed significantly from DE + M. Significance accepted at $p < 0.05$.

Table 2. Liver toxicity markers

Group	SGPT (U/L)	SGOT (U/L)	ALP(U/L)
N	43.05±2.67	54.09±1.75	8.53±.28
N+M	43.04±2.05	54.68±3.63	8.40±.35
DE	83.76±4.61[a]	102.96±1.75[a]	19.71±.42[a]
DE+M	53.51±2.97[b]	70.97±2.67[b]	10.36±.42[b]
DE+Met	62.12±4.58[b,c]	76.78±1.75[b,c]	12.29±.58[b,c]

DE + Met ('c' depicts values that varied significantly from DE+M. Significance accepted at $p < 0.05$.

3.4 Cholinergic Function

ChAT and AchE activities are depicted in Figure 4. Compared to the normal control rats, DE animals exhibited a noticeable reduction in ChAT activity and an upsurge in AchE activity. In STZ-induced DE rats, ChAT activity was considerably ($p < 0.05$) elevated, and AchE activity was lowered following menthol or metformin therapy. Both menthol and metformin produced comparable effects.

3.5 Neurotransmitters

Monoaminergic neurotransmitter systems are crucial for neuronal function and behaviour. They were significantly affected by the disruption of glucose homeostasis. In our study, monoaminergic neurotransmitters such as dopamine, serotonin, and norepinephrine declined in DE rats more than in normal groups. However, menthol

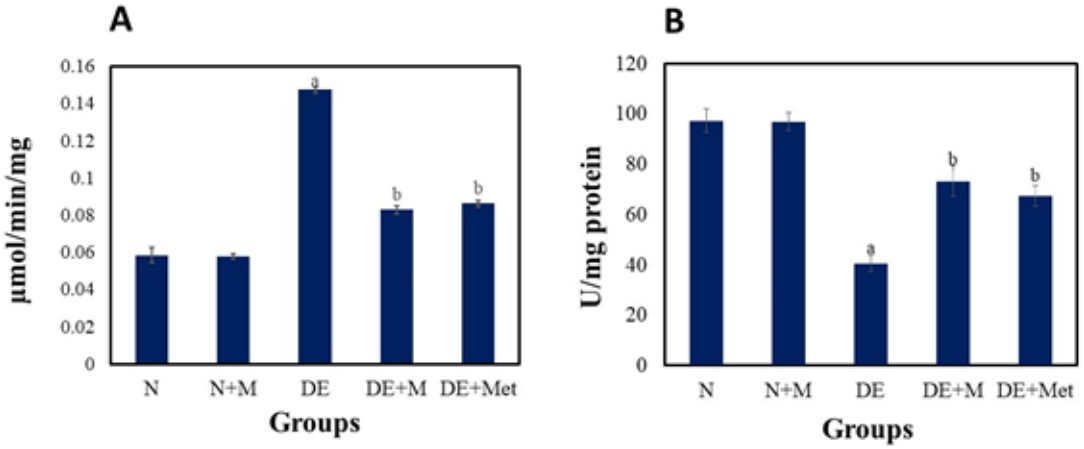

Figure 4. **(A)** Acetylcholinesterase, **(B)** Choline acetyl transferase. Values are represented as mean ± SE (n = 6). 'a' depicts values that differed significantly from N, DE is contrasted to DE+M and DE+ Met ('b' depicts values that varied significantly from DE). Significance accepted at $p < 0.05$.

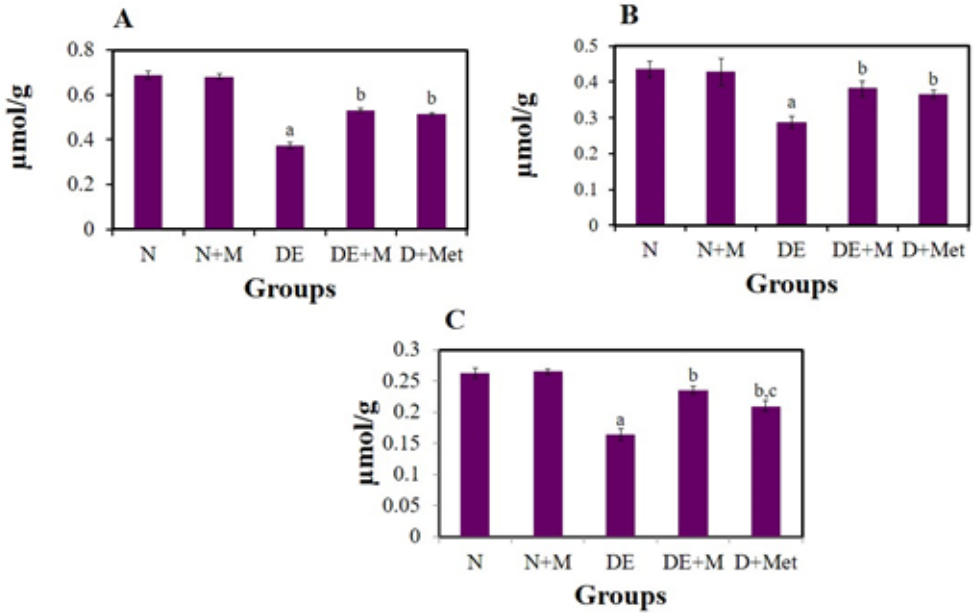

Figure 5. **(A)** Dopamine, **(B)** Serotonin, **(C)** Norepinephrine. Values are represented as mean ± SE (n = 6). 'a' depicts values that differed significantly from N, DE is contrasted to DE + M and DE + Met ('b' depicts values that differed significantly from DE, and DE + M is a contrasted with DE + Met ('c' depicts values that differed significantly from DE + M. Significance accepted at $p < 0.05$.

and metformin treatment markedly raised the level of these neurotransmitters (Figure 5). Both menthol and metformin displayed comparable effects except for norepinephrine, where menthol displayed a superior effect.

3.6 mRNA Expressions of MAO A and MAO B

In the present work, DE rats exhibited upregulated MAO A and MAO B expressions compared to normal rats. However, MAO A and B expressions were markedly reduced after menthol and metformin administration.

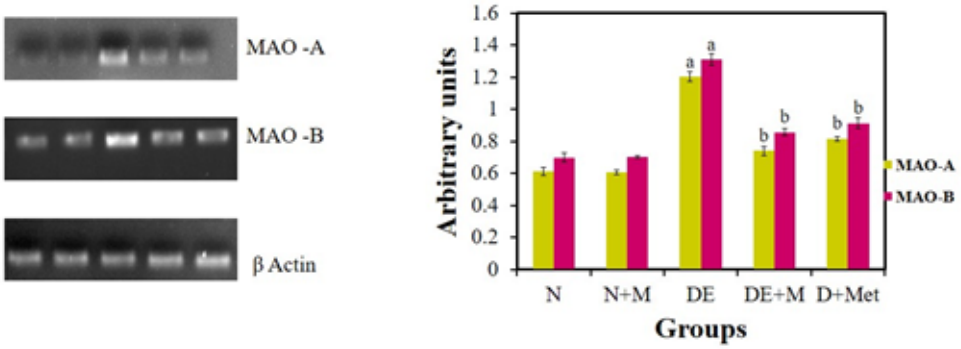

Figure 6. mRNA expressions of monoamine oxidase A and B. Values are represented as mean ± SE (n = 6). 'a' depicts values that differed significantly from N, DE is contrasted to DE + M and DE + Met ('b' depicts values that differed significantly from DE). Significance accepted at $p < 0.05$.

In DE rats treated with menthol and metformin, there was no discernible difference between the expressions of MAO A and MAO B (Figure 6).

4. Discussion

DM is a metabolic condition marked by persistent hyperglycemia resulting from poor insulin production, insulin action, or both. One of the most alarming microvascular complications of DM is diabetic encephalopathy[24]. It is distinguished by cognitive impairment caused by neurochemical and structural alterations.

Treatment of CNS complications by modern medicine has several shortcomings because of their strong side effects and higher chances of reoccurrence. As a result, it is essential to look for safer and more effective drugs, and phytochemicals may be preferable. Secondary plant metabolites with various structural and functional properties have recently received much attention as potential therapeutic leads for the therapy and avoidance of dementia caused by diabetes. In the current study, the neuroprotective effect of dietary monoterpene menthol in STZ-induced diabetic rats was determined.

MWM is a popular tool for evaluating spatial learning and memory[25]. Increased escape latency values in DE rats in the current study revealed that diabetes considerably reduced learning and memory function, which is in agreement with the outcomes of earlier investigations[26,27]. However, the escape latency values of DE rats fed with menthol and metformin were diminished significantly compared with the DE rats. These findings suggested that menthol administration improves learning and memory in DE rats. The ameliorative potential of menthol might be attributable to an improvement in acetylcholine in the brain[28].

The hallmark of T2D is chronic hyperglycemia, caused by insulin resistance and beta cell malfunction and linked to long-term consequences such as retinopathy, nephropathy, and micro- and macro-vascular disorders. Blood sugar, HbA1c, and insulin are the three leading glycemic indicators of diabetes[29]. In the present investigation, blood glucose and HbA1c levels were significantly raised, and plasma insulin levels declined in DE groups than in normal rats administered with menthol. Menthol and metformin supplementation decreased blood glucose and HbA1c levels. Furthermore, they also elevated plasma insulin levels (Figure 3). Menthol showed a superior effect on both blood glucose and plasma insulin. Nevertheless, for HbA1c, the results were comparable for menthol and metformin. These outcomes align with previous studies by Muruganathan *et al.*, which revealed that menthol supplementation stimulated insulin release from the rejuvenated pancreatic β-cells, which consequently raises glucose consumption by the tissues[14].

The cholinergic system is crucial in promoting neuronal plasticity and is essential for neuronal function in memory, learning, and cognition[30]. Cognitive processes are greatly influenced by normal cholinergic

function, maintained by the constant presence of acetylcholine, either by sustained synthesis or reduced acetylcholinesterase activity[31]. The two distinct indicators of cholinergic neurons in the hippocampus and cerebral cortex, AChE, and ChAT, are crucial for controlling the cholinergic pathway. The outcomes of the present investigation demonstrated an upsurge in AchE and a decline in ChAT activity in the diabetic encephalopathic group, representing inadequate cholinergic action because of the depletion of acetylcholine in the brain. Rats in the menthol-supplemented group exhibited diminished AChE activity and augmented ChAT activity. Similar results were seen in the metformin-treated group as well. Our results agree with the previous study by Mao *et al.*, where huperzine A ameliorates cognitive deficits by augmented ChAT activity and decreased AChE activity in STZ-induced diabetic rats[32]. The observed reduction in AChE activity and an upsurge in ChAT activity could avert the quick degradation of acetylcholine, thereby increasing its availability for nerve impulse transmission. It is crucial to inhibit AChE to maintain memory function as its improved activity leads to the development of amyloid plaques, another mechanism causing cognitive decline[33,34].

Neurotransmitters are chemical molecules that may convey and amplify impulses and are essential for transferring information throughout the neurological system[35]. One of the central neurotransmitters in the mammalian nervous system, dopamine, enhances learning, locomotion, sexual fulfilment, motivation, attention, and arousal[36]. Additionally, it plays a role in sustaining homeostasis and acts as a precursor for norepinephrine and epinephrine[37]. Serotonin regulates brain function, behaviour, vasoconstriction, peristalsis, gastrointestinal secretion, sleep and waking states, and respiration[38]. Norepinephrine is recognized to play a role in waking-state arousal and alertness, emotion regulation, sensory signal recognition, memory, learning, and attention. DA, 5-HT, and NE are monoaminergic neurotransmitters that are crucial for neuronal behaviour and function. Neuropsychiatric and neurodegenerative illnesses, often known as monoamine neurotransmitter disorders, are caused by the dysregulation of these systems[39]. The current study observed that diabetic rats had lower amounts of monoaminergic neurotransmitters such as DA, 5-HT, and NE than normal control rats. These outcomes align with the previous study by Ezzeldin *et al*[40]. On the other hand, menthol and metformin treatment significantly improved the level of DA, 5-HT, and NE and thus played an essential role in solving many neuropathologies associated with DE. Our findings agree with those of Wang *et al.*, who found that the modulation of the 5-hydroxytryptaminergic, GABAergic, and dopaminergic systems by the antidepressant-like actions of menthol results in the maintenance of neurotransmitters[41].

Monoamine oxidases play a pivotal function in the brain through the metabolic control of monoamine neurotransmitters[42]. They catalyze the oxidative deamination of epinephrine, norepinephrine, and dopamine and produce hydrogen peroxide (H_2O_2)[43]. Combined with metal ions, H_2O_2 will produce Reactive Oxygen Species (ROS) in the brain of AD rats[44]. Increased ROS-mediated oxidative stress brought on by Aβ accumulation and deposition will eventually cause neurons to die[45]. In our study, mRNA expressions of monoamine oxidases (A and B) were significantly upregulated in the diabetic encephalopathic group. This was consistent with the study conducted by Adefegha *et al*[46]. This finding could suggest that DA, 5-HT, and NE were destroyed in DE rats' brains. However, compared to DE rats, menthol and metformin administration in DE rats significantly downregulated the expression of MAO A and B. This downregulated MAO-A and B expression suggested that menthol had MAO inhibitory effects and may confer neuroprotection. In addition, our results concur with those of Emory and Mizrahi, who found that inhibiting MAO reduced dopamine deficiency in diabetic patients and depression[47]. The findings of the current investigation suggest that menthol could improve brain cholinergic function and regulate the level of neurotransmitters in experimental diabetic rats.

5. Conclusion

To date, cognitive deterioration unquestionably represents a novel and developing long-term DM consequence, resulting in poor glycemic control and a lack of diabetes self-management. Scientists, researchers, and pharmaceutical companies are increasingly searching for bioactive compounds to discover and develop targeted novel anti-diabetic drugs. Compared to conventional diabetes medications, these compounds may be less likely to cause side effects. The present

study revealed that menthol treatment ameliorates high glucose-induced cognitive impairment and attenuates cholinergic dysfunction and neurotransmitter imbalance in experimental diabetes. Thus, menthol seems to be a promising compound to prevent the complications associated with diabetic encephalopathy.

6. Acknowledgments

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