

ORIGINAL RESEARCH ARTICLE

Anoectochilus roxburghii aqueous extract improves scopolamine-induced cognitive impairment in mice: Associations with gut microbiota and fecal metabolomics

Hui Yin^{1†}, Jiayi Zhang^{1†}, Tao Yang^{2†}, Min Feng¹, Ting Yang¹, Ning Zhang¹, Feiyue Cheng¹, Xiao Zhang¹, Yonghong Liang¹, Ming Yang¹, Chang Peng^{2*} , and Haiyan Zhang^{1,3*} 

¹State Key Laboratory for the Modernization of Classical and Famous Prescriptions of Chinese Medicine, Jiangxi University of Chinese Medicine, Nanchang, Jiangxi, China

²Discipline of Chinese and Western Integrative Medicine, College of Traditional Chinese Medicine, Jiangxi University of Chinese Medicine, Nanchang, Jiangxi, China

³Jiangzhong Pharmaceutical Co., Ltd., Nanchang, Jiangxi, China

[†]These authors contributed equally to this work.

*Corresponding authors:

Haiyan Zhang
(20070930@jxutcm.edu.cn);
Chang Peng
(pengchang@jxutcm.edu.cn)

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Abstract

Cognitive dysfunction (CD) associated with cholinergic deficits is a hallmark of Alzheimer's disease (AD) and related disorders. The natural edible herb *Anoectochilus roxburghii* has various pharmacological activities, including anti-inflammatory, antioxidant, and neuroprotective effects. However, the underlying mechanism by which *A. roxburghii* alleviates CD via the microbiota–gut–brain (MGB) axis remains unclear. In this study, a scopolamine-induced CD mouse model was used to evaluate the therapeutic effect of *A. roxburghii* aqueous extract (ARAE) through behavioral tests, intestinal and brain histopathology, ELISA quantitative assays, gut microbiota sequencing, and metabolomics analysis. ARAE effectively improved cognitive and memory abilities in the mouse model, as demonstrated by significantly reduced escape latency and increased crossings over the target platform in the Morris water maze test. Mechanistic studies elucidated that ARAE effectively reduced the release of inflammatory factors, alleviated oxidative stress, and corrected cholinergic imbalance. Furthermore, ARAE reshaped the gut microbiota and upregulated the expression of tight junction proteins claudin-1, occludin, and zonula occludens-1 (ZO-1), as well as mucin-2 (MUC-2), thereby contributing to the integrity of the intestinal barrier. Meanwhile, ARAE increased the levels of propionic acid and butyric acid, concurrently restoring the disordered host–microbiota co-metabolism by targeting the tryptophan metabolism pathway. In summary, ARAE alleviates scopolamine-induced CD via the MGB axis through anti-inflammatory, antioxidant, cholinergic, and gut barrier-enhancing mechanisms. These findings indicate that *A. roxburghii* may be a promising candidate for the treatment of CD associated with cholinergic dysfunction and lay a foundation for further research in more comprehensive AD models.

Keywords: *Anoectochilus roxburghii*; Alzheimer's disease; Cognitive dysfunction; Gut microbiota; Metabolomics

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease, characterized by progressive cognitive dysfunction (CD), memory loss, and behavioral and psychological symptoms, which severely impact quality of life and exert a significant burden on global healthcare systems.¹ Although cholinesterase inhibitors, such as donepezil (DON), rivastigmine, and galantamine, and N-methyl-D-aspartic acid (NMDA) receptor antagonists, such as memantine, are currently used to alleviate symptoms, there are still no curative treatments capable of halting the progression of AD.^{2,3} This has made the exploration of new therapeutic strategies a major focus of current research. In recent years, a growing body of evidence suggests that the onset of neurodegenerative diseases is not only associated with traditional pathological mechanisms but may also be closely linked to dysbiosis of the gut microbiota.^{4,5} Research on the microbiota–gut–brain (MGB) axis offers a novel perspective, revealing a potential link between gut microbiota dysbiosis and cognitive decline.^{6,7} In this context, disruption of the intestinal barrier is considered a key mechanism leading to systemic inflammatory responses and neurodegenerative changes. Therefore, restoring intestinal barrier function may be a new approach for the treatment of neurodegenerative diseases.

Anoectochilus roxburghii (Wall.) Lindl. (*Jin xian lian*), a native medicinal herb of Fujian province, is a well-documented dual-purpose plant with medicinal and edible values in traditional folk remedies. Rich in various nutrients, it is commonly used as a dietary supplement and has been clinically applied to treat inflammatory conditions such as type 2 diabetes and hepatitis.⁸ Modern pharmacological studies indicate that *A. roxburghii* extracts possess multiple biological activities, including antioxidant, anti-inflammatory, and neuroprotective effects, suggesting its potential value in the treatment of AD.^{9–11} Notably, recent studies have found that *A. roxburghii* extract can improve learning and memory abilities in animal models of CD, further corroborating its neuroprotective potential.^{12,13} However, existing mechanistic studies on *A. roxburghii* are largely limited to its antioxidative stress activity, and experimental evidence supporting its neuroprotective effects via modulation of the MGB axis and restoration of intestinal barrier integrity remains scarce.

The integrity of the intestinal barrier primarily depends on the interaction between tight junction (TJ) proteins and the mucus layer, which together maintain the barrier function of the intestine and prevent harmful substances from entering the bloodstream.^{14–16} When this barrier is compromised, a leaky gut forms, allowing pro-inflammatory

substances, such as lipopolysaccharides, to translocate into the bloodstream, thereby triggering systemic low-grade inflammation.¹⁷ Systemic inflammation can further breach the blood–brain barrier, activating microglia to release large amounts of inflammatory mediators and reactive oxygen species, thereby promoting β -amyloid deposition and tau protein hyperphosphorylation, ultimately causing neuronal dysfunction and loss.¹⁸ Recent studies have indicated that herbal medicines and their active components can indirectly repair the intestinal barrier by reshaping the gut microbiota, increasing beneficial metabolites such as short-chain fatty acids (SCFAs), thereby furnishing colonic epithelial cells with energy and upregulating the expression of TJ proteins.^{19–21} However, it has not yet been conclusively demonstrated whether *A. roxburghii* can regulate the intestinal barrier through similar mechanisms and further improve cognitive function. Therefore, we hypothesized that *A. roxburghii* may indirectly influence central nervous system function by directly enhancing the integrity of the intestinal barrier, with a mechanism of action involving upregulation of intestinal TJ protein expression.

Against this backdrop, this study aims to use a scopolamine (SCOP)-induced mouse model of CD, combined with behavioral, pathological, and omics-based techniques, to preliminarily investigate the modulatory effects of *A. roxburghii* aqueous extract (ARAE) on the MGB axis and the intestinal barrier. Through this study, we hope to provide new evidence supporting *A. roxburghii* as a potential candidate for ameliorating CD related to cholinergic dysfunction, and to offer new insights into the application of traditional herbal medicines in the treatment of neurodegenerative diseases.

2. Materials and methods

2.1. Materials

The following chemicals and reagents were used: hydrochloride DON tablets (705240702, Ouyi Pharmaceutical, China), scopolamine hydrobromide (SCOP; purity $\geq$ 98%, C17276010, Macklin Biochemical, China), anhydrous glucose (purity $\geq$ 99%; D810594, Macklin Biochemical), rutin (purity $\geq$ 98%; B20771, Yuanye Biotechnology, China), kinsenoside (purity $\geq$ 98%; B29395, Yuanye Biotechnology), mouse acetylcholine (ACh) enzyme-linked immunosorbent assay (ELISA) kits (MM-0520M1, Meimian, China), mouse acetylcholinesterase (AChE) ELISA kits (MM-0674M2, Meimian), mouse choline acetyltransferase (ChAT) ELISA kits (MM-44492M1, Meimian), mouse tumor necrosis factor- α (TNF- α) ELISA kits (E-EL-M3063, Eritai Biotechnology, China), mouse interleukin-1 β (IL-1 β) ELISA kits (E-EL-M0037, Eritai Biotechnology), mouse

IL-6 ELISA kits (E-EL-M0044, Eritai Biotechnology), malondialdehyde (MDA) assay kit (A003-1-1, Jiancheng Biological Engineering, China), superoxide dismutase (SOD) assay kit (A001-3-2, Jiancheng Biological Engineering), bicinchoninic acid (BCA) protein assay kit (P0012, Beyotime Biotechnology, China), claudin-1 (1:500; GB15032, Servicebio, China), mucin-2 (MUC-2, 1:500; GB11344, Servicebio), occludin (1:500; GB111401, Servicebio), zonula occludens-1 (ZO-1, 1:500; GB151981, Servicebio), horseradish peroxidase (HRP)-conjugated rabbit anti-goat immunoglobulin G (IgG) (H + L) (1:200; GB23204, Servicebio), and cyanine 3 (Cy3)-conjugated goat anti-rabbit IgG (H + L) (1:300; GB21303, Servicebio).

2.2. Preparation of *Anoectochilus roxburghii* aqueous extract

Dried *A. roxburghii* herbal slices were purchased from Shangrao Guangxin Yuntian Agriculture Co., Ltd. (20230715, China). The botanical material was authenticated by Associate Professor Yonghong Liang of Jiangxi University of Chinese Medicine as the dried whole herb of *A. roxburghii* (Wall.) Lindl. (Orchidaceae). The preparation process was as follows: 200 g of pulverized herbal powder was accurately weighed and suspended in distilled water at a solid–liquid ratio of 1:10 (w/v). The mixture was soaked for 30 min and then decocted for 1 h. After the first filtration, the residual herbal dregs were supplemented with 8-fold volumes of distilled water for a second 1-h decoction, followed by another filtration step. The two filtrates were then combined, concentrated by rotary evaporation at 60 °C, and finally freeze-dried to obtain the ARAE, which was stored in a vacuum desiccator for later use.

2.3. Liquid chromatography–mass spectrometry analysis of ARAE

The chemical composition of ARAE was analyzed using high-performance liquid chromatography (LC) coupled with a triple quadrupole mass spectrometer (HPLC-TQ-MS; Vanquish Flex/SCIEX TRIPLE QUAD, Thermo Fisher Scientific, United States of America [USA]). The parameters were: (i) chromatographic column: Evosphere AQUA C18 column (2.1 mm × 100 mm, 1.7 μm); (ii) mobile phase: 0.1% formic acid aqueous solution (A) and 0.1% formic acid acetonitrile (B); (iii) gradient elution: 0 min, 5% B; 2 min, 5% B; 42 min, 95% B; 47 min, 95% B; 47.1 min, 5% B; 50 min, 5% B; (iv) flow rate: 0.3 mL/min; (v) column temperature: 40 °C; and (vi) injection volume: 2 μL.

The mass spectrometry (MS) used an electrospray ionization (ESI) source, with a full-scan, data-dependent secondary scan (Full MS-ddMS2), a scanning range of

m/z 100–1,500, a first-level MS resolution of Full Scan 70,000, and a second-level MS resolution of tandem mass spectrometry (MS/MS) 17,500. The collision energy was adjusted to 20–50 eV; the sheath gas flow rate, spray voltage, and capillary temperature were set to 25 mL/min, 3.5 kV and 320 °C, respectively.

The ARAE powder was subjected to ultrasonic extraction using 60% ethanol for 45 min. The resultant mixture was centrifuged at 13,000 rpm and 4 °C for 15 min. The supernatant was filtered through a 0.22 μm microporous membrane and transferred into an autosampler vial prior to instrumental analysis.

2.4. Determination of polysaccharide and total flavonoid content

The ARAE samples were analyzed for polysaccharide and total flavonoid content using the phenol–sulfuric acid method and the aluminum nitrate–sodium nitrite colorimetric method, with detection wavelengths of 488 nm and 470 nm, respectively.^{22,23}

2.5. Determination of the content of kinsenoside

Chromatographic separation was carried out on a Wondasil C18 Superb column (4.6 mm × 250 mm, 5 μm; Shimadzu, Japan). Isocratic elution was adopted using a mobile phase consisting of methanol–water (10:90, v/v) at a flow rate of 0.5 mL/min, with the column temperature maintained at 30 °C. The nebulization temperature of the evaporative light scattering detector was 70 °C, with a carrier gas flow rate of 2.5 L/min. The injection volume was 10 μL.

2.6. Animal information

A total of 60 specific pathogen-free (SPF)-grade male C57BL/6J mice (20 ± 2 g) were purchased from Tianqin Biotechnology Co., Ltd. (China). All animals were housed in the animal facility at Jiangxi University of Chinese Medicine under controlled conditions of 50–60% relative humidity, 23 ± 2 °C, and a natural light–dark cycle. All animal experiments adhered to the ethical standards for animal research (Ethics No.: HLK-20250428-002).

2.7. Animal grouping and modeling methods

After being housed under standard conditions in the animal facility for one week, the mice were randomly assigned to the following groups ($n = 10/\text{group}$): control group (CON), SCOP model group (SCOP), DON group (DON), low-dose ARAE group (ARAE-L), medium-dose ARAE group (ARAE-M), and high-dose ARAE group (ARAE-H). The drug-treated mice were administered 3 mg/kg DON, 0.3, 0.6, or 1.2 g/kg ARAE, respectively, via gavage, while the CON and SCOP groups received an equal volume of 0.9% saline via gavage. Treatments were given once daily for 28

days. Two weeks after administration, all groups except the CON group received 3 mg/kg SCOP via intraperitoneal injection once daily for 14 days. All solutions were prepared fresh every three days and stored in the dark at 4 °C to ensure solution stability. No animals were excluded during the experiment. All behavioral scoring and image analyses were conducted by an experimenter blinded to the group allocation.

2.8. Open field test

On the 21st day, an open field test (OFT) was conducted to assess the spontaneous activity and anxiety-like behavior of the mice.²⁴ Eight mice per group were used for behavioral testing. Behavioral testing was performed in a black opaque square plastic box (50 cm × 50 cm × 40 cm). Each mouse was gently positioned at a box corner and given 5 min of unrestricted exploration. The experimental process was recorded by a camera, and automatic analysis was performed with ANY-maze software (version 6.34, Stoelting Co., USA). After each mouse completed the experiment, the box was thoroughly cleaned.

2.9. Morris water maze test

Starting on the 22nd day, the Morris water maze (MWM) was conducted to assess the spatial learning and memory abilities of the mice.²⁵ The same eight mice per group from the OFT were used. The system consisted of a circular pool with a diameter of 100 cm, filled with opaque white water at a depth of approximately 30 cm and a temperature of 22–25 °C. The pool was divided into four quadrants, with a transparent circular platform submerged 1 cm beneath the water surface at the center of quadrant IV. Lighting and curtains were adjusted to minimize environmental interference during the experiment. Mice were dried and returned to their cages after each trial. The experiment lasted six days and included both a training phase and a spatial exploration phase:

- (i) Training phase: From day 1 to day 5, mice were released facing the pool wall from distinct starting quadrants to search for the submerged platform. Training sessions were conducted twice daily, with a 1-h interval between sessions, and escape latency was recorded. Animals that did not find the platform within 60 s were manually guided to it and permitted to rest for 5 s.
- (ii) Spatial exploration phase: On day 6, the hidden platform was removed. Each mouse was placed in quadrant II and allowed to swim freely for 60 s. The recorded indicators include the number of platform crossings, the average swimming speed, and the duration spent in the target quadrant. The swimming trajectory was captured by an overhead camera and

analyzed using the ANY-maze behavior tracking software.

2.10. Hematoxylin-eosin staining

The tissue collection was performed the day after the behavioral tests concluded. Three mice from each group were randomly selected from the eight mice used for behavioral testing for histological analyses. Following heart perfusion with 0.9% saline and 4% paraformaldehyde, brain and colon tissues were collected. The colonic tissues were gently flushed with saline to remove fecal contents. All tissue samples were fixed in 4% paraformaldehyde for 48 h, then embedded in paraffin and sectioned to 5 µm thickness. The sections were deparaffinized, rehydrated, and stained with 2.10. hematoxylin-eosin (HE) for 30 min, followed by rinsing with distilled water. The sections were differentiated using a graded ethanol series (75%, 85%, and 95%), cleared, and mounted with neutral resin. Observations and image acquisition were performed under a light microscope (Eclipse E100, Nikon, Japan).

2.11. Nissl staining

Fixed brain tissue was retrieved from 4% paraformaldehyde solution, followed by dehydration, embedding, and sectioning. The sections were then deparaffinized and stained with toluidine blue. After staining, Nissl bodies appeared as deep blue granules, the cell nuclei appeared pale blue, and the background was light blue. Microscopic observation was performed to assess neuronal morphology.

2.12. Immunohistochemical analysis

Deparaffinized paraffin sections were rehydrated and heat-retrieved in ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0, 90 °C, 30 min). After H₂O₂ and bovine serum albumin (BSA) blocking, sections were incubated overnight at 4 °C with claudin-1, occludin, and ZO-1 primary antibodies, followed by HRP-labeled secondary antibodies (room temperature, 50 min). 3,3'-diaminobenzidine (DAB) was used as a chromogen, followed by counterstaining with hematoxylin to visualize nuclei. Slides were dehydrated, mounted, and observed under bright-field microscopy (ECLIPSE CI-L, Nikon, Japan). Five random fields per section were photographed, and positive staining area ratios were quantified via ImageJ (National Institutes of Health, USA).

2.13. Immunofluorescence staining

Paraffin slices were deparaffinized, rehydrated, and antigen-retrieved in citrate buffer (pH 6.0) for 30 min. Following BSA blocking, slices were incubated overnight at 4 °C with MUC-2 primary antibody, then Cy3-conjugated secondary antibody in dark conditions for 50

min, with 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstaining. Autofluorescence was eliminated before anti-fade mounting. Fluorescent images were obtained with a fluorescence microscope (Eclipse C1, Nikon, Japan), and fluorescence intensity was analyzed using ImageJ.

2.14. Determination of hippocampal and cortical ACh, AChE and ChAT contents via ELISA

Eight mice from each group (the same animals used for behavioral testing) were selected, and the bilateral hippocampal and cortical tissues were homogenized using a tissue grinder. The mixture was then centrifuged at 5,000 rpm for 10 min to obtain supernatants. The levels of ACh, AChE, and ChAT were quantified using commercial ELISA kits at 450 nm according to standard operating protocols.

2.15. Detection of inflammatory factors and oxidative stress markers

The hippocampal and cortical tissues were ground and processed, as referred to the ELISA kit manuals, to determine the levels of the inflammatory cytokines TNF- α , IL-1 β , and IL-6. The detection wavelength was 450 nm. Similarly, the levels of MDA and SOD were measured following the instructions of the respective assay kits, with detection wavelengths of 532 nm and 550 nm, respectively.

2.16. 16S ribosomal RNA sequencing

The 16S ribosomal RNA (rRNA) sequencing was performed with the assistance of Ouyi Biomedical Technology Co., Ltd (China). According to the prior pharmacodynamic outcomes, mice were randomly selected from the CON, SCOP, and ARAE-H groups for gut microbiota sequencing ($n = 6$ /group from the 8 behaviorally tested animals). Total genomic DNA was isolated from colon contents using commercial kits, and DNA integrity and concentration were verified by agarose electrophoresis and a NanoDrop 2000 (Thermo Fisher Scientific). The V3–V4 hypervariable regions of 16S rRNA were amplified using barcoded primers 343F-TACGGRAGGCAGCAG and 798R-AGGGTATCTAATCCT. Purified polymerase chain reaction (PCR) amplicons were quantified with Qubit (Thermo Fisher Scientific), normalized by concentration, and sequenced on the Illumina MiSeq platform (USA).

Raw sequencing reads were preprocessed in QIIME2 (2020.11, USA). Primer sequences were removed via cutadapt, followed by DADA2-based quality filtering, denoising, read merging, and chimera elimination to generate amplicon sequence variant (ASV) feature tables and representative sequences. Taxonomic annotation of representative ASVs was performed against the Silva 138 database using q2-feature-classifier. Alpha and Beta diversity metrics were calculated to evaluate gut microbial

diversity. Principal coordinates analysis (PCoA) visualized intergroup discrepancies in community composition, whereas linear discriminant analysis effect size (LEfSe) analysis screened significantly different taxa across groups.

2.17. Untargeted metabolomics analysis

Fecal samples from six mice per CON, SCOP and ARAE-H group were subjected to untargeted LC-MS metabolomics detection. Metabolites were extracted and measured with an ACQUITY UPLC HSS T3 column (100 mm $\times$ 2.1 mm, 1.8 μ m; Waters Corporation, USA). Mobile phases consisted of 0.1% aqueous formic acid (A) and acetonitrile (B), eluted under a multi-segment gradient at a flow rate of 0.35 mL/min; injection volume was 3.0 μ L, and column temperature was maintained at 45 °C. Dual positive/negative ESI modes were adopted for MS acquisition, with corresponding spray voltages of 3,800 V and –3,600 V. Ion transfer tube and capillary temperatures were set to 350 °C and 320 °C, respectively, with sheath gas at 35 arb, an auxiliary gas at 8 arb, and a m/z scanning range of 70–1,050. Raw chromatogram data were processed via XCMS v4.6.3 for subsequent metabolomic profiling.

2.18. Determination of short-chain fatty acid levels

Qualitative and quantitative analyses of fecal metabolites from the CON, SCOP, and ARAE groups were conducted using ultra-high-performance LC-ESI-MS/MS. Fecal samples were collected from the same mice used in the untargeted metabolomics experiment. Chromatographic detection was carried out with a mobile phase of 0.1% aqueous formic acid and acetonitrile-methanol mixture (2:1), with a flow rate of 0.35 mL/min, injection volume of 1 μ L, and column temperature of 40 °C. A gradient elution procedure was applied for metabolite separation. For MS detection in negative-ion mode, the spray voltage was –4,500 V, and the ion source temperature was 450 °C.

2.19. Statistical analysis

All statistical analyses and graph generation were conducted using GraphPad Prism 10.1.2 (GraphPad Software, USA). Data are presented as mean $\pm$ standard deviation (SD). Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA) with Tukey's post hoc test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Composition of ARAE

To ensure the quality of the extract, LC-MS was first used to identify the main chemical constituents of ARAE, yielding 45 compounds (Table 1). The base peak chromatograms in

Table 1. Detection and characterization of phytochemical compounds in ARAE using LC-MS in positive- and negative-ionization modes

No.	Compound	Molecular formula	RT (min)	Experimental value (<i>m/z</i>)	Theoretical value (<i>m/z</i>)	Ion
1	Kinsenoside	C ₁₀ H ₁₆ O ₈	1.24	265.1211	265.0918	[M+H] ⁺
2	Uridine	C ₉ H ₁₂ N ₂ O ₆	3.35	245.1290	245.0768	[M+H] ⁺
3	Gallic acid	C ₇ H ₆ O ₅	3.43	171.0292	171.0288	[M+H] ⁺
4	Adenine	C ₅ H ₅ N ₅	3.48	136.0619	136.0617	[M+H] ⁺
5	<i>p</i> -hydroxy benzoic acid	C ₇ H ₆ O ₃	3.57	137.1075	137.0224	[M-H] ⁻
6	Phenylalanine	C ₉ H ₁₀ NO ₂	3.94	163.0506	163.0639	[M-H] ⁻
7	Coumaric acid	C ₉ H ₈ O ₃	3.94	163.0502	163.0400	[M-H] ⁻
8	Trigonelline	C ₇ H ₇ NO ₂	3.95	138.0273	138.0549	[M+H] ⁺
9	Methyl-quercetin	C ₁₆ H ₁₁ O ₇	4.27	314.1599	314.0432	[M-H] ⁻
10	Hypoxanthine	C ₅ H ₄ N ₄ O	4.61	137.0444	137.0457	[M+H] ⁺
11	Tyrosine	C ₉ H ₁₁ NO ₃	4.68	180.1024	180.0666	[M-H] ⁻
12	Aceto-vanillone	C ₉ H ₁₀ O ₃	5.01	167.0724	167.0702	[M+H] ⁺
13	<i>bis</i> (2-ethylhexyl) phthalate	C ₂₄ H ₃₈ O ₄	5.44	391.1514	391.2847	[M+H] ⁺
14	Kaempferol-7-O-β-glucopyranoside	C ₂₁ H ₂₀ O ₁₁	5.63	447.1800	447.0933	[M-H] ⁻
15	Orientin	C ₂₁ H ₂₀ O ₁₁	5.83	447.1764	447.0933	[M-H] ⁻
16	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	6.11	353.1719	353.0878	[M-H] ⁻
17	3',4',7-trimethoxy-3,5-dihydroxyflavone	C ₁₈ H ₁₆ O ₇	6.49	343.1300	343.0823	[M-H] ⁻
18	Quercetin	C ₁₅ H ₁₀ O ₇	6.51	301.1202	301.0354	[M-H] ⁻
19	Methyl-quercetin-hexose	C ₂₂ H ₂₁ O ₁₂	6.59	476.1900	476.0960	[M-H] ⁻
20	Kaempferol-3-O-β-rutinoside	C ₂₁ H ₁₈ O ₁₂	6.6	461.2008	461.0725	[M-H] ⁻
21	Guanine	C ₅ H ₅ N ₅ O	6.76	152.0170	152.0566	[M+H] ⁺
22	Cytidine	C ₉ H ₁₃ N ₃ O ₅	7.04	244.0972	244.0927	[M+H] ⁺

(Cont'd...)

Table 1. (Continued)

No.	Compound	Molecular formula	RT (min)	Experimental value (<i>m/z</i>)	Theoretical value (<i>m/z</i>)	Ion
23	Quinic acid	C ₇ H ₁₂ O ₆	7.28	191.0815	191.0561	[M-H] ⁻
24	Caffeic acid	C ₉ H ₈ O ₄	7.63	179.1438	179.0349	[M-H] ⁻
25	Narcissoside	C ₂₈ H ₃₂ O ₁₆	7.69	623.1603	623.1618	[M-H] ⁻
26	Ionsine	C ₁₀ H ₁₂ N ₄ O ₅	7.74	269.1287	269.0880	[M+H] ⁺
27	Caffeic acid	C ₉ H ₈ O ₄	8.26	181.0496	181.0495	[M+H] ⁺
28	Quercetin-7-O-β-D-glucopyranoside	C ₂₁ H ₂₀ O ₁₂	8.27	463.2139	463.0882	[M-H] ⁻
29	Vanillic acid	C ₈ H ₈ O ₄	8.33	169.0764	169.0495	[M+H] ⁺
30	Gallic acid	C ₇ H ₆ O ₅	8.45	169.0761	169.0142	[M-H] ⁻
31	Palmitic acid	C ₁₆ H ₃₂ O ₂	8.47	255.1128	255.2329	[M-H] ⁻
32	Hyperin	C ₂₁ H ₂₀ O ₁₂	9.25	465.1670	465.1027	[M+H] ⁺
33	Gibberellic acid	C ₁₉ H ₂₂ O ₆	9.83	345.1820	345.1343	[M-H] ⁻
34	Quercetin-3'-O-glucoside	C ₂₁ H ₂₀ O ₁₂	10.72	463.2200	463.0882	[M-H] ⁻
35	Isoquercitrin	C ₂₁ H ₂₀ O ₁₂	10.72	463.2176	463.0881	[M-H] ⁻
36	5,7-Dihydroxy-3'-methoxyflavonol-3, 4'-O-β-D-diglucoside	C ₂₈ H ₃₂ O ₁₇	11.28	641.2400	641.1712	[M+H] ⁺
37	Isorhamnetin-7-O-glucoside	C ₂₂ H ₂₂ O ₁₂	11.48	479.1186	477.1038	[M-H] ⁻
38	Methyl-OCH3-2deoxyhexose	C ₂₉ H ₃₃ O ₁₄	11.65	604.2600	604.1798	[M-H] ⁻
39	Isorhamnetin 3-O-neohesperidoside	C ₂₈ H ₃₂ O ₁₆	14.51	625.2967	625.1763	[M+H] ⁺
40	Methyl palmitate	C ₁₇ H ₃₄ O ₂	14.95	269.2257	269.2486	[M-H] ⁻
41	Kinsendioside A	C ₁₆ H ₂₆ O ₁₃	15.59	425.2542	425.1301	[M-H] ⁻
42	Isorhamnetin 7-O-β-glucopyranoside	C ₂₂ H ₂₂ O ₁₂	16.93	477.2130	477.1038	[M-H] ⁻
43	<i>trans</i> -zeatin	C ₁₀ H ₁₃ N ₅ O	20.84	220.1473	220.1192	[M+H] ⁺
44	Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	25.34	268.2636	268.1040	[M+H] ⁺
45	Linoleic acid	C ₁₈ H ₃₂ O ₂	32.52	279.0933	279.2329	[M-H] ⁻

Abbreviations: ARAE: *Anoectochilus roxburghii* aqueous extract; LC-MS: Liquid chromatography-mass spectrometry; RT: Retention time.

positive-ion and negative-ion modes are presented in Figure 1A–1B. Subsequently, the contents of polysaccharides, total flavonoids, and kinsenoside in ARAE were determined to be 30.45%, 4.07%, and 27.38%, respectively (Table 2). The chromatograms of the kinsenoside standard and the sample are shown in Figure 1C–1D.

3.2. Effects of ARAE on anxiety behavior in the scopolamine-induced mouse model

Next, OFT was used to evaluate anxiety-like behavior in mice. The results showed that mice in the SCOP group preferred to move along the walls, with a significant reduction in the total distance traveled ($p < 0.0001$; Figure 2B) and in the number of entries into the central area ($p = 0.0059$; Figure 2C) compared with those in the CON group. In contrast, mice in the ARAE groups exhibited stronger exploratory behavior and more relaxed actions. Notably, the total distance moved in the ARAE-L ($p = 0.0111$), ARAE-M ($p = 0.0053$), and ARAE-H ($p = 0.0015$) groups increased significantly to varying degrees compared with the SCOP group.

3.3. Regulatory effects of ARAE on spatial learning and memory in the scopolamine-induced mouse model

The MWM test was adopted to assess ARAE's influence on spatial cognition in SCOP-induced mice. Over the five-day training phase, the escape latency of all groups of mice declined rapidly, whereas the SCOP group showed a negligible decline (Figure 2D). The escape latency of mice in the ARAE-M, ARAE-H, and DON groups showed the most significant decrease compared with those in the SCOP group (all $p < 0.0001$; Figure 2E).

No obvious intergroup differences in average swimming speed were observed during the probe trial, suggesting intact motor function in all animals (Figure 2F). Mice in

the SCOP group spent significantly less time in the target quadrant ($p = 0.0006$) and had fewer crossings over the platform location ($p = 0.0006$) compared with those in the CON group. In contrast, the ARAE-H group showed a significant increase in these measures ($p = 0.017$, 0.0061 , respectively) compared with the SCOP group (Figure 2G–2H). The swimming trajectory maps of the mice also support these findings: the SCOP group's trajectories were disorganized and lacked purpose, whereas the ARAE groups' trajectories exhibited more purposeful swimming and increased sensitivity to the location of the platform (Figure 2I).

3.4. Effects of ARAE on hippocampal pathological damage in the scopolamine-induced mouse model

To assess morphological alterations in the hippocampal tissue of mice, we performed HE staining. The hippocampal tissues of the CON group exhibited intact morphological structures, with neurons appearing rich, uniformly shaped, and arranged in an orderly manner (Figure 3A). In contrast, the SCOP group showed apparent damage to the hippocampal tissues, characterized by deep staining of nuclear condensation and disordered arrangement. The hippocampal tissues of the DON and ARAE groups appeared less damaged, with a qualitative improvement in neuronal morphology and arrangement.

3.5. Effects of ARAE on neuronal morphology in the hippocampal CA1 region of the scopolamine-induced mouse model

Nissl staining was employed to evaluate neuronal morphology in the cognitive-related areas of the hippocampus. Neuronal damage in the hippocampal CA1 region of the SCOP group was evident (Figure 3B), characterized by irregular morphology, disordered arrangement, nuclear condensation, widened intercellular

Table 2. The chemical constituent contents of ARAE

Component	Content (%)	Standard substance	Calibration curve equation	R ² value
Polysaccharide	30.45	Glucose	$y = 4.6316x - 0.0069$	0.9991
Total flavonoids	4.07	Rutin	$y = 1.7856x - 0.002$	0.9990
Kinsenoside	27.38	Kinsenoside	$y = 1.3052x + 3.1022$	0.9992

Abbreviation: ARAE: *Anoectochilus roxburghii* aqueous extract.

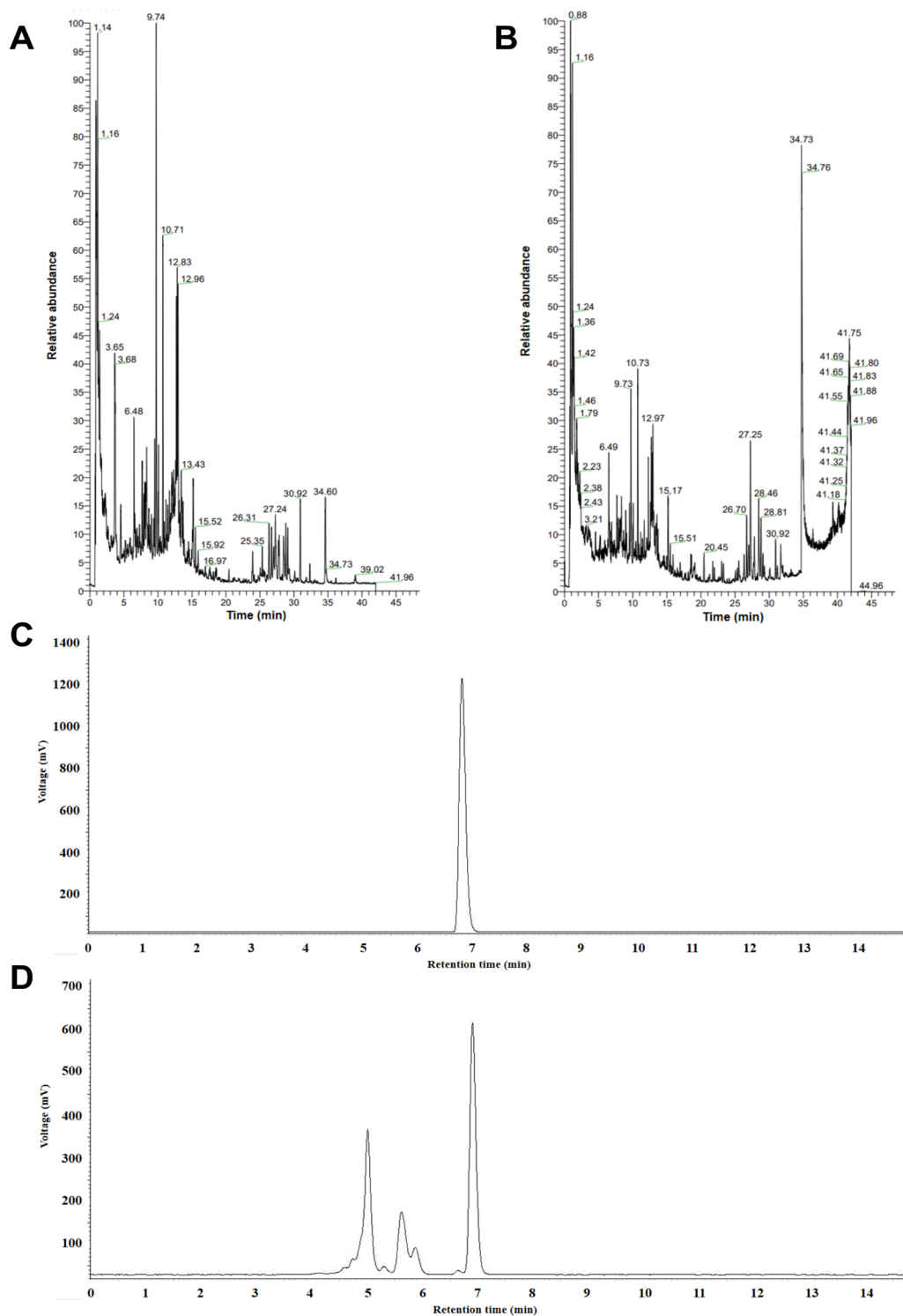


Figure 1. Quality control of *Anoectochilus roxburghii* aqueous extract (ARAE). (A) Positive-mode total ion chromatogram (TIC). (B) Negative-mode TIC. (C) Chromatogram of kinsenoside reference standard. (D) Chromatogram of ARAE sample.

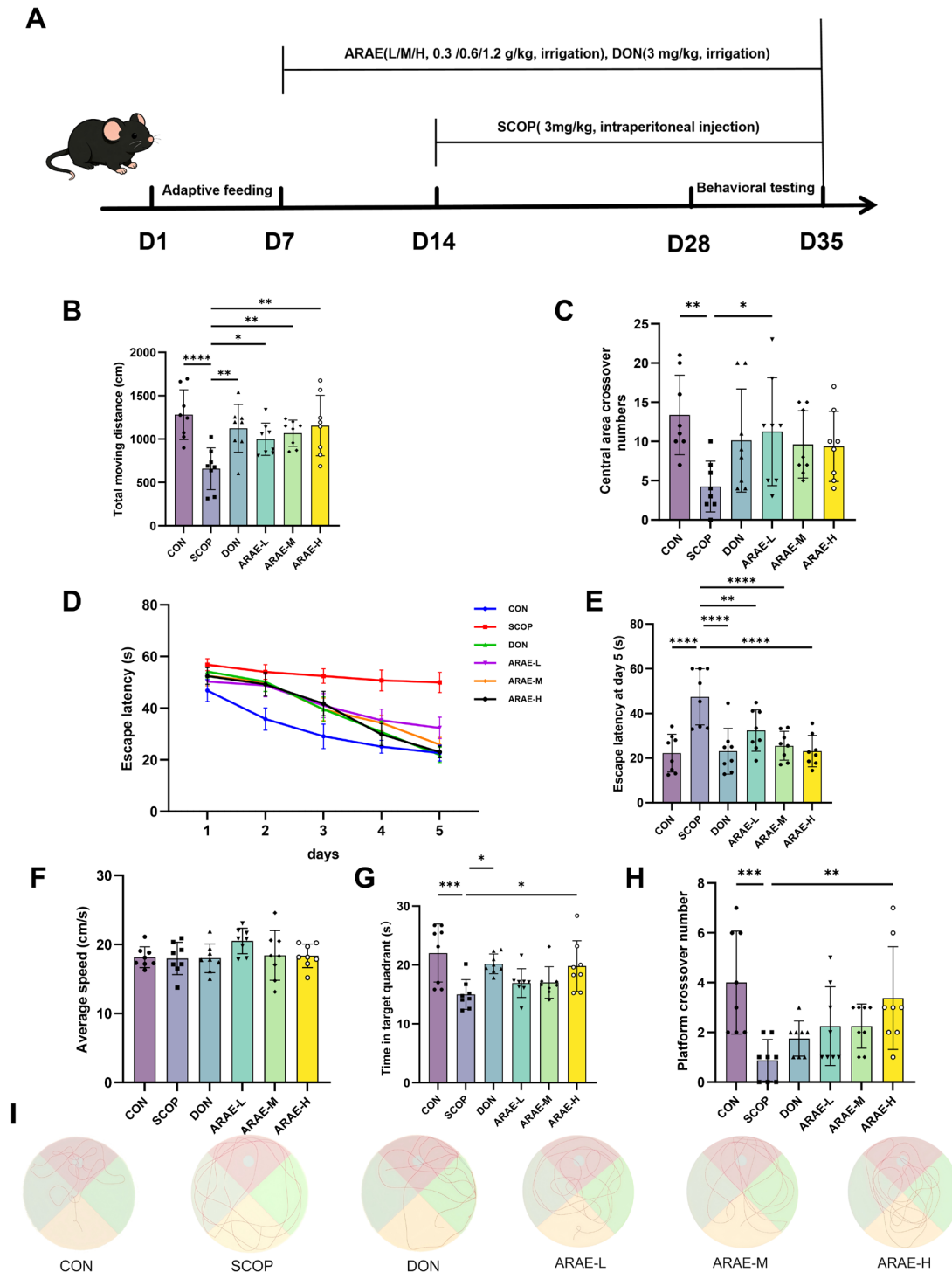


Figure 2. Effects of *Anoectochilus roxburghii* aqueous extract (ARAE) on the behavior of scopolamine-induced memory impairment mice. (A) Experimental procedure. (B) Total distance travelled in the open field test (OFT). (C) Central area crossover number in OFT. (D) Escape latency over five days of the training phase in the Morris water maze (MWM). (E) Escape latency at day 5 of the training phase in MWM. (F) Average swimming speed in MWM. (G) Time spent in the target quadrant in MWM. (H) The number of platform crossings in MWM. (I) Swimming trajectories in MWM.

Notes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. the SCOP group ($n = 8/\text{group}$).

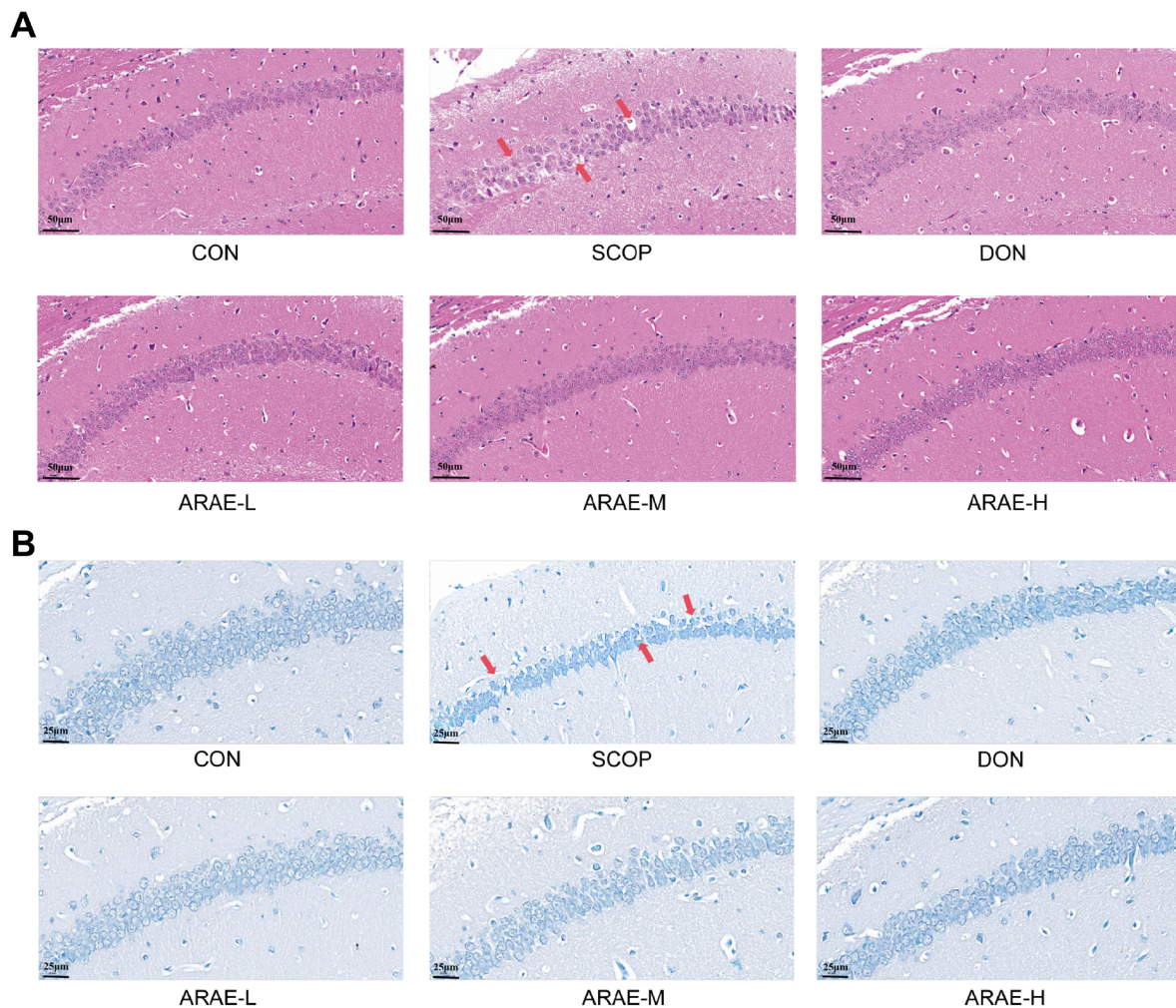


Figure 3. Pathological analysis of hippocampal CA1 damage in scopolamine-induced mouse model treated with *Anoectochilus roxburghii* aqueous extract (ARAE). (A) Hematoxylin and eosin (HE) staining (scale bars: 50 μm; magnifications: 600×). (B) Nissl staining (scale bars: 25 μm; magnifications: 400×). Note: Red arrows indicate the damaged sites in the hippocampal CA1 region.

spaces, and severe Nissl body dissolution compared to the CON group. In contrast, the mice in the ARAE and DON groups showed more regular neuronal and nuclear morphology, improved alignment, and partial preservation of Nissl bodies, with the attenuation of neuronal damage being more pronounced in the ARAE-H group.

3.6. ARAE attenuates colonic injury and improves intestinal barrier function in the scopolamine-induced mouse model

The results of HE staining (Figure 4A) revealed that mice in the SCOP group exhibited severely disrupted

crypt architecture, reduced goblet cells, and pronounced inflammatory cell infiltration. In the ARAE-H group, crypt structure was markedly improved, goblet cell counts were increased, and inflammatory infiltration was significantly alleviated. Although the ARAE-L and ARAE-M groups still showed some degree of crypt disruption and inflammatory infiltration, these manifestations were less severe than in the SCOP group.

To evaluate the effects of ARAE on the intestinal mucosal barrier in the CD mouse model, this study employed immunohistochemistry and immunofluorescence techniques (Figure 4B–4C). Compared with the SCOP

group, ARAE intervention resulted in a significant increase in the expression levels of the TJ proteins, including claudin-1, occludin, and ZO-1 (Figure 4D–4F), except that claudin-1 levels in the ARAE-L and ARAE-M groups showed an increasing but not statistically significant trend. Concurrently, immunofluorescence experiments showed that the MUC-2 expression of the ARAE-H group increased significantly compared with the SCOP group (Figure 4G), suggesting its potential to restore the mucus protective layer. In summary, the CD mouse model exhibits significant intestinal mucosal barrier damage, and ARAE can effectively promote the structural reconstruction and functional repair of the intestinal epithelial barrier by synergistically enhancing the expression of key TJ proteins and MUC-2.

3.7. Effects of ARAE on cholinergic levels in the scopolamine-induced mouse model

The hippocampus is typically closely linked to learning and memory functions, while the cortex is involved in executive functions and cognitive processing. The levels of ACh, AChE, and ChAT were measured in the hippocampal and cortical tissues (Figure 5A–5C). In the hippocampal tissue, the SCOP group exhibited a significant decrease in ACh ($p < 0.0001$) and ChAT concentrations ($p = 0.0022$), along with a notable increase in the AChE level ($p < 0.0001$), compared with those of the CON group. In contrast, the changes in ACh and AChE in the ARAE and DON groups were completely opposite to those in the SCOP group. We also found that ChAT activity in the ARAE-H group was significantly higher than in the SCOP group ($p = 0.0012$). Similar results were observed in the cortical tissues (Figure 5D–5F), though the extent of change was less pronounced than in the hippocampal tissues.

3.8. Effects of ARAE on inflammatory factor levels and oxidative stress damage in the scopolamine-induced mouse model

Studies have shown that CD is associated with neuroinflammation and oxidative stress, underscoring the importance of assessing relevant indicators to understand their effects. The results of neuroinflammation are presented in Figure 5G–5I). The levels of IL-6, IL-1 β , and TNF- α were significantly elevated in the hippocampal tissues of the SCOP group compared with those of the CON group ($p = 0.0087, 0.0039, 0.0071$, respectively). The treatment groups showed varying degrees of inhibitory effects on elevations of IL-6, IL-1 β , and TNF- α in the hippocampus, with the ARAE-H group displaying the most pronounced anti-inflammatory capabilities ($p = 0.0048, 0.0059, 0.0044$, respectively, compared with those of the SCOP group). A significant increase in these inflammatory factor levels was

also observed in the cortical tissues of the SCOP group ($p = 0.0001, 0.0276, 0.0107$, respectively, compared with those of the CON group), and all treatment groups were able to downregulate the levels of inflammatory factors in the cortex to different extents (Figure 5J–5L).

Similarly, we analyzed the changes in MDA and SOD levels in the hippocampal and cortical tissues (Figure 5M–5P). Compared with the CON group, SOD activity was significantly reduced in both hippocampal and cortical tissues of the SCOP group ($p = 0.0488, 0.0284$, respectively), while the MDA contents in both regions showed a marked increase (both $p < 0.0001$). Compared with the SCOP group, both the ARAE and DON groups upregulated SOD activity and downregulated MDA levels, with the changes in the ARAE-H group being particularly pronounced ($p = 0.0079, p < 0.0001$, respectively).

3.9. Effects of ARAE on gut microbiota in the scopolamine-induced mouse model

Gut microbiota plays a crucial role in regulating the gut–brain axis, and CD has been associated with gut microbiota imbalance.²⁶ To explore the regulatory effect of ARAE on gut microbiota, we conducted a systematic evaluation in mice across different treatment groups using 16S rRNA gene sequencing. A total of 460, 352, and 487 ASVs were identified in the CON, SCOP and ARAE-H groups, respectively, among which 375 ASVs were common across all three groups (Figure 6A). The Simpson index dilution curves indicated that all curves tended to stabilize with increasing sequencing depth (Figure 6B), suggesting that the amount of sequencing data was reasonable and sufficient to reflect the diversity of the current samples. A PCoA analysis based on the Bray–Curtis distance was performed to evaluate β -diversity, reflecting differences in the microbial community structure among groups. The data (Figure 6C) showed that the CON and SCOP groups were largely separated in the coordinate plot, while the ARAE-H group exhibited a closer clustering with the CON group, indicating intergroup differences. Next, relative abundance histograms were generated at the phylum and genus levels to further elucidate the effect of ARAE on specific microbial abundances in the SCOP-induced mouse model (Figure 6D–6E). At the phylum level, Bacteroidetes, Firmicutes, and Proteobacteria were major phyla. The Firmicutes/Bacteroidetes (F/B) ratio in the SCOP group was higher than in the CON group, whereas the ARAE-H group showed a partial decrease compared to the SCOP group, although the difference was not statistically significant (Figure 6F). Additionally, the levels of Proteobacteria and Desulfobacterota were significantly elevated in the SCOP group compared with the CON group ($p = 0.0430, 0.0069$, respectively), whereas

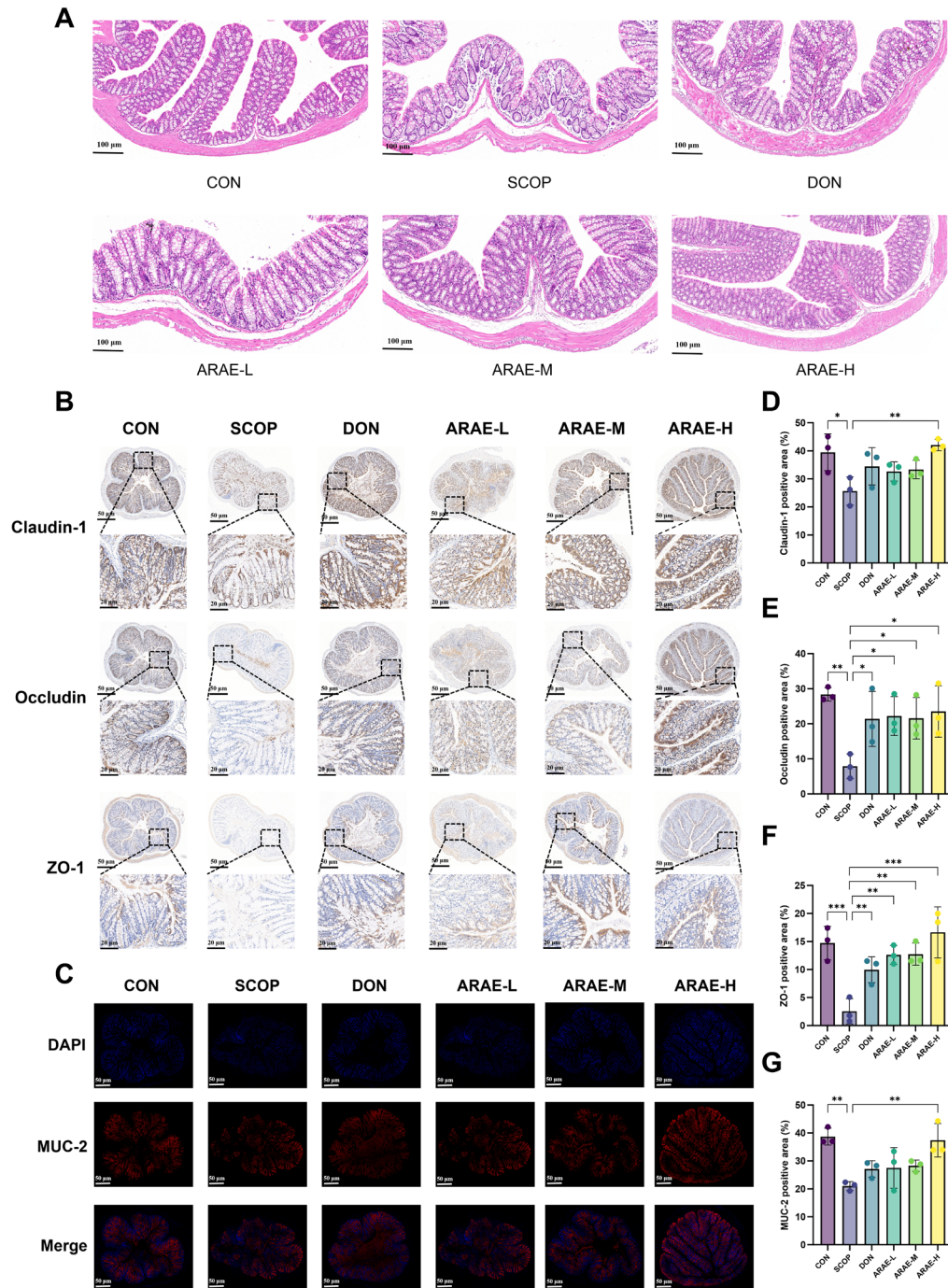


Figure 4. Effects of *Anoectochilus roxburghii* aqueous extract (ARAE) on intestinal mucosal barrier function in the scopolamine (SCOP)-induced mouse model. (A) Hematoxylin and eosin (HE) staining of mouse colon tissue (scale bars: 100 μ m; magnifications: 100 $\times$). (B) Immunohistochemical detection of claudin-1, occludin, and zonula occludens-1 (ZO-1) (scale bars: 50 μ m; magnifications: 200 $\times$; for inset: scale bars: 20 μ m; magnifications: 500 $\times$). (C) Immunofluorescent staining of mucin-2 (MUC-2) (scale bars: 50 μ m; magnifications: 200 $\times$). (D–G) Areas of positive staining of (D) claudin-1, (E) occludin, (F) ZO-1, and (G) MUC-2.

Notes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the SCOP group ($n = 3/\text{group}$).

Abbreviation: DAPI: 4',6-diamidino-2-phenylindole.

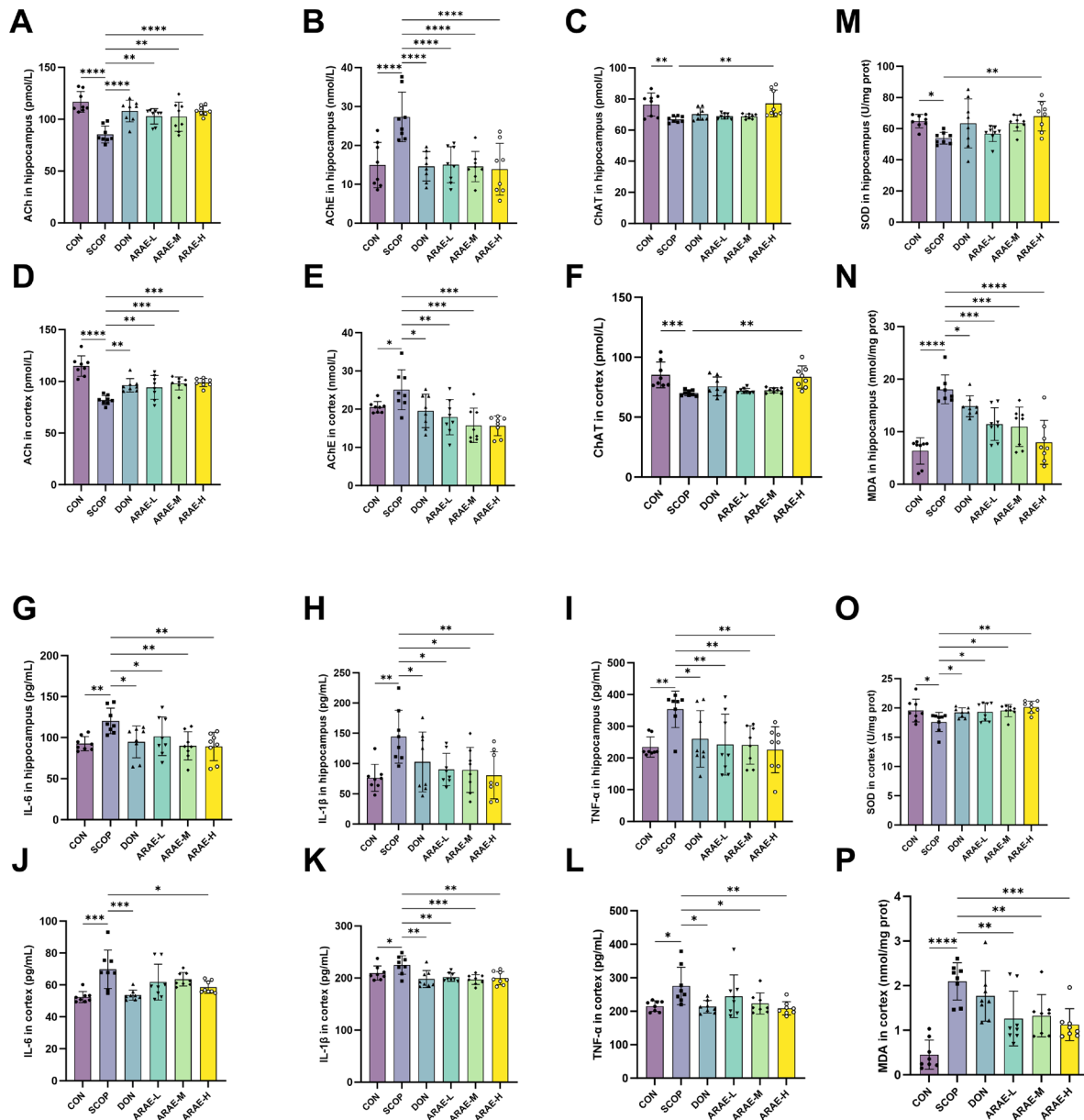


Figure 5. *Anoectochilus roxburghii* aqueous extract (ARAE) regulates the cholinergic system, inhibits neuroinflammation, and alleviates oxidative stress damage. (A–L) The enzyme-linked immunosorbent assay (ELISA) method was used to measure levels of hippocampal (A) acetylcholine (ACh), (B) acetylcholinesterase (AChE), and (C) choline acetyltransferase (ChAT); cortical (D) ACh, (E) AChE, and (F) ChAT; hippocampal (G) interleukin (IL)-6, (H) IL-1 β , and (I) tumor necrosis factor- α (TNF- α); and cortical (J) IL-6, (K) IL-1 β , and (L) TNF- α . (M–P) The activities of hippocampal (M) superoxide dismutase (SOD) and (N) malondialdehyde (MDA), and cortical (O) SOD and (P) MDA.

Notes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. the SCOP group ($n = 8$ /group).

the ARAE-H intervention effectively reduced this change ($p = 0.0482, 0.0077$, respectively, compared with the SCOP group), restoring levels close to normal (Figure 6G–6H).

At the genus level, compared with the CON group, the SCOP group showed varying degrees of decrease in genera such as *Alloprevotella*, *Prevotellaceae* NK3B31 group, *Lactobacillus*, and *Parasutterella*, while showing

increases in *Escherichia-Shigella*, *Alistipes*, *Treponema*, *Bacteroides*, *Helicobacter*, and *Muribaculum*. Conversely, the ARAE-H group demonstrated opposite trends, as most of these genera shifted toward levels comparable to those in the CON group (Figure 6I–6M). This is consistent with the clustering heatmap of genus-level species abundance (Figure 6N). Furthermore, the differences in microbial

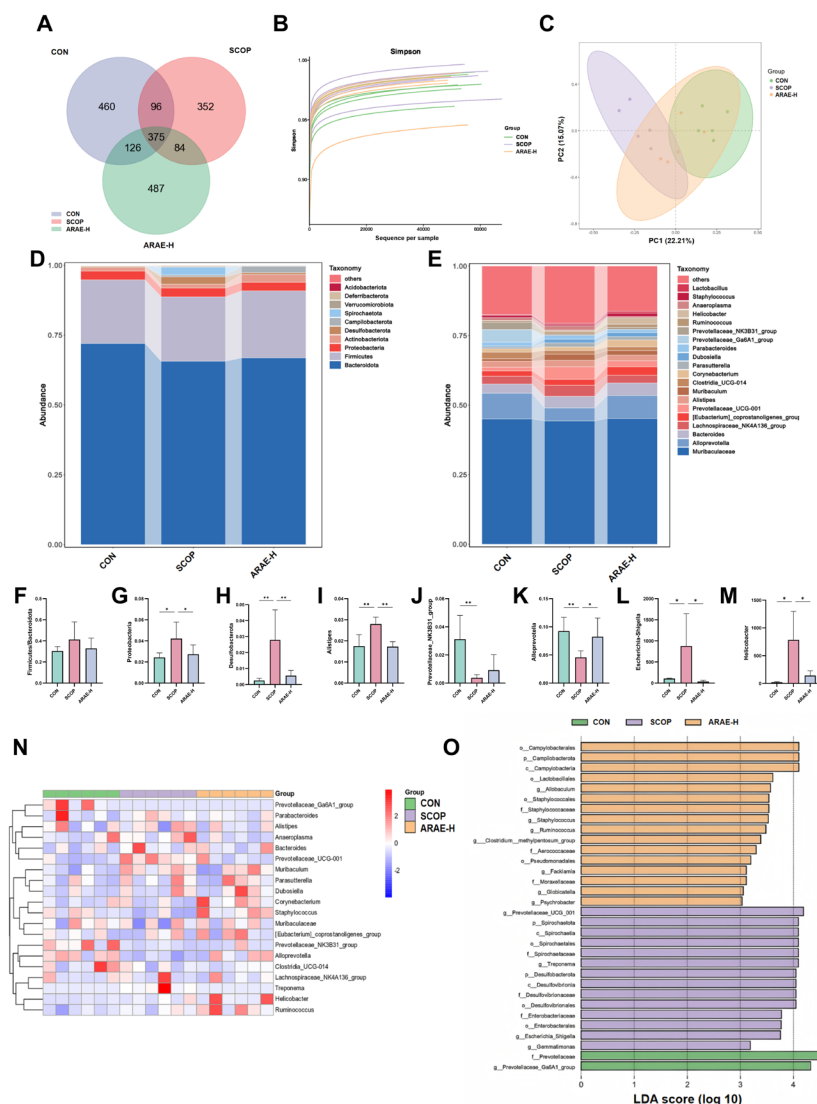


Figure 6. Regulation of gut microbiota by *Anoectochilus roxburghii* aqueous extract (ARAE). (A) amplicon sequence variant (ASV)–Venn diagram. (B) Simpson curve. (C) Principal coordinates analysis (PCoA) analysis of Bray–Curtis distances between groups. (D) Microbial community composition at the phylum level and (E) the genus level. (F–M) Relative abundance of (F) Firmicutes/Bacteroidetes; representative phyla: (G) Bacteroidetes, (G) Proteobacteria, and (H) Desulfobacteriia; and (I) *Alistipes*, (J) *Prevotellaceae* NK3B31 group, (K) *Alloprevotella*, (L) *Escherichia-Shigella*, and (M) *Helicobacter*. (N) Heatmap of the top 20 genera. (O) Linear discriminant analysis (LDA) scores for the SCOP, ARAE-H, and CON groups.

Notes: * $p < 0.05$, ** $p < 0.01$ ($n = 6/\text{group}$).

community composition were further evaluated among groups using linear discriminant analysis effect size (LEfSe). The LEfSe analysis indicated (Figure 6O) that significantly enriched taxa in the SCOP group included pSpirochaetota, fSpirochaetaceae, oSpirochaetales, cSpirochaetia, g*Treponema*, pDesulfobacterota, cDesulfovibrionia, fDesulfovibrionaceae, and oDesulfovibrionales (linear discriminant analysis [LDA] > 3.6), which are often associated with intestinal inflammation and infection. In contrast, the ARAE-H group showed significant enrichment of lineages including oLactobacillales and g*Allobaculum* (LDA > 3.6), which are potential probiotics.

3.10. Metabolic influence of ARAE in the scopolamine-induced mouse model

Principal component analysis (PCA) and partial least squares discriminant analysis were first used to observe overall distribution trends and inter-group separation (Figure 7A–7B). The results revealed significant distinctions in fecal metabolites between the SCOP and CON groups, while the ARAE-H group was closer to the normal composition. Additionally, a Venn diagram was used to visualize differentially expressed metabolites across comparison groups (Figure 7C). The data showed that ARAE administration restored 172 core disrupted metabolites in the CD model, but it also induced new metabolic changes, and some differences from the healthy model remained unrestored. Subsequently, differential metabolites in each group were screened based on criteria of variable importance in projection (VIP) > 1, $p < 0.05$, and fold change (FC) ≥ 2.0 or $\text{FC} \leq 0.5$. Compared with the SCOP group, the CON group exhibited 567 upregulated and 278 downregulated metabolites, while the ARAE-H group showed 203 upregulated and 761 downregulated metabolites (Figure 7D–7E). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed on the differential metabolites between the ARAE-H and SCOP groups, identifying major affected pathways including tryptophan metabolism, primary bile acid biosynthesis, steroid hormone biosynthesis, serotonergic synapse, peroxisome proliferator-activated receptor (PPAR) signaling pathway, sphingolipid signaling pathway, neuroactive ligand-receptor interaction, and apoptosis (Figure 7F–7G). Based on these related pathways, further screening of differential metabolites revealed that, compared with the SCOP group, ARAE-H significantly upregulated taurine, acetyl-D-carnitine, N-acetylserotonin, taurocholic acid 3-sulfate, chenodeoxycholicysteine, deoxycholytyrosine, and 20-hydroxy-eicosanoic acid, while downregulating anandamides, sphingosine 1-phosphate (d16:1-P), and 5 α -tetrahydrocortisol (Figure 8A–8J). To investigate the

intrinsic relationship between changes in gut microbiota and differentially expressed metabolites, a Spearman correlation analysis was conducted at the genus level for gut microbes and differential metabolites in the SCOP and ARAE-H groups. The focus was on the relationships between the 15 most abundant bacterial genera and significantly altered metabolites. In the comparison between the ARAE-H and SCOP groups, the microbe-metabolite association network exhibited a clear clustering structure (Figure 8K). Microbes were mainly divided into two clusters: the first centered on *Aliiprevotella*; the second included Muribaculaceae and *Bacteroides*. Differential metabolites were also divided into two clusters: the first included eight metabolites, such as sphingosine 1-phosphate, N-acetylserotonin, and taurine, showing extensive associations with the aforementioned microbial clusters; the second consisted solely of taurocholic acid 3-sulfate, with a relatively independent association pattern.

To further validate the regulatory effects of ARAE on microbial metabolic functions, targeted metabolomics was used to assess SCFA levels in mouse feces. The results showed that ARAE intervention markedly increased levels of propionic acid and butyric acid, with an elevation in acetic acid levels that did not reach statistical significance (Figure 8L–8N).

4. Discussion

This research provides evidence that ARAE can improve CD in a SCOP-induced mouse model, potentially by regulating the MGB axis. Pharmacological studies showed that high doses of ARAE significantly improved the condition of SCOP-induced CD mice. ARAE intervention not only enhanced learning and memory in mice and reduced neuronal damage in the hippocampal CA1 region but also exerted central neuroprotective effects through multiple pathways, including modulation of the cholinergic system, suppression of neuroinflammation, and alleviation of oxidative stress. Notably, systematic studies on whether the central neuroprotective effects of ARAE stem from its regulation of the gut microbiota are currently lacking. In recent years, the introduction of the MGB axis framework has provided a new perspective for understanding the pathogenesis of neurodegenerative diseases.²⁷ The gut microbiota communicates bidirectionally with the central nervous system via neural, immune, and endocrine pathways.²⁸ SCFAs, as key mediators of microbial metabolism, play a crucial role in regulating neuroinflammation, oxidative stress, and the integrity of the blood-brain barrier.²⁹ Based on this, we further assessed the effects of ARAE on gut microbiota composition and metabolism.

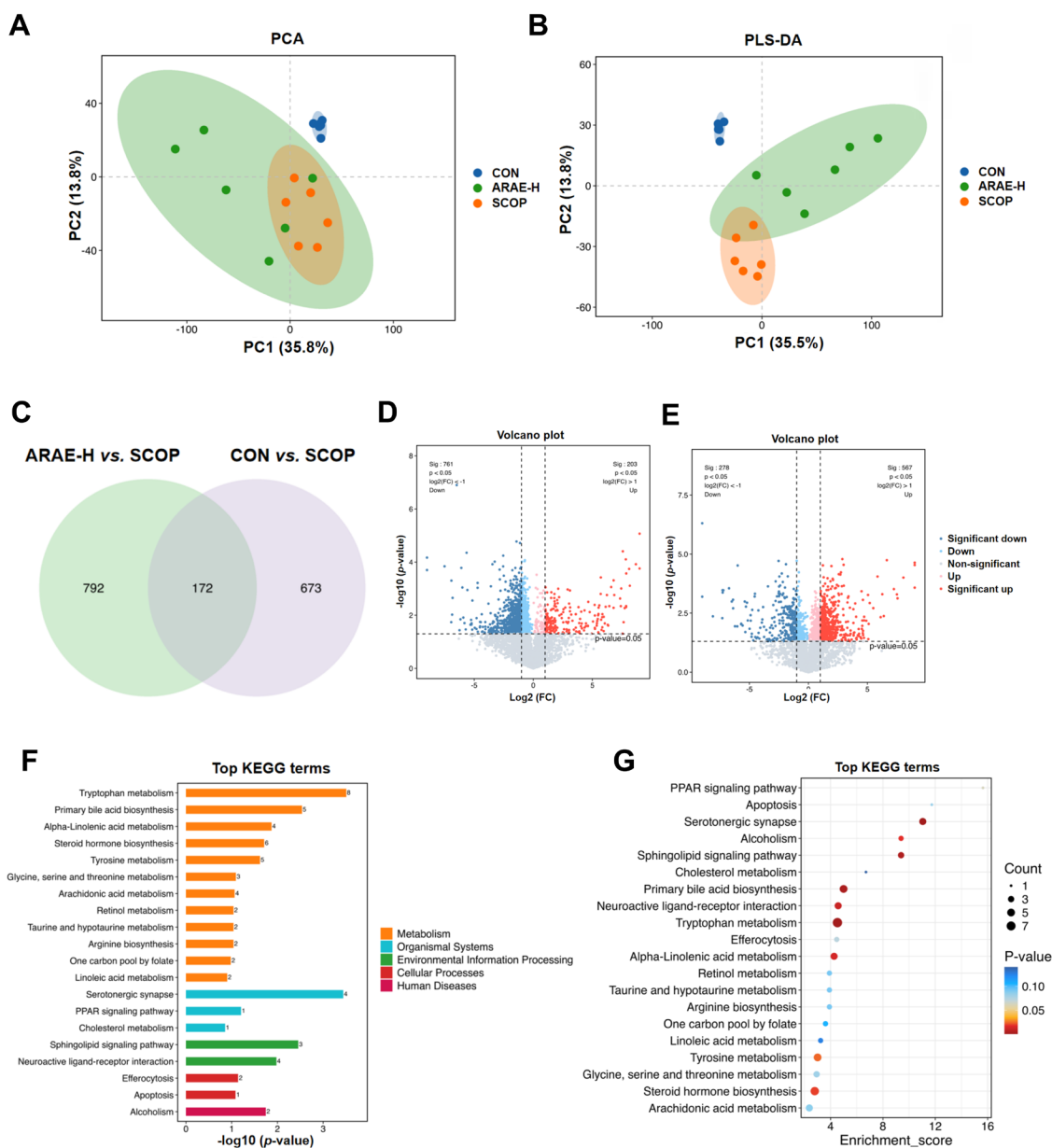


Figure 7. *Anoectochilus roxburghii* aqueous extract (ARAE) alleviates scopolamine (SCOP)-induced metabolic disorders. (A) Principal component analysis (PCA). (B) Partial least squares (PLS) analysis. (C) Count of differential metabolites among groups. (D–E) Volcano plots: (D) ARAE-H vs. SCOP; (E) CON vs. SCOP. (F) Overall Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation for the ARAE-H group vs. the SCOP group. (G) KEGG pathway enrichment analysis for the ARAE-H group vs. the SCOP group. (N = 6).

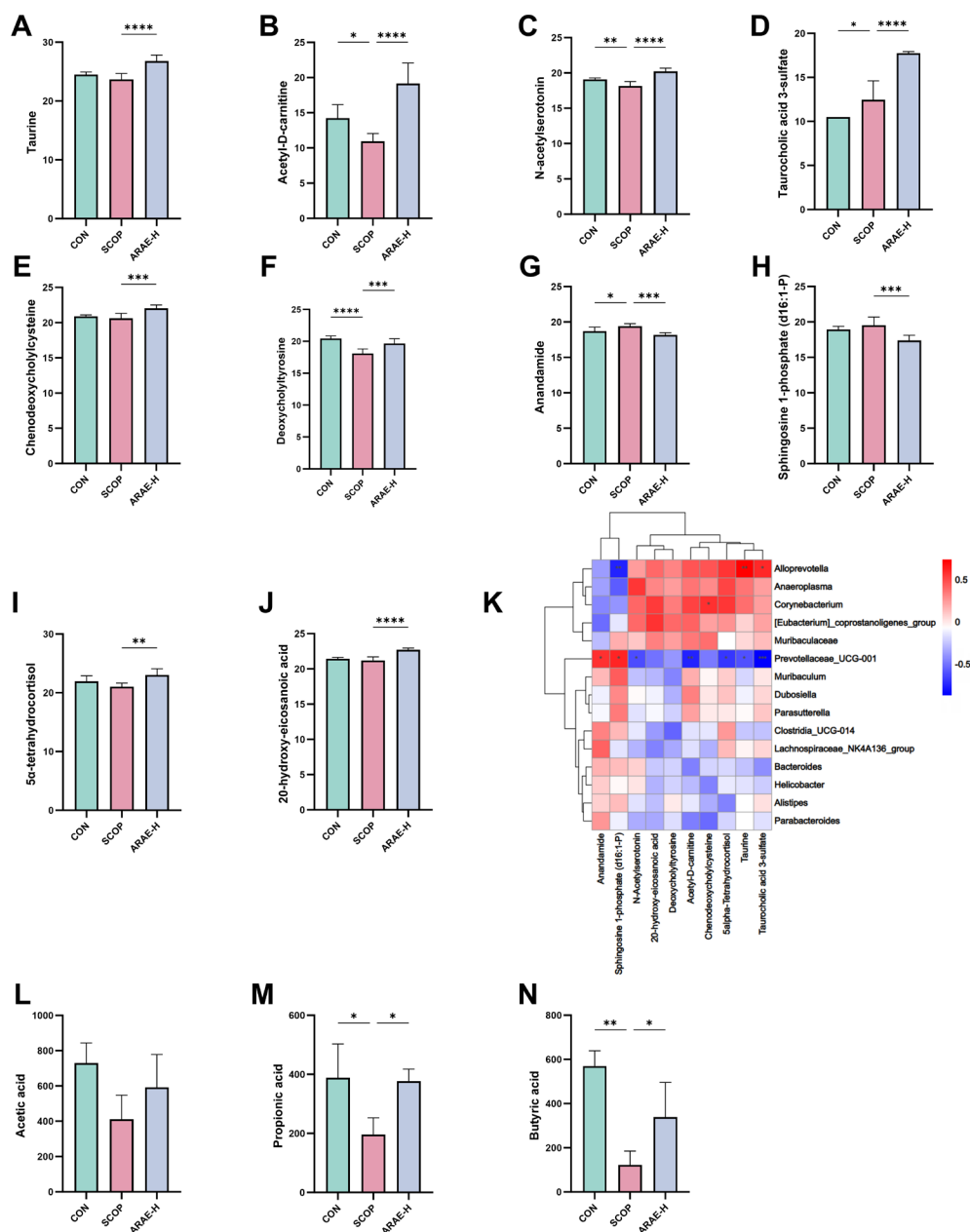


Figure 8. Correlation analysis between the 15 microbial communities with the greatest abundance changes and the metabolites that differ significantly. (A–J) Relative levels of (A) taurine, (B) acetyl-*D*-carnitine, (C) N-acetylserotonin, (D) taurocholic acid 3-sulfate, (E) chenodeoxycholicysteine, (F) deoxycholytyrosine, (G) anandamide, (H) sphingosine 1-phosphate (d16:1-P), (I) 5 α -tetrahydrocortisol, and (J) 20-hydroxy-eicosanoic acid in feces. (K) Correlation analysis between the species-level microbiome and differential metabolites. (L–N) Relative concentrations of (L) acetate, (M) propionate, and (N) butyrate in gut contents.

Notes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ ($n = 6/\text{group}$).

Analysis of 16S rRNA sequencing data revealed that SCOP-induced CD was accompanied by significant disruption of gut microbiota structure, manifested by reduced α -diversity, increased abundance of Proteobacteria and Desulfobacterota, and a higher Firmicutes-to-Bacteroides ratio. These changes closely resemble the microbiota characteristics observed in AD.³⁰ ARAE intervention effectively reversed the aforementioned dysbiosis and significantly enriched SCFA-producing bacteria, such as *Alloprevotella*, *Lactobacillus*, and *Prevotellaceae* NK3B31 group.³¹ At the same time, it also inhibited opportunistic pathogens, such as *Escherichia-Shigella*.³² The neuroprotective potential of gut microbiota has been documented in recent studies. Specific bacterial strains, such as *Lactobacillus buchneri* KU200793 and *Lactobacillus plantarum* GD2, have been shown to protect neuronal cells against neurotoxic insults through antioxidant and anti-apoptotic mechanisms.^{33,34} In the present study, the enrichment of beneficial genera, such as *Lactobacillus*, following ARAE treatment is consistent with these reported neuroprotective profiles, providing a plausible link between gut microbiota remodeling and the attenuation of neuronal damage observed in our model. Research has also found that an increase in the abundance of *Lactobacillus* species is closely associated with elevated levels of SCFAs in the gut and that SCFAs help maintain the integrity of the intestinal barrier.³⁵

Analysis of SCFA levels revealed that the total concentration of SCFAs in the feces of mice in the SCOP group was significantly reduced, consistent with previous studies showing decreased numbers of SCFA-producing colonic bacteria and lower SCFA levels in patients with AD.³⁶ Following ARAE intervention, the levels of acetic acid, propionic acid, and butyric acid in feces all showed a trend toward recovery, with the most significant increases observed in propionic acid and butyric acid. Propionic acid can maintain mitochondrial fission and autophagy homeostasis in the pathological process of AD by activating the G-protein-coupled receptor 41 (GPR41) and GPR43 receptors.³⁷ Butyric acid is the primary product of dietary fiber fermentation by the gut microbiota and serves as an important energy source for colonic epithelial cells.³⁸ The correlation observed in this study between elevated SCFA levels and subsequent upregulation of TJ proteins provides preliminary support for the existence of this regulatory pathway.

The integrity of the intestinal barrier depends on the synergistic action of TJ proteins and the mucus layer. Studies have found that the expression of TJ proteins claudin-1, occludin, and ZO-1 was significantly downregulated in colonic tissue from CD mice, and MUC-2, a major

component of the mucus layer, also showed a decreasing trend, suggesting impaired intestinal barrier function. ARAE intervention significantly reversed these changes, restoring TJ protein expression and mucus layer integrity. Studies indicate that SCFA deficiency can impair intestinal immune function and epithelial integrity, whereas butyrate supplementation promotes mucus secretion, reduces endotoxin absorption, suppresses epithelial inflammation, and upregulates TJ protein expression.^{39,40} Given the correlation observed in this study between elevated SCFA levels and improved barrier function, it is hypothesized that ARAE may repair the intestinal barrier by promoting SCFA production, thereby preventing pro-inflammatory factors such as lipopolysaccharides from entering the systemic circulation and causing neuroinflammation.

Untargeted metabolomics analysis further revealed the modulatory effects of ARAE on the overall metabolic profile. ARAE intervention reversed metabolic dysregulation in the CD mouse model and specifically regulated pathways closely associated with neuropsychiatric function, including tryptophan metabolism, bile acid metabolism, and neuroactive ligand-receptor interactions. Additionally, ARAE upregulated metabolites with neuroprotective potential, such as taurine and N-acetylserotonin.^{41,42} It also downregulated sphingosine-1-phosphate, which is associated with neuroinflammation and apoptosis. Spearman correlation network analysis also revealed that variations in these metabolites were closely correlated with changes in the abundance of specific bacterial genera.

Taken together, this study proposes a potential mechanism by which ARAE exerts neuroprotective effects via the MGB axis: the active components in ARAE first act on the gut microbiota, promoting the proliferation of SCFA-producing bacteria, inhibiting pathogenic bacteria, and rehabilitating microbial equilibrium. Changes in microbial metabolic function subsequently increase the production of SCFAs such as propionate and butyrate. SCFAs not only provide energy to intestinal epithelial cells but may also upregulate the expression of TJ proteins via the GPR41/43-HDAC signalling pathway, thereby repairing the intestinal barrier and preventing pro-inflammatory factors such as lipopolysaccharides from entering the bloodstream. Reduced peripheral endotoxin levels mitigate attacks on the blood-brain barrier and the overactivation of central microglia, thereby lowering levels of neuroinflammation and oxidative stress in the brain, protecting the structure and function of hippocampal neurons, and ultimately improving cognitive behavior. However, the current data are largely correlative, and direct evidence for this causal chain is still lacking.

This study has several limitations. First, the SCOP-induced model primarily reflects cholinergic dysregulation rather than classic AD pathology, which presents certain limitations. Second, the association between improved intestinal barrier function and reduced neuroinflammation remains poorly elucidated and warrants further exploration. Third, the current data primarily reflect correlational findings; the causal relationship between changes in the microbiota and neuronal protection remains to be investigated. Finally, current assessments of neuroprotective effects rely primarily on histological observations, without analysis of synaptic or neuronal functional markers. Future research should incorporate immunohistochemical or biochemical analyses of synaptic and neuronal functional markers to confirm the neuroprotective effects suggested by current histological observations.

5. Conclusion

Overall, this study established that ARAE alleviates SCOP-induced CD through multiple mechanisms, including modulation of the cholinergic system, suppression of neuroinflammation, and attenuation of oxidative stress. Furthermore, ARAE reshaped the gut microbiota, enriched SCFA-producing bacteria in the colon, and increased levels of propionate and butyrate in feces. These changes may be linked to the repair of the intestinal TJ and the reduction of neuroinflammation. These findings suggest that ARAE may improve CD, at least in part through changes in the MGB axis, and warrant further studies in AD models.

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Conflict of interest

Haiyan Zhang is an employee of Jiangzhong Pharmaceutical Co., Ltd., but was not involved in any activities that could constitute a conflict of interest in relation to this study. The authors declare they have no competing interests.

Author contributions

Conceptualization: Hui Yin, Chang Peng, Haiyan Zhang

Formal analysis: Hui Yin, Xiao Zhang, Yonghong Liang, Ming Yang

Investigation: Hui Yin, Jiayi Zhang, Tao Yang

Methodology: Hui Yin, Min Feng, Ting Yang, Ning Zhang, Feiyue Cheng

Writing—original draft: Hui Yin, Jiayi Zhang, Tao Yang

Writing—review & editing: Hui Yin, Jiayi Zhang, Chang Peng, Haiyan Zhang

Ethics approval and consent to participate

All experimental procedures were conducted in accordance with the guidelines established by the Ministry of Health, and were approved by the Laboratory Animal Ethics Committee of Wuhan Hualianke Biotechnology Co., Ltd. (Ethics No. HLK-2025-0428-002).

Consent for publication

Not applicable.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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