Isolation of Sialic Acid Using Affinity Bead Technology (ABT) and In Vitro/In Vivo Evaluation of Nutrient Delivery System (NDS) As an Alternative Food/Drug to Improve Brain Function

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ABSTRACT: The objective of this study was to produce a nutrient delivery system (NDS) as an alternative food and drugs using sialic acid, a N-acetylneuraminic acid, isolated from edible bird's nest (EBN) by affinity bead technology (ABT). Additionally, this study aims to investigate the in vivo ability of sialic acid and NDS to improve memory impairment induced by scopolamine using a cell of in vitro assays, such as antioxidant and acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition, and in vivo animal models, including elevated plus maze, Morris water maze, passive avoidance, and novel object recognition tests. Sialic acid caused a concentration dependent inhibition of AChE and BuChE enzymes with IC50 values of 55 and 52 µg/mL, respectively, and antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 20-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) with IC50 values 170 and 220 µg/mL, respectively. NDS reversed the scopolamine induced amnesia as indicated by a dose dependent decrease in escape latency, path length, and passing frequency in the Morris water maze test compared with the relevant control. The compound also significantly increased the discrimination index in a dose dependent manner in NORT and decreased transfer latency in EPM, reflective of its memory enhancing effect. Furthermore, NDS also caused significant dose dependent elevation in the step-down latency (SDL) in the passive avoidance test. The results indicated that NDS might be a helpful memory restorative mediator (as an alternative food and drugs using sialic acid) in treating cognitive disorders such as Alzheimer 's disease and Parkinson's disease.

KEYWORDS: sialic acid; N-acetylneuraminic acid; dementia, Alzheimer's disease, Parkinson's disease, edible bird's nest; affinity bead technology (ABT); nutrient delivery system (NDS); alternative food; scopolamine induced mice; brain function test; ABTS; DPPH

I. INTRODUCTION

Dementia also includes other common types, such as Alzheimer 's disease (AD), dementia with Lewy bodies, cerebrovascular dementia, Parkinson's disease, and frontotemporal dementia [1,2]. The most common type is AD, which is responsible for 50–60% of all cases). AD is a neurodegenerative disease with slow onset and progressive impairment of cognitive and behavioral functions, including memory attention, comprehension, reasoning, language, and judgment [3]. The initial symptom is usually memory loss.In AD, the most acceptable therapeutic approach has been the usage of acetylcholinesterase inhibitors that normally block the activity of acetylcholinesterase breakdown acetylcholine (ACh). This enzyme inhibition prevents the breakdown of the ACh that is known to be deficient in the AD brain. Although these drugs will be effective in the provision of symptomatic treatment, these drugs are non-selective. They may lead to the overstimulation of cholinergic systems throughout the body and cause a variety of cholinergic manifestations [4].

The currently available acetylcholine esterase AChEIs have uncertain clinical significance and are associated with serious adverse effects [5]. Thus, these therapeutic limitations in managing Alzheimer 's disease necessitate discovering and developing new and better drugs or alternative foodfor this devastating disorder.

An edible bird's nest(EBN) is a nest made of saliva and feathers secreted by a small petrel called 'Aerodramus funiphagus', which mainly lives in Southeast Asia. It has been a traditional Chinese food consumed since the Ming Dynasty, and petrels

build nests on coastal cliffs. The materials used vary depending on the number of nests they make. This EBN is not only rich in vitamins, hormones, and fatty acids, but also contains various nutritional components such as proteins, carbohydrates, mineral salts, and amino acids [6-8]. In particular, the carbohydrate component of EBN has various pharmacological effects on the human body. Among them, sialic acid, contained at a concentration of about 60-160 mg/kg, is a neuraminic acid with a nine-carbon skeleton. It is a general term for acyl derivatives of acid, and more than 20 types are currently known in the animal world [8].

Sialic acid, also known as N-acetylneuraminic acid, is considered to be an acidic sugar involved in important physiological functions such as receptor function for viruses, immunological recognition sites for cells and molecules, cell adhesion, or cancer metastasis. It only exists temporarily in a free form during the metabolic process, but mostly exists in the form of oligosaccharides by being bound to the non-reducing end of the sugar chain as a glycoside [9-11]. It has been reported that it not only helps infants' intellectual abilities, but also has anti-inflammatory activity that regulates physiological and pathological processes. In general, sialic acid forms N-acetylgalactosamine (GalNAc) through a glycosidic bond, and this compound plays an important role in the proper function of synapses and is known to help improve memory [6,7,12-14]. In a previous study, it was confirmed that EBN prevents lipopolysaccharide (LPS)-induced neuroinflammation and reverse LPS-induced memory impairment in a dose-proportional manner, showing that EBN sialic acid is not only a functional food for neurodegenerative diseases, but also memory impairment. It has been reported that it can potentially play a neuroprotective role by promoting and inhibiting neuroinflammatory responses and oxidative stress processes [12]. As EBN becomes known through high-end Chinese restaurants, online distribution, and home shopping, interest in and consumption as various processed foods and food ingredients is rapidly increasing, but most of them are dried and processed products [15]. Therefore, in producing bird's nest extract as a functional raw material, the importance of researching efficient extraction methods for these functional substances is increasing.

A nutrient delivery system (NDS) is a system that effectively delivers nutrients from food to target points in the body [13,14,16]. There are cases where ingested nutrients are not easily delivered to specific organ tissues or cells after being absorbed into the body, making it difficult to fully deliver their efficacy to the target point in the body, so a delivery vehicle is used. As a similar delivery system, research has been conducted on the drug delivery system (DDS) in the pharmaceutical industry and the transdermal drug delivery system (TDDS) in the cosmetics industry [17-19]. Accordingly, our research team conducted research on NDS substances to realize formulations that increase the body's absorption rate of food nutrients. In this study, focusing on micelle nanoparticles with excellent absorption rate and efficacy in the body using bile acid, bile acid-sialic acid micelle nanoparticles were manufactured using the NDS system with excellent tissue penetration and absorption rate and used for research for the first time [13,14].

Affinity beads technology (ABT) is an eco-friendly separation and purification method that uses non-polar polymer beads to adsorb target components onto non-polar polymer beads. After separation, a adsorbent is added to remove the target components from the non-polar polymer beads. It is a detachment technology. It has the advantage of being able to selectively separate and purify using a simple method, and has high productivity using low energy and is easy to apply in a repetitive production process. In addition, ABT technology is environmentally friendly because it uses non-polar polymer beads with lower manipulation energy than ionic polymer beads, minimizing the use of organic solvents that cause environmental pollution [10,11].

Previous findings indicated that sialic acid (N-acetylneuraminic acid), the most active component of EBN, could have neuroprotective effects against Alzheimer's disease (AD) via inhibiting the activity of AChE and modulating oxidative stress [20]. A recent study on sialic acid showed potent urease [21], weak α-glucosidase enzyme inhibition, and antioxidant effects.

Since there is no report, to the best of our knowledge, available on the scopolamine induced memory impairment in mice concerning NDS, which prompted the investigation of the isolated compound [22], the present study aimed to evaluate the possible memory-enhancing properties of the compound using various in vitro assays, in vivo, and in silico assays.

II. MATERIALS AND METHODS
1. Chemicals
Acetylcholine iodide (Sigma-Aldrich, Gillingham, UK), butyrylcholine iodide (Sigma-Aldrich, Schaffhausen, Switzerland), and 5,5-dithio-

bis-nitrobenzoic acid (DTNB) (Sigma-Aldrich, Taufkirchen, Germany) were purchased from local suppliers. Electric eel acetyl-cholinesterase (type-VI-S) and equine butyrylcholinesterase (Sigma-Aldrich, St. Louis, MO, USA), DPPH (2,2-diphenyl-1-picrylhydrazyl) from (Sigma-Aldrich, USA) and ABTS (2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma-Aldrich, Germany and HPLC grade methanol was purchased from (Sigma-Aldrich, Gillingham, UK). Donepezil and scopolamine were purchased from Sigma (St. Louis, MO, USA). All chemicals and solvents used were of analytical grades.

2. Animals
A selected number of healthy albino mice weighing up to 20–30 g was used in the study. The animals were housed in several groups in individual cages made from stainless steel with softwood shavings as bedding and were provided with water and a standard pellet diet. They were maintained under normal laboratory conditions and at 12 h light-dark cycle. Activities were carried out according to the accepted guidelines of the Animal (Scientific Procedures) Act UK 1986 [23].

3. Sample Collectionof EBN Extract,Sialic Acid, NDS and Identification
For the preparation of EBN extract, bird's nest concentrate sold under the trade name The King's Morning[TM] manufactured by Nestural Ltd, Pathum Thani, Thailand was used. The separation and purification process of sialic acid using the EBN extraction method and ABT, measurement of sialic acid purity, and NDS production (Figure 1) were performed with reference to our previous research. [10,11,13,14].Voucher specimens (SAE, SA, NDS) were deposited in the herbarium of R&D Center of KimJungMoon Aloe and Oriental Medicine Biotechnology Center of Kyung Hee University.
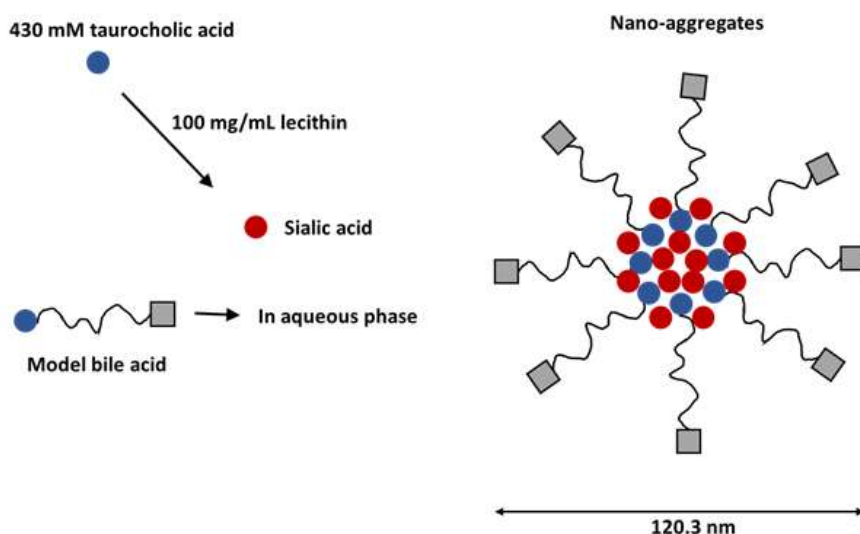

Figure 1. Nutrient delivery system which is consisted of sialic acid-bile acid conjugated micelle nano particles.

4. Assessment of Sialic Acidin InVitro Assays
4-1. DPPH Radical Scavenging Assay
The compound's total free radical scavenging capacity was carried out according to the previously reported method [24] with a slight modification by using stable DPPH (2,2diphenyl-1-picrylhydrazyl radical), which has a maximum of 515 nm of absorption. By dissolving 2.4 mg of DPPH, a solution of the radical was prepared in 100 mL of methanol. A test solution of (5 μL) was added to the 3.995 mL of a methanolic DPPH. The mixture was vigorously shaken and then kept at room temperature for about 30 min in the dark. Absorbance at 515 nm of the reaction mixture was measured spectrophotometrically. Measurement of the absorbance of DPPH radical, without antioxidant, i.e., blank, was also performed. All the determinations were performed in triplicate form.The scavenging capability of the DPPH radical was also calculated by using a specific equation [25].

The formula for scavenging free radicals by a compound:

Radical scavenging (%) =A − B / A x 100

A = control absorbance; B = sample absorbance
Control value: A = 0.723, Abs. on UV–visible spectrophotometer

4-2. ABTS Radical Scavenging Assay

Free radical scavenging, the compound's activity was determined by 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) or ABTS radical, cation decolorization activity/assay [14]. ABTS+, between 7 mM ABTS cation radical, was produced by the reaction in the water and 2.45 mM of the potassium persulfate (1:1) stored at room temperature in the dark for about 12–16 h before use. A dilution of ABTS solution with methanol was prepared to obtain 0.700 of absorbance at 734 nm. After adding 5 µL of the compound to the 3.995 mL diluted ABTS solution, 30 min after the starting and initial mixing, the absorbance was measured. In each assay, an appropriate solvent of blank was run. All the measurements were carried out in triplicate. Absorbance at about 734 nm percent inhibition was calculated by using the specific formula [26].
The formula for scavenging free radicals by a compound:

$$\text{Radical scavenging (\%)} = A - B / A \times 100$$

A = control absorbance; B = sample absorbance
Control value: A = 0.735, Abs. on UV–visible spectrophotometer

4-3. Anti-Cholinesterase Assays

In these assays, the compound was analysed spectrophotometrically for the inhibition potential of AChE and BuChE inhibition. As a substrate, the butyrylcholine iodide and acetylcholine iodide were used by following Ellman's assay [27]. Dilutions of the compound were added in this assay to a cuvette that contained 5 µL of acetylcholinesterase in (0.03 µ/mL) and butyrylcholinesterase in (0.01 U/mL) in a 30 ◦ C temperature water bath; about 5 µL of the DTNB catalyst was kept. Then, this was incubated for about 15 min. After the incubation phase, 5 µL of the substrate was added to the mixture to start the reaction. At 412 nm for about 4 min, absorbance was measured by using the double-beam spectrophotometer. 5-thio-2-nitrobenzoate anion was formed by a reaction between the DTNB and the thiocholines in yellow colour. NDS was used as a control, while the reaction mixture had all of the above components. Percent enzymatic inhibition and percent enzymatic activity was calculated by a specific formula [27].

$$V = \Delta Abs / \Delta t$$

Enzyme activity (%) = V / Vmax x 100
Enzyme inhibition (%) = 100 − Enzyme activity (%)

(V (sample) shows the rate of reaction in the presence of an inhibitor and Vmax (control) shows the rate of reaction without inhibition)
Control values: A1 = 0.734, A2 = 0.884, Abs. on UV–visible spectrophotometer

5. Assessment of Behavioural Parameters in In Vivo Assays
5-1. Assessment of Acute Toxicity Study of NDS

NDS was administered to five groups, each containing six animals. Group I served as a control, whereas groups II-V were given the compound at doses of 50, 100, 200, and 300 mg/kg, i.p.Behavioural properties of the NDS were noted for the individual animal at 0, 30, 60, and 120 min, 24, 48, and 72 h, and one week after the administration of the drug. During the study, no acute toxicity signs were observed (evident from the absence of any effects on mortality, respiratory discomfort (cyanosis or gasping), altered reflex actions, as well as the lack of convulsions). A slight degree of sedation was observed at 200 and 300 mg/kg doses. Otherwise, all the animals seemed good 24 h to 1 week after the administration of the agents and no prominent alterations in the behaviour, activities, and appearance were noted.

5-2. Assessment of Antiamnesic Activity

The treatment/administration of standard, drug, and test compounds in several groups was per protocol (Table 1). Randomly, the animals were put into six different groups, each group containing eight animals (n = 8). Each animal trial was marked with a permanent marker for easy identification. The volume of dose administrations was adjusted for all animals. For each behavioural experiment performed in this study, vehicle, NDS, and the reference drug were administered to animals 1 h before each trial. The vehicle and the amnesic agent were administered 30 min before each trial [28].

Table 1. Various treatment groups were used in the study.

Group	Group Category	Test Solution	Route
1	Normal control	Vehicle 8 mL/kg	p.o.
2	Negative control	Scopolamine 1 mg/kg	i.p.
3	Positive control	Donepezil 2 mg/kg + scopolamine 1 mg/kg	p.o/i.p
4	Treatment-1	NDS 1 mg/kg + scopolamine 1 mg/kg	p.o/i.p
5	Treatment-2	NDS5 mg/kg + scopolamine 1 mg/kg	p.o/i.p
6	Treatment-3	NDS 10 mg/kg + scopolamine 1 mg/kg	p.o/i.p

5-3. Novel Object Recognition Test (NORT)

According to the procedure of Rodroze and his coworkers, the novel object recognition task (NORT) was performed [29]. The apparatus was made of a white-coloured box (40 cm, × 40 cm, ×66 cm) with a complex floor, which could be easily cleaned with 70% v/v of ethanol after each trial. A 60 W light was suspended in the apparatus, 50 cm over the wreck. NORT activity consisted of the habituation, sample, and test phases. In the sample phase, an individual mouse was positioned in an open field chamber with two identical objects (blue bottle) for about five minutes. Then, the mouse was returned to the home cage. The objects and the arena were cleaned with 70% v/v of ethanol in the middle of the trials to escape the olfactory cues. The test phase was conducted 24 h after the exposure of the sample phase. In the test phase, each mouse was again located in an open field chamber where one identical object had been exchanged with the novel object (red bottle). The object was placed in the arena so that 1/2 of the animals in each group looked at the novel object placed on the left side of the box arena, and the further half looked at the novel object on another or right side of the box arena, to remove preference of the sides. The time spent exploring each object in each phase was manually noted using a stopwatch. An animal was counted as exploring when its head was focused on the object within the expanse of about 2 cm or when the nose interacted with the specific object. Parameters assessed included the duration (in seconds) spent exploring the familiar object (TF), the time (in seconds) spent exploring the novel object (TN), and the total time (in seconds) spent exploring both objects (TF + TN). The percentage of the discrimination index (DI) was determined by using the following equation:

$$DI\ (\%) = TN / (TN + TF) \times 100$$

5-4. Assessment of Morris Water Maze Test (MWMT)

The Morris water maze test was used to test learning, including the acquisition of spatial memory, according to the method described previously [30,31]. By adding titanium dioxide, the water was made opaque. The tank was divided into four equal quadrants by using the two threads fixed at right angles on the rim of the pool to each other. At the center of the pool, the submerged platform was placed inside the tank, which was 1 cm below the water level. Throughout the training session, the position of the platform was unaltered. Each animal followed four consecutive trials each day with the different points within 5 min gaps between each of the trials for about four consecutive days, during which escape on the hidden platform was allowed and the animal was allowed to remain there for about 20 s. The animal was gently placed during the training session in the water from different locations and allowed 120 s to locate the submerged platform. If within 120 s the animal failed to locate the platform, it was guided on the platform gently and for 20 s the animal was allowed to remain there. On the retrieval day (last day) of the training session (i.e., Day 5), the platform was removed and the animal was placed into the pool from any point and allowed to explore the targeted quadrant for 300 s. In the center of the pool, the mean time spent searching for the missing platform by the rat in the center of the pool, which is an index of memory retrieval, was recorded.

5-5. Assessment in Elevated Plus Maze (EPM)

The Elevated plus maze is used extensively to evaluate cognition in rodents. The EPM arena or apparatus is based on the innate aversion of

animals/rodents to open and high space. The procedure, technique, and end point for testing learning and memory were followed as per the parameters described earlier [32,33]. On the first day, each animal was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was defined as the time required by the animal to move from the open arm into one of the closed arms with all four legs. TL was measured on the 1st day (i.e., 6th day of drug administration) for each animal (learning). If the animal did not enter into one of the closed arms within 90 s, it was gently pushed into one of the two closed arms and the animal was assigned the TL of 90 s. The animal was further allowed to explore the maze for another 2 min and then returned to its home cage. Retention of this learned task (memory) was evaluated after 24 h on the 7th day (trial day). A decline in the transfer latency of the animal serves as an indication of the memory-enhancing effect of the drug [34].

5-6. Assessment in Passive Avoidance Test

In two equal-sized light and dark compartments, the passive avoidance test was carried out with the electrifiable floor grid in nature [35]. A guillotine door acted as the separation of the two compartments. In the acquisition trial phase, the mouse was initially placed in and allowed to explore the light compartment for about 40s. Upon opening the door into the dark compartment, the mouse moved and automatically the door was closed. The training trial was performed 24 h after the acquisition trial. Mice were allowed to explore the light compartment for about 40 s and then the guillotine door was opened. The door closed automatically as soon as the mice entered the dark compartment and, through the grid floor, an electric foot shock (0.1 mA/10 g body weight for 2 s) was delivered. All the drugs were administered after training to avoid modifying memory storage processes. A test trial was performed 24 h after the training trial using the same training program. The latency time was estimated by measuring the time before the mice entered the dark compartment after the opening of the door within the time of 180 s. If mice did not enter the dark compartment within 180 s, a latency time of 180 s was recorded.

5-7. Statistical Analysis

Graphpad prism® was used for the statistical analysis in version 5 (Graph-Pad. Software Inc, San Diego, CA, USA). A Students–Newman–Keuls test for statistical significance calculation was followed by one-way ANOVA. Each value was expressed as the mean ± SEM and consideration of statistical significance was performed at $p < 0.05$.

III. RESULTS
1. Assessment of Free Radical Scavenging Activities
1-1. ABTS Free Radical Activity

In ABTS free radical scavenging assay, sialic acid showed % ABTS inhibition of 70.96 ± 0.37, 67.56 ± 0.58, 49.72 ± 0.67, 42.12 ± 1.05, 28.99 ± 0.36, and 11.68 ± 0.56 with their IC50 value range of 210 μg/mL at a concentration of 1000, 500, 250, 125, 62.5, and 31.25 μg/mL. % ABTS inhibition of the sialic acid was compared with the positive control, which was (ascorbic acid), and revealed a concentration, dependent reaction. Ascorbic acid indicated 83.94 ± 0.37 inhibitions at a concentration of 1000 μg/mL against the ABTS with the IC50 value range of 130 μg/mL (Table 2).

Table 2. ABTS assay activity results. Values represent means ± standard error of mean of n = 3.

Sample	Concentration (μg/mL)	ABTS Scavenging Mean (%) ± SEM	IC50 (μg/mL)
Sialic Acid	1000 μg/mL	70.96 ± 0.37	170
	500 μg/mL	67.56 ± 0.5	
	250 μg/mL	49.72 ± 0.67	
	125 μg/mL	42.12 ± 1.05	
	62.5 μg/mL	28.99 ± 0.36	
	31.25 μg/mL	11.68 ± 0.56	

Ascorbic acid	1000 µg/mL	83.94 ± 0.47	130
	500 µg/mL	74.68 ± 0.28	
	250 µg/mL	63.98 ± 1.00	
	125 µg/mL	49.67 ± 0.57	
	62.5 µg/mL	45.79 ± 1.09	
	31.25 µg/mL	39.73 ± 0.65	

1-2. DPPH Free Radical Activity

The DPPH free radical scavenging potential of the sialic acid was 74.98 ± 0.29, 64.53 ± 0.18, 44.9 ± 0.25, 39.68 ± 0.19, 34.03 ± 0.19, and 29.87 ± 0.18 with the IC50 value range of about 270 µg/mL in the concentration range of about 1000, 500, 250, 125, 62.5, and 31.25 µg/mL (Table 3). The ascorbic acid indicated 86.78 ± 0.39 inhibitions at about 1000 µg/mL concentration against the DPPH with the IC50 value range of 63 µg/mL (Table 3).

1-3. Inhibition of AChE Activity

Table 4 shows the results of the AChE inhibition by the various doses of the sialic acid and the donepezil. Sialic acid dose-dependently inhibited the AChE enzyme with the IC50 value range of 65 and 72 µg/mL, correspondingly. Likewise, the donepezil also inhibited the AChE enzyme with an IC50 value range of 60 and 67 µg/mL against the AChE, respectively (Table 4).

1-4. Inhibition of BuChE Activity

Table 5 shows the results of the BuChE inhibition by the various doses of sialic acid and donepezil. Sialic acid dose-dependently inhibited the BuChE enzyme with the IC50 value range of 65 and 72 µg/mL, correspondingly. Likewise, the donepezil also inhibited the BuChE enzyme with an IC50 value range of 60 and 67 µg/mL against the BuChE, respectively (Table 5).

Percent enzymatic activity and percent enzymatic inhibition were calculated using the following formulas:

$$V = \Delta Abs / \Delta t$$

Enzyme activity (%) = V / V max x 100
Enzyme inhibition (%) = 100 − Enzyme activity (%)

(V (sample) shows the rate of reaction in the presence of an inhibitor and Vmax (control) shows the rate of reaction without inhibition)
Control values: A1 = 0.587, A2 = 0.745, Abs. on UV–visible spectrophotometer

2. In Vivo Pharmacological Assessment
2-1. Effects of NDS on the Acute Toxicity of Animals and Their General Behaviours

There was no significant toxic effect in acute toxicity testing, as was evident from the absence of respiratory discomfort (cyanosis or gasping), altered reflex actions, and the lack of convulsions. In four out of the six animals/mice, the escaping behaviour and the spontaneous action were observed to be greater at doses of about 10 and 50 mg/kg. Additionally, there was an elevation in an allergic reaction (assessed as aggressive behaviour during the treatment and showed a high increase in irritability) and the escape performance was seen as greater in the same animals. Five of the six animals/mice were observed to be slightly sleepy at 300 mg per kg. All the animals seemed good 24 h to 1 week after the administration/injection and no prominent alterations in the behaviour, activities, and appearance were noted.

2-2. Effect of NDS in Novel Object Recognition Test

The results obtained with the novel object recognition test are shown in Figure 2. In the sample phase, no significant difference was observed in the total time spent exploring both objects (Figure 2A). Furthermore, no significant difference was found between NDS and scopolamine-treated groups in exploring each identical object.

However, in the test phase, the group treated with the NDS at the doses of 100 and 200 mg/kg + scopolamine (1 mg/kg) and donepezil (2 mg/kg) and scopolamine (1 mg/kg) spent a significantly longer time with the novel object than

the familiar one compared with the scopolamine-treated group only (* $p < 0.05$; ** $p < 0 0.01$ respectively) (Figure 2B).

A significant dose-dependent increase was noted in the percentage discrimination index (% DI) with NDS at the doses of 100, 150, and 200 mg/kg. There was also a significant dose-dependent increase in the percentage discrimination index (% DI) with NDS at 100 and 200 mg/kg ($p < 0.05$; $p < 0 0.01$, respectively) compared with scopolamine. Donepezil (2 mg/kg) also caused a significant increase in the % DI ($p < 0.01$) (Figure 2C).

2-3. Effect of NDS in Morris Water Maze Test (MWMT)

Morris's water maze test is profound in assessing dysfunction in spatial memory and learning. All groups' mice performance was improved through the training phase, as shown by the reduced escape latency over the consecutive days (Figure 3A). A significant modification was found in mean latency between the training days ($F (4, 116) = 34.32, p < 0.001$) and between treatments ($F (5, 119) = 26.31, p < 0.001$). Still, there was no interaction observed between the training day and the groups ($F (20, 016) = 0.738, p > 0.05$), proposing that alterations among the different groups were dependent on treatment.

Table 3. DPPH assay activity results. Values represent means ± standard error of mean of n = 3.

Sample	Concentration (µg/mL)	DPPH Scavenging Mean (%) ± SEM (%)	IC50 (µg/mL)
Sialic Acid	1000 µg/mL	74.98 ± 0.29	220
	500 µg/mL	64.53 ± 0.18	
	250 µg/mL	44.9 ± 0.25	
	125 µg/mL	39.68 ± 0.19	
	62.5 µg/mL	34.03 ± 0.19	
	31.25 µg/mL	29.87 ± 0.18	
Ascorbic acid	1000 µg/mL	86.78 ± 0.40	63
	500 µg/mL	74.9 ± 0.35	
	250 µg/mL	67.98 ± 0.37	
	125 µg/mL	52.87 ± 0.86	
	62.5 µg/mL	46.95 ± 0.18	
	31.25 µg/mL	30.29 ± 1.10	

Table 4. AChE assay activity results. Values represent means ± standard error of mean of n = 3.

Sample	Concentration (µg/mL)	AChEI Scavenging Mean (%) ± SEM	IC50 (µg/mL)
Sialic Acid	1000 µg/mL	86.91 ± 0.43	55
	500 µg/mL	84.21 ± 0.32	
	250 µg/mL	76.98 ± 0.35	
	125 µg/mL	61.89 ± 0.66	

	62.5 µg/mL	48.88 ± 0.54	
	31.25 µg/mL	30.73 ± 0.33	
Donepezil	1000 µg/mL	96.08 ± 0.43	60
	500 µg/mL	94.03 ± 0.32	
	250 µg/mL	84.08 ± 0.23	
	125 µg/mL	72.09 ± 0.16	
	62.5 µg/mL	49.06 ± 0.15	
	31.25 µg/mL	19.50 ± 0.23	

Table 5. BuChE assay activity results. Values represent means ± standard error of mean of n = 3.

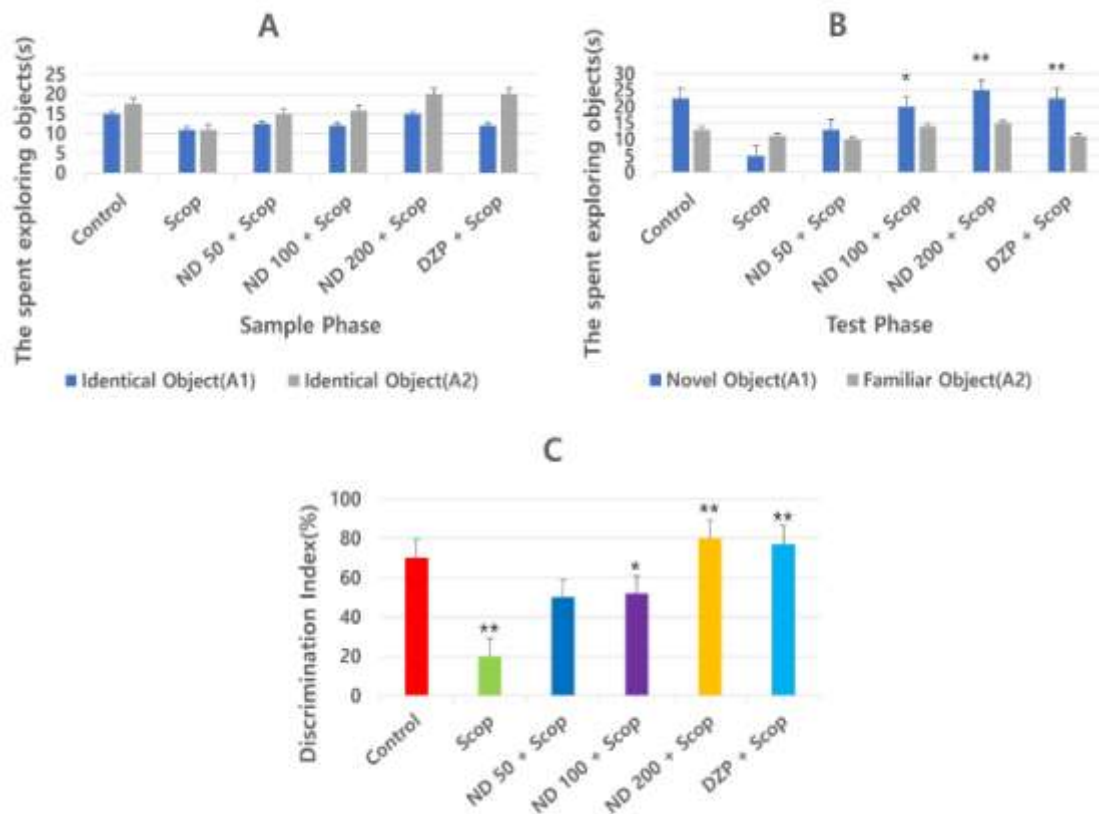| Sample	Concentration (µg/mL)	BuChEI Scavenging Mean (%) ± SEM	IC50 (µg/mL)
Sialic Acid	1000 µg/mL	79.8 ± 0.39	52
	500 µg/mL	68.9 ± 0.30	
	250 µg/mL	61.73 ± 0.39	
	125 µg/mL	52.68 ± 0.69	
	62.5 µg/mL	37.98 ± 0.38	
	31.25 µg/mL	24.93 ± 0.29	
Donepezil	1000 µg/mL	93.39 ± 0.48	67
	500 µg/mL	88.95 ± 0.30	
	250 µg/mL	79.9 ± 0.29	
	125 µg/mL	68.9 ± 0.58	
	62.5 µg/mL	41.34 ± 0.48	
	31.25 µg/mL	24.93 ± 0.32	

Figure 2. Effect of NDS (50, 100, and 200 mg/kg) in a short-term memory loss NORT. (A) exploration duration in sample phase, (B) exploration duration in test phase, (C) the discrimination index (DI). * $p < 0.01$. vs. the control group and * p, < 0.05, ** p. < 0.01 vs. the scopolamine 1 mg/kg.

The hidden platform–swimming trials showed their escape latency, and their path length in the scopolamine-induced group animal/mice was significantly increased when compared with the control group on the fifth and sixth testing days ($p < 0.01$, n = 8; Figure 3A, B). In contrast, treatment with the NDS significantly decreased the escape latency and path length at the doses of 50, 100, and 200 mg/kg on the fifth and sixth day ($p < 0.05$, $p < 0.01$, n = 8) when compared with the scopolamine treated group. Similar effects were observed with donepezil at 2 mg/kg. On the sixth day after the last training trial, the mice were subjected to the probe test, in which the platform was removed from the pool to conclude whether the mice could recall the platform's location. In comparison with the control group animal/mice, the spatial memory of the scopolamine-induced mice was significantly damaged, passing through the less target zone where the hidden platform was located, compared with the control group (Figure 3C, ### $p < 0.05$). In contrast, the NDS and donepezil-treated groups spent significantly less time in the target quadrant than the scopolamine-treated amnesic animals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 8).

2-4. Assessment of NDS inElevated PlusMaze Test (EPM)

The transfer latency (TL) on a second day reveals the retaining of the learned task or the memory. Scopolamine (1 mg/kg, i.p.) given before the training significantly increased ($p < 0.01$) the transfer latency (TL) on the first and second day, showing impairment in both learning and cognition or memory compared with the control (* $p < 0.05$). All animals treated with the NDS in doses of (50, 100, and 200 mg/kg; i.p.) revealed a dose-dependent decrease in TL on the sixth day compared with the scopolamine group (learning) (# $p < 0.05$, ## $p < 0.01$) (Figure 4). Donepezil also caused a significant decrease in the TL compared with the scopolamine-treated group. NDS and donepezil also significantly decreased the TL time on the seventh day (memory) of drug administration compared with the scopolamine-treated group, indicating a memory-enhancing effect (# $p < 0.05$, ## $p < 0.01$).
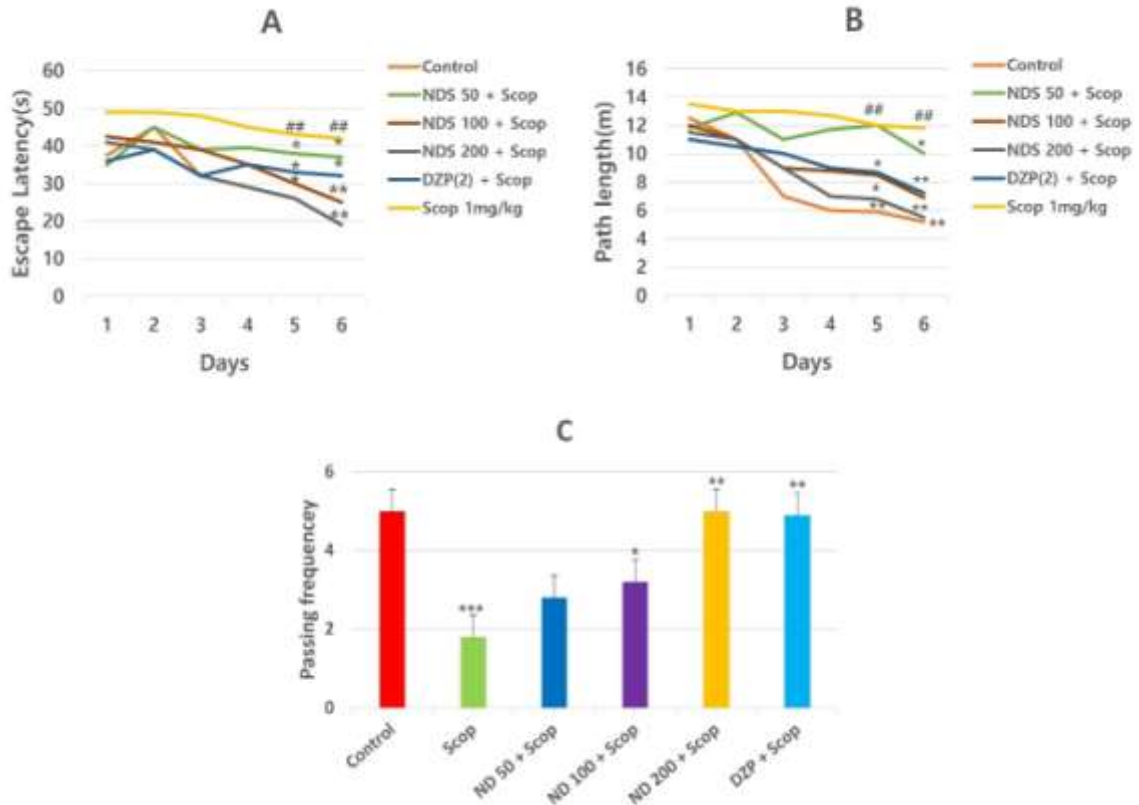
Figure 3. Effects of the NDS (50, 100, and 200 mg/kg) on scopolamine-induced cognitive/memory impairment in mice. Memory impairment was carried out by the administration of scopolamine at the dose of (1 mg/kg, i.p). (A) Mice escape latency in the hidden platform (tests for 6 consecutive days); (B) mice path length in the hidden platform tests (6 consecutive days); (C) mice frequency passing through hidden platform location. * $p < 0.05$; ** $p < 0.01$; against the scopolamine administered group. ## $p < 0.01$, ### $p < 0.05$; against the control group.
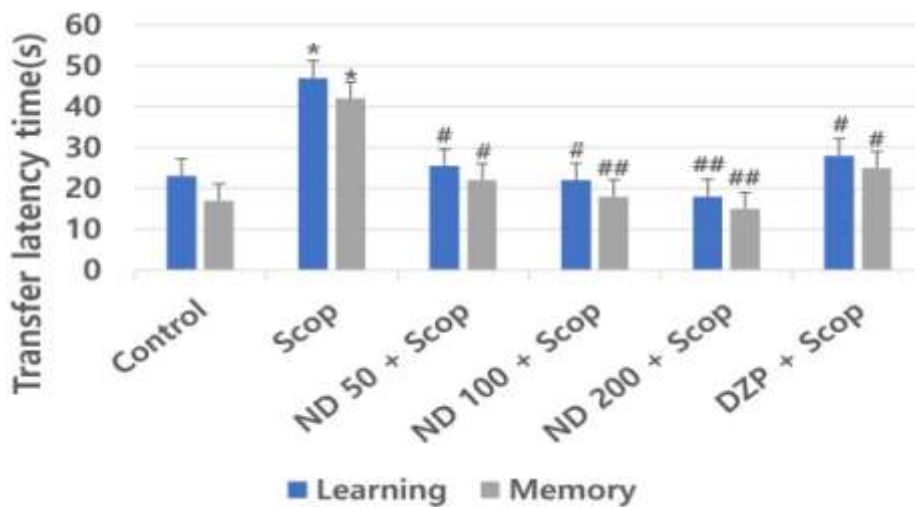

Figure 4. Effect of NDS on learning and memory in EPM. Donepezil (2 mg/kg) was used as a standard drug. * $p < 0.01$ compared with control animals, # $p < 0.0$, ## $p < 0.01$ compared with scopolamine-treated animals. Values are mean ± SEM (n = 8), ANOVA followed by Tukey–Kramer test.

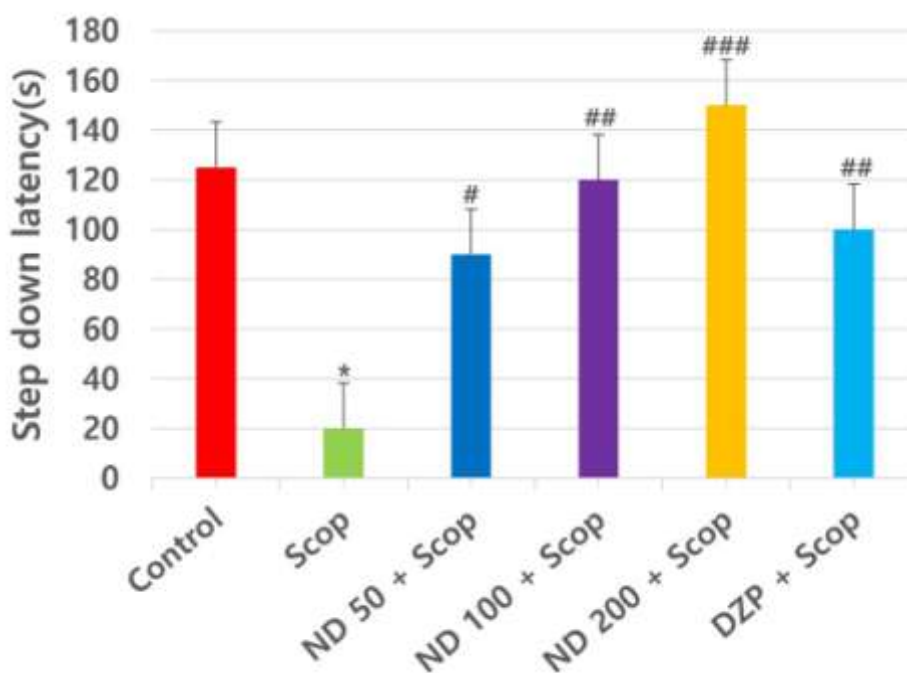**Figure 5.**Effect of NDS on the SDL in passive avoidance test. *p < 0.05 compared with scopolamine, # p < 0.05, ## p < 0.01, ### p < 0.001 compared with scopolamine. Value ranges are mean ± SEM (n = 8), ANOVA; followed by the Tukey–Kramer test. Donepezil (2 mg/kg) was used as a standard reference drug.

On the first day, TL was noted (training session) for each animal. The animal was permitted to discover the elevated plus maze for another two minutes and then reverted to its home cage. Twenty-four hours later (trial day), the animals were tested again for transfer latency. The transfer latency was measured when the mouse or animal moved from the open arms into the closed arms with all four legs. A substantial decrease in the TL indicated an improvement in cognition or memory.

2-5. Effect of NDS on Passive Avoidance Test (PAT)
Step-down latency (SDL) on the second day echoed the long-term memory of the animals/mice. Various doses of the NDS (50, 100, and 200 mg/kg) administered to the mice showed a dose-dependent elevation in the SDL value range; they significantly overturned the memory deficits due to the amnesia induced by scopolamine (p < 0.05, p < 0.01, p < 0.001) compared with the scopolamine-treated group. (Figure 5). Animals treated with donepezil (2 mg/kg) also showed significant enhancement in scopolamine-induced memory deficits (## p < 0.01).

IV. DISCUSSION
Several authors have described sialic acid as having an inhibitory action on the acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) [13,14,36,37] that results in the elevation of long-term recognition or memory. Thus, the agents having an inhibitory effect on these enzymes may be beneficial for the treatment of memory dysfunctions, including Alzheimer 's disease. In the treatment of Alzheimer 's disorder and other dementia disorders, choline esterase reversible inhibitors have been used as a cognitive enhancer.

Sialic acid has been shown to cross the blood–brain barrier, stabilizing brain activities and withdrawing the memory and cognitive weakness effect of scopolamine [13,14,38]. In this research study, NDS, a sialic acid isolated from the EBN, inhibited the AChE and BuChE with the IC50 of 55 and 52 µg/mL, respectively. The IC50 value ranges of the sialic acid and donepezil were not significantly different against the acetylcholinesterase (AChE) and the butyrylcholinesterase (BuChE). In age-related cognitive and memory deficits, oxygen free radicals are involved, and they may be accountable for memory deterioration in Alzheimer's disease that occurs in old age people [13,14].Antioxidants are

known to counteract the attack of free radicals and may be beneficial to increase cognitive or memory functions in Alzheimer's disease. Currently, research has been focussed on the use of antioxidants from natural sources to protect the human body, especially the oxidative damage of the brain tissues caused by free radicals. Sialic acid, as apparent from the findings of the current study, indicates antioxidant potential in the DPPH and the ABTS free radical scavenging assay.

In experimental animals and humans, scopolamine causes memory impairment, causing impairment in giving out information, keeping consideration, and gaining novel knowledge [39]. Thus, in test subjects, scopolamine-prompted amnesia is a generally referred model for the simulation of human dementia as an imperative and investigational Alzheimer's disease [40].

Scopolamine-induced memory impairment is most commonly used to assess the antiamnesic effect potential of therapeutic substances in experimental animals. For example, ginkgo ketoester tablets (GT) and donepezil—a clinically used combination for the treatment of AD—have been evaluated and validated using the scopolamine-induced memory impairment model [41]. Similarly, other agents have also been found to possess significant improvements in memory impairment in this model [42]. Scopolamine induces learning and memory deficits by blocking cholinergic signalling [43].

In this study, to confirm the memory-enhancing effect of sialic acid on scopolamine-induced memory impairment in mice, sialic acid-bile acid conjugated micelle nano particles (NDS) suggested in a previous study were prepared, and these were ingested as NDS. In some cases, they were transported to the brain to exert direct memory-enhancing effects, and tests such as the Morris water maze, elevated plus maze, passive avoidance, and novel object recognition were performed.The escape latency of repeated trial tests for six days and the time spent in the target quadrant in the probe test were investigated in the Morris water maze test. The results obtained in the MWM tests indicated that NDS significantly attenuated scopolamine-induced memory impairment and improved long-term spatial memory in the MWM test.Similarly, in EPM, NDS reversed scopolamine-induced decreases in transfer latency, thus suggesting improvement in learning and memory. The passive avoidance test is based on the principle that the animal learns to avoid an aversive stimulus such as an electrical foot shock. Administration of NDS ameliorated the scopolamine-induced memory deficit by elevating the SDL in the passive avoidance test, suggesting that it may improve long-term memory. The results obtained in the NORT were consistent with the findings of the MWM, EPM, and passive avoidance tests. NDS caused a dose-dependent increase in the exploration time of the novel object in the test phase and discrimination index, whereas scopolamine failed the novelty test, indicating that NDS prevents the memory impairment induced by scopolamine.

The memory-enhancing effects of NDS observed in this study may be due to the inhibition of the AChE and BuChE enzymes involved in the degradation of ACh. Thus, agents inhibiting these enzymes can cause an elevation in the level of ACh. Furthermore, the effects observed in this study were similar to donepezil, a standard AChE inhibitor, suggesting that the mechanism (s) of action involved in the effect of NDS may be identical to the mechanisms of donepezil [44].

V. CONCLUSIONS

In conclusion, NDS exerts antioxidant effects, inhibits choline esterase enzymes, and exhibits significant antiamnesic effects in a battery of in vivo behavioural paradigms. Thus, NDS could be a useful novel agent for the development of therapeutic alternatives (as an alternative food and drugs using sialic acid) for treating memory dysfunction in Alzheimer 's disease.

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