



## 곽향의 cyclophosphamide 유도 인지장애 개선 효과: 실험적 검증 및 성분-표적 예측 연구

정희영<sup>1#</sup> · 김영미<sup>2#</sup> · 이아영<sup>3</sup> · 서윤수<sup>4</sup> · 김정민<sup>5</sup> · 이예지<sup>6</sup> · 고희철<sup>7</sup> · 송지훈<sup>8</sup> · 정소희<sup>9</sup> · 문창종<sup>10</sup> · 임데레사<sup>11</sup>  
김중선<sup>12†</sup> · 이수은<sup>13‡</sup>

### Protective Effects of *Agastache rugosa* Against Cyclophosphamide-Induced Cognitive Impairment in Mice: Experimental Validation and Compound-Target Prediction

Huiyeong Jeong<sup>1#</sup>, Youngmee Kim<sup>2#</sup>, A Yeong Lee<sup>3</sup>, Yun-Soo Seo<sup>4</sup>, Jungman Kim<sup>5</sup>, Yeji Lee<sup>6</sup>, Hee Chul Ko<sup>7</sup>,  
Ji Hoon Song<sup>8</sup>, Sohee Jeong<sup>9</sup>, Changjong Moon<sup>10</sup>, Teresa Yim<sup>11</sup>, Joong-Sun Kim<sup>12†</sup>, and Sueun Lee<sup>13‡</sup>

#### ABSTRACT

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**Background:** The chemotherapeutic and immunosuppressive agent cyclophosphamide (CYP), which is widely used in cancer treatment, can cause oxidative damage and inflammation in the brain, resulting in behavioral abnormalities, including cognitive impairment. *Agastache rugosa* (AR) has been traditionally used to treat digestive disorders and it has several physiological activities.

**Methods and Results:** We investigated the neuroprotective effects of an AR ethanol extract against CYP-induced neurodegeneration and cognitive dysfunction. Free radical scavenging assays confirmed the antioxidant activity of the AR extract. C57BL/6 mice were orally administered an AR extract (100 or 200 mg/kg) for seven days, followed by an intraperitoneal injection of CYP (50 mg/kg). Mice exhibited memory impairment in a contextual fear conditioning test 24 h after CYP treatment. However, AR pretreatment notably improved behavioral deficits. Histological analysis demonstrated that CYP administration reduced the immunoreactivity of Ki67 (a proliferative cell marker) and doublecortin (an immature progenitor cell marker) in the dentate gyrus of the hippocampus, whereas AR pretreatment considerably normalized the levels of these markers. Subsequently, network pharmacology was used to investigate the potential genes involved in the ameliorative action of AR on CYP-induced neurodegeneration and memory impairment, which suggested *BCL2*, *JUN*, and *MAPK3* as key candidates.

**Conclusions:** These findings demonstrate that AR pretreatment protects against CYP-induced neurodegeneration and cognitive impairment. In addition, network pharmacology analysis suggests that AR compounds may provide neuroprotection against CYP-induced neuroinflammation by modulating key target genes such as *BCL2*, *JUN*, and *MAPK3*.

**Key Words:** *Agastache rugosa*, Cyclophosphamide, Cognitive Impairment, Antioxidant Activity, Network Pharmacology Analysis

<sup>†</sup>Corresponding author: (Phone) +82-62-530-2815 (E-mail) centraline@jnu.ac.kr

<sup>‡</sup>Co-corresponding author: (Phone) +82-61-338-7151 (E-mail) leese@kiom.re.kr

<sup>#</sup>Huiyeong Jeong and Youngmee Kim are contributed equally to this paper.

<sup>1</sup>전남대학교 수의학과 석사과정생 / Master's student, College of Veterinary Medicine and BK21 FOUR Program, Chonnam National University, Gwangju 61186, Korea

<sup>2</sup>제주한의약연구원 선임연구원 / Senior researcher, Jeju Institute of Korean Medicine, Jeju 63309, Korea

<sup>3</sup>한국한의약연구원 한의약데이터부 책임연구원 / Principal researcher, KM Data Division, Korea Institute of Oriental Medicine, Daejeon 34054, Korea

<sup>4</sup>한국한의약연구원 한약자원연구센터 선임연구원 / Senior researcher, Herbal Medicine Resource Research Center, Korea Institute of Oriental Medicine, Naju 58245, Korea

<sup>5</sup>제주한의약연구원 연구원 / Researcher, Jeju Institute of Korean Medicine, Jeju 63309, Korea

<sup>6</sup>제주한의약연구원 연구원 / Researcher, Jeju Institute of Korean Medicine, Jeju 63309, Korea

<sup>7</sup>제주한의약연구원 연구개발팀장 / Research and development manager, Jeju Institute of Korean Medicine, Jeju 63309, Korea

<sup>8</sup>제주한의약연구원 연구원 / Researcher, Jeju Institute of Korean Medicine, Jeju 63309, Korea

<sup>9</sup>전남대학교 수의학과 석박통합과정생 / Integrated Master's and Doctoral student, College of Veterinary Medicine and BK21 FOUR Program, Chonnam National University, Gwangju 61186, Korea

<sup>10</sup>전남대학교 수의학과 교수 / Professor, College of Veterinary Medicine and BK21 FOUR Program, Chonnam National University, Gwangju 61186, Korea

<sup>11</sup>글로벌푸른친구들 대표 / CEO, Global GreenFriends Co., Seoul 06569, Republic of Korea

<sup>12</sup>전남대학교 수의학과 교수 / Professor, College of Veterinary Medicine and BK21 FOUR Program, Chonnam National University, Gwangju 61186, Korea

<sup>13</sup>한국한의약연구원 한약자원연구센터 선임연구원 / Senior researcher, Herbal Medicine Resource Research Center, Korea Institute of Oriental Medicine, Naju 58245, Korea

## INTRODUCTION

Cyclophosphamide (CYP) has been extensively used as an anticancer and immunosuppressive agent (de Jonge *et al.*, 2005). Although the chemotherapeutic agent was initially thought not to cross the blood-brain barrier when administered at standard clinically relevant doses, 17 - 75% of patients, who received the chemotherapy, have reported several adverse effects related to learning and memory such as concentration difficulties and cognitive impairment (Silberfarb, 1983; Hodgson *et al.*, 2013; Lange *et al.*, 2014; Matsos and Johnston, 2019). Furthermore, the extent of cognitive dysfunction was proportional to the dose of chemotherapy (Ahles and Saykin, 2001). Several molecular mechanisms underlying the CYP-induced deficits in learning and memory function have been studied, which include oxidative stress, inflammation, neuronal cell death, and interference with the function of neurotransmitters and neurotrophic factors (Bagnall-Moreau *et al.*, 2019).

*Agastache rugosa* (AR), also known as Korean mint, is a member of the Lamiaceae family (Rinik *et al.*, 2024), and it is widely distributed across East Asia, including Korea, China, Japan, and Russia. AR, a major component of the traditional formula Gwakyangjeonggi-san, has been historically used for digestive disorders, including diarrhea, abdominal discomfort, and vomiting (Ko *et al.*, 2013). However, recent accumulating pharmacological evidence suggests that AR may broaden the therapeutic potential beyond classical indications by its antioxidant, anti-inflammatory, anti-aging, anti-bacterial, and anti-tumor activities. These effects are largely attributed to its bioactive constituents, including flavonoids (e.g., acacetin, tiliarin), phenylpropanoids (e.g., rosmarinic acid), lignans, and terpenoids (Nam *et al.*, 2020; Song *et al.*, 2024). In this study, we aimed to broaden the indications of AR treatment by investigating its ameliorative effects in a mouse model of neurodegeneration and memory impairment induced by CYP administration. Moreover, we examined the possible target genes under the protective effects of AR against CYP-induced neurotoxicity by using network pharmacology analysis, which has been widely applied to explain the multi-component, multi-target, and multi-pathway behavior of medicinal herbs (Song *et al.*, 2024). To this end, we performed the analysis using encephalitis (a clinical diagnosis broadly regarded as reflecting neuroinflammatory conditions) as a keyword, allowing us to identify the predicted target genes potentially related to neuroinflammatory processes.

## MATERIALS AND METHODS

### 1. 70% ethanol extract of AR

The AR extract was prepared as previously published (Kang *et al.*, 2024). Briefly, the whole shoot part of AR was obtained from Jeju Island (Seongsan-eup, Seogwipo-si, Republic of Korea [33°27'47.5" N, 126°54'48.9" E, altitude 17 m]). Total 100 g of the dried AR plant was included in 1 L of 20% EtOH (v/v) solution, and refluxed for 2 h at 30°C. The extract was filtered, concentrated with a rotary air evaporator, and lyophilized using a freeze dryer. The final extract was stored at -80°C until use.

### 2. DPPH and ABTS radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical scavenging activities for measurement of antioxidant capacities were determined according to report (n = 5 per each group) (Seo *et al.*, 2025). DPPH solution dissolved in methanol and 100 µL AR extract (0, 5, 10, 20, 100, and 500 mg/mL) reacted at 37°C in an incubator for 30 min. The absorbance of 100 µL DPPH radical was measured at 517 nm. For the ABTS assay, the radical solution reacted with phosphate was adjusted to a final absorbance of  $0.7 \pm 0.02$  at 734 nm. Each scavenging rate of DPPH and ABTS of the AR extract was calculated according to the following formula:

$$\text{Scavenging rate (\%)} = [(Ab_{\text{free}} - Ab_{\text{sample}})/Ab_{\text{free}}] \times 100$$

### 3. Animals

Seven-week-old male C57BL/6 mice ( $18 \pm 2$  g) were obtained from Dooyeol Biotech (Seocho-gu, Seoul, Korea). The mice were housed in standard mouse cages at a temperature of  $23 \pm 2^\circ\text{C}$ , humidity of  $50 \pm 10\%$ , air ventilation frequency of 10 to 20 times/h, and light intensity of 105 to 300 Lux from 08:00 to 20:00, with food and water provided randomly. All mice were housed in accordance with the guidelines set forth in the Animal Welfare Regulation, and the experimental procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (CNU IACUC-YB-2024-112) and Korea Institute of Oriental Medicine (20-061, 01 September 2020).

### 4. CYP and AR extract administration

Following an acclimatization period, the mice were divided

into four groups ( $n = 6$  per each group). The first group served as control (control group), and the second group was administered only CYP (CYP group). Group 3 was administered CYP and AR extract at a dose of 100 mg/kg (CYP+AR100), whereas group 4 received the same treatment but with a higher dose of AR extract of 200 mg/kg (CYP+AR200). The AR treatment groups were administered 100 or 200 mg/kg/day of the extract for 7 days. Mice in groups 2, 3, and 4 were intraperitoneally injected with CYP (50 mg/kg) on day 7, whereas group 1 was administered phosphate-buffered saline (PBS) instead of CYP.

### 5. Contextual fear conditioning test

Contextual fear conditioning paradigm was conducted by using a TruScan Photobeam Activity Monitoring System ( $n = 6$  per each group) (Coulbourn Instruments, Whitehall, PA, USA). Each mouse was placed in the conditioning chamber ( $26 \times 26 \times 40$  cm) with 23 stainless steel grids on the floor, and allowed to explore the space for 2 min before an electronic impact. Subsequently, the subjected mouse received a single shock (0.5 mA for 2 s) and was permitted to remain in the conditioning chamber for 30 s. 24 hours following the training, the mice were returned to the conditioning chamber for 2 min to assess the percentage of the complete absence of movement behavior (defined as freezing) except for respiration. The duration of the freezing behavior was recorded when the mouse exhibited sustained freezing for a minimum of 2 seconds.

### 6. Immunohistochemical analysis

Mice were euthanized by the combination of alfaxalone and xylazine, and the brains were removed immediately, and fixed in 4% paraformaldehyde for paraffin embedding ( $n = 3$  per each group). The immunohistochemical analysis was conducted in accordance with previously described protocols in the literature (Lee *et al.*, 2022). Paraffin sections (thickness, 4  $\mu$ m) were cut with a microtome, and deparaffinized using routine protocols, and tissue sections were treated with primary antibodies. The primary antibodies used were rabbit anti-Ki67 (1:100; #DRM004, OriGene Technologies Inc., Beijing, China) and rabbit anti-DCX (1:1,000; #4604, Cell Signaling Technology, Beverly, MA, USA), diluted in antibody dilution buffer (Invitrogen, Carlsbad, CA, USA). Subsequently, the sections were washed with PBS and incubated with biotinylated goat anti-rabbit IgG (Vector ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). The specific binding was detected with the Vector ABC Elite Kit (Vector Laboratories) employing

an avidin-biotin-peroxidase complex, and stained utilizing a diaminobenzidine (DAB; Vector Laboratories) substrate. The sections were then counterstained with hematoxylin prior to mounting, and the DAB-positive cells were analyzed using a microscope platform (BX53 apparatus; Olympus, Tokyo, Japan).

## 7. Network pharmacology analysis

### 7.1. Active small molecule screening and target genes

The sample used in this study has been mentioned in the previous reports, and the components used in the network analysis were only detected by liquid chromatography and gas chromatography (Nam *et al.*, 2020; Song *et al.*, 2024). These components were selected using the *in silico* integrative Absorption, Distribution, Metabolism, and Excretion (ADME) model, based on which the components were screened for Oral Bioavailability (Impey *et al.*, 1999)  $\geq 30.0\%$  and  $-0.3 <$  Blood-Brain Barrier (BBB) according to Traditional Chinese Medicine System Pharmacology Database (TCMSP) (<https://www.tcmspe.com/index.php>). Drug Likeness (Sekeris *et al.*, 2021) values were 'yes' according to SwissADME databases (<http://www.swissadme.ch/>). The chemical information of all components was confirmed at PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and ChemSpider (<https://www.chemspider.com/>) databases.

Information on the proteins related to active components in AR was collected from the Search Tool for Interactions of Chemicals and Proteins (STITCH) database (<http://stitch.embl.de/>, ver. 5.0) with '*Homo sapiens*' and SwissTargetPrediction (<http://www.swisstargetprediction.ch/>) databases (Park *et al.*, 2022). Information on genes was verified in the UniProt database (<https://www.uniprot.org/>). The Venn diagram was drawn using Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>).

### 7.2. Potential target genes and Protein-Protein Interaction (PPI) network

Potential target genes were defined as proteins that overlapped with the protein-derived active components and proteins linked to encephalitis. Information on encephalitis-derived proteins was searched for the GeneCards: The Human Gene Database (<https://www.genecards.org/>, version 5.19). The PPI network was built after searching the STRING database (<https://string-db.org/>, version 12) with '*Homo sapiens*', and the number of first shell interactors did not exceed 50 with a confidence score  $\geq 0.400$  (medium confidence) (Park *et al.*, 2022). Topology analysis including degree, closeness, and betweenness centrality

of the PPI network was conducted using Cytoscape version 3.10.1 (<https://cytoscape.org/>).

### 7.3. Analysis of signaling pathways

Signaling pathways were analyzed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/>, updated January 12, 2024 version) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg/>) with  $p < 0.05$ . The network was visualized using Cytoscape version 3.10.1 (Cytoscape, Boston, MA, USA).

## 8. Statistical analysis

The analysis parameters are presented as mean  $\pm$  standard error (SE). Statistical significance was identified by one-way analysis of variance, followed by Holm-Sidak post hoc test using GraphPad Prism 10 (GraphPad software, San Diego, CA, USA). Statistical significance was set at  $p < 0.05$ .

## RESULTS

### 1. The antioxidative activities of AR extract in DPPH- and ABTS-scavenging assays

To determine the antioxidant activity of AR extract, we measured free radical scavenging rates in DPPH and ABTS assays at different concentrations of the extracts (0, 5, 10, 20, 100, and 500 mg/mL; Fig. 1). AR extracts at concentrations  $\geq 20$  mg/mL exerted significantly higher antioxidative activities in DPPH ( $p = 0.610$  and  $p < 0.001$  for the other comparisons) and ABTS ( $p = 0.009$  and  $p < 0.001$  for remaining comparisons) assays compared with AR 5 mg/mL-treated group. At concen-

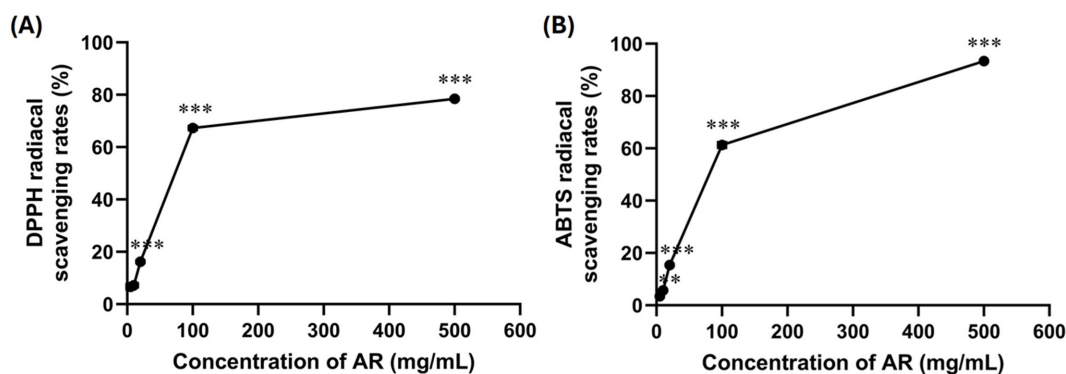
trations below 100 mg/mL, the AR extract exhibited a steeply increasing antioxidant effect in a dose-dependent manner, whereas the slope of scavenging rates became gentler above 100 mg/mL.

### 2. Protective effects of AR extract in the CYP-induced cognitive impairment

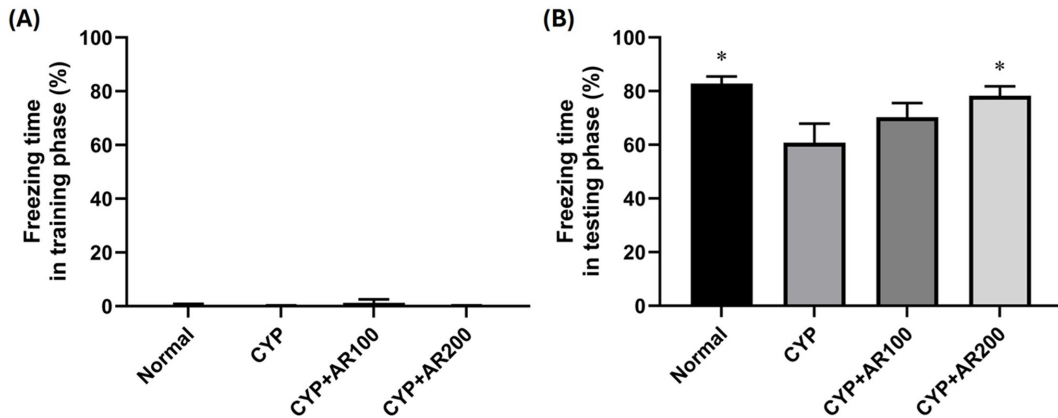
We evaluated memory and cognitive performance by measuring the freezing time during the contextual fear conditioning test (Fig. 2). There were no differences between groups in the training phase, but in the testing phase, the CYP group showed a significantly lower freezing time than that of the control group ( $p = 0.015$ ). However, AR pretreatment before CYP administration tended to increase the freezing time, although the significance was observed in only CYP+AR200 group ( $p = 0.040$ ).

### 3. Ameliorative effect of AR extract against CYP-induced suppression of hippocampal neurogenesis

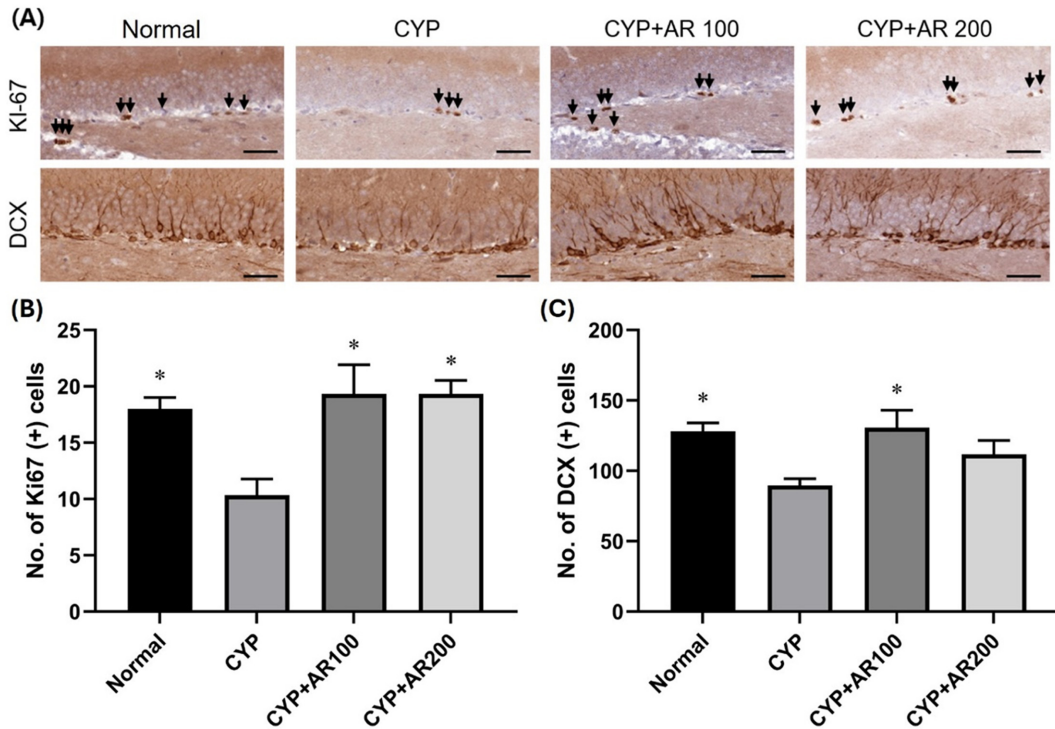
Proliferating progenitor cells and immature neurons in the dentate gyrus (DG) of adult mouse hippocampus were quantified based on the number of Ki67- and DCX-positive cells, respectively (Fig. 3). Representative microscopic images are presented in Fig. 3A. CYP administration significantly decreased the positive cells of Ki67- (Fig. 3B) and DCX-positive cells (Fig. 3C) compared with the control group ( $p = 0.016$  and  $p = 0.033$ , respectively). However, pretreatment with AR at 100 and 200 mg/kg ameliorated the reduction of Ki67-positive cells (both  $p = 0.016$ ), and 100 mg/kg of AR significantly increased the expression of DCX-positive cells ( $p = 0.033$ ).



**Fig. 1. The percentage of DPPH (A) and ABTS (B) radical scavenging activity showed the antioxidant benefits of AR.** All data are presented as mean  $\pm$  standard error (SE). \*\* $p < 0.01$  and \*\*\* $p < 0.001$  indicates a significant difference compared to AR 5 mg/mL-treated group. DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); AR, *Agastache rugosa* extract.



**Fig. 2. Freezing time (%) during training (A) and testing (B) phases of the contextual fear conditioning test.** All data are presented as mean  $\pm$  standard error (SE).  $p < 0.05$  indicates a significant difference compared to CYP group. CYP, cyclophosphamide treated group; CYP+AR100, cyclophosphamide and *Agastache rugosa* extract at a dose of 100 mg/kg treated group; CYP+AR200, cyclophosphamide and *Agastache rugosa* extract at a dose of 200 mg/kg treated group.



**Fig. 3. Pretreatment of AR extract improved the decrease in the number of Ki67- and DCX-positive cells in the subgranular zone of the hippocampus after CYP treatment.** (A) Representative microscopic images of Ki67- (arrows) and DCX-positive cells in the hippocampal DG. Quantitative analysis of Ki67-positive cells (B) and DCX-positive cells (C). Scale bar is 100  $\mu$ m. All data were expressed as mean  $\pm$  standard error (SE).  $*p < 0.05$  indicates a significant difference compared to CYP group. CYP, cyclophosphamide treated group; CYP+AR100, cyclophosphamide and *Agastache rugosa* extract at a dose of 100 mg/kg treated group; CYP+AR200, cyclophosphamide and *Agastache rugosa* extract at a dose of 200 mg/kg treated group. DCX: Doublecortin.

#### 4. Active small molecules and target genes

The literature search was conducted for 11 active components identified through liquid and gas chromatographic analy-

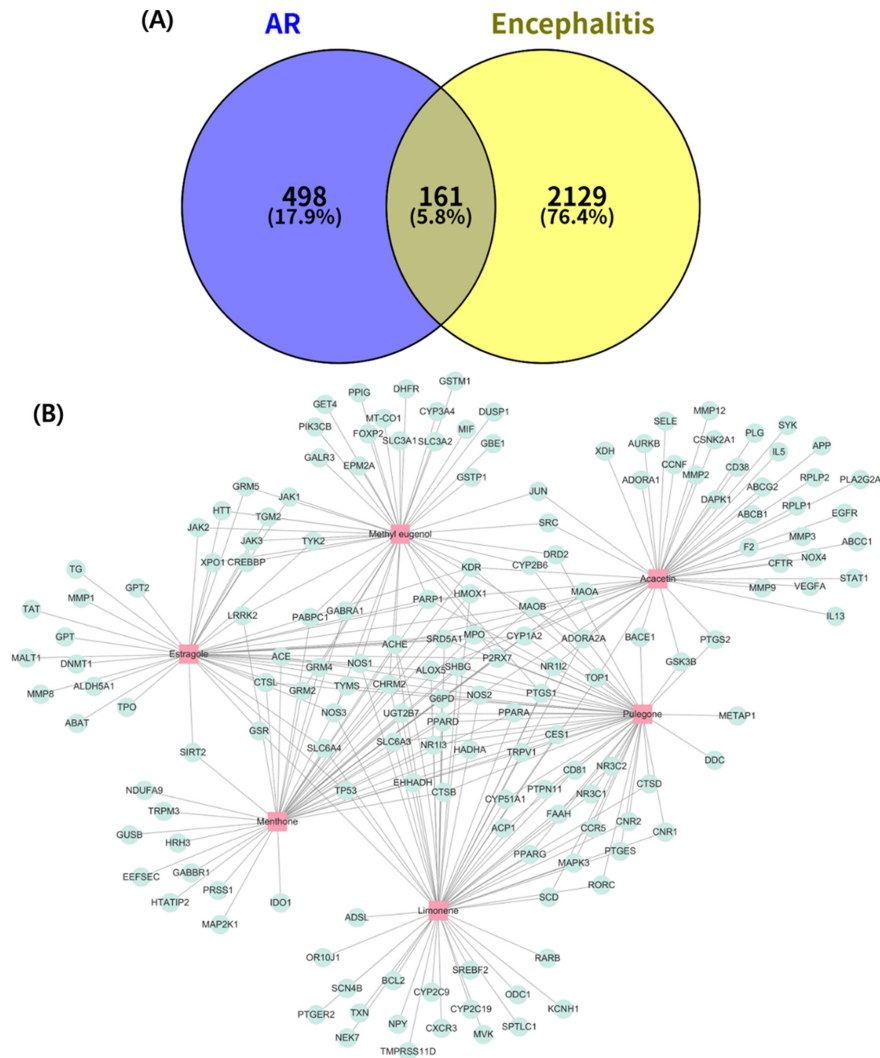
ses in a 70% ethanol extract of AR (Nam *et al.*, 2020; Song *et al.*, 2024). These components were rosmarinic acid, tilianin, acacetin 7-O-(6"-O-malonyl)-beta-D-glucopyranoside, isoagasta-

**Table 1.** Information related to active components was collected from public databases.

Components	TCMSP		SwissTargetPrediction
	OB (%)	BBB	DL
Acacetin	34.97	-0.05	Yes
Limonene	39.84	2.12	Yes
Menthone	57.90	1.75	Yes
Estragole	36.59	1.83	Yes
Pulegone	51.60	1.74	Yes
Methyl eugenol	73.39	1.41	Yes

TCMSP, traditional Chinese medicine system pharmacology database; OB, oral bioavailability; BBB, blood-brain barrier; DL, drug likeness.

choside, acacetin 7-O-(2''-O-acetyl-6''-malony)-beta-D-glucopyranoside, acacetin, limonene, menthone, estragole, pulegone, and methyl eugenol. Rosmarinic acid and tilianin did not pass the ADME criteria according to public databases. Acacetin 7-O-(6''-O-malonyl)-beta-D-glucopyranoside, isoagastachoside, and acacetin 7-O-(2''-O-acetyl-6''-malony)-beta-D-glucopyranoside were excluded because of lack of related ADME data from public databases. Finally, six components including acacetin, limonene, menthone, estragole, pulegone, and methyl eugenol were selected. Their OB was  $\geq 30\%$  and  $-0.3 < BBB$  according to TCMSP. DL values were 'yes' according to SwissADME databases. Structurally, these components were classified as one flavonoid (acacetin) and five monoterpenes (limonene, menthone, estragole,



**Fig. 4.** Network of six active components–161 potential target proteins related to encephalitis. (A) Venn diagram showing the intersection of 659 proteins associated with AR and 2,290 proteins involved in encephalitis. (B) The network comprised 167 nodes and 285 edges. Pink rectangles are active components, and light blue ovals represent potential target proteins. AR, *Agastache rugosa* extract.

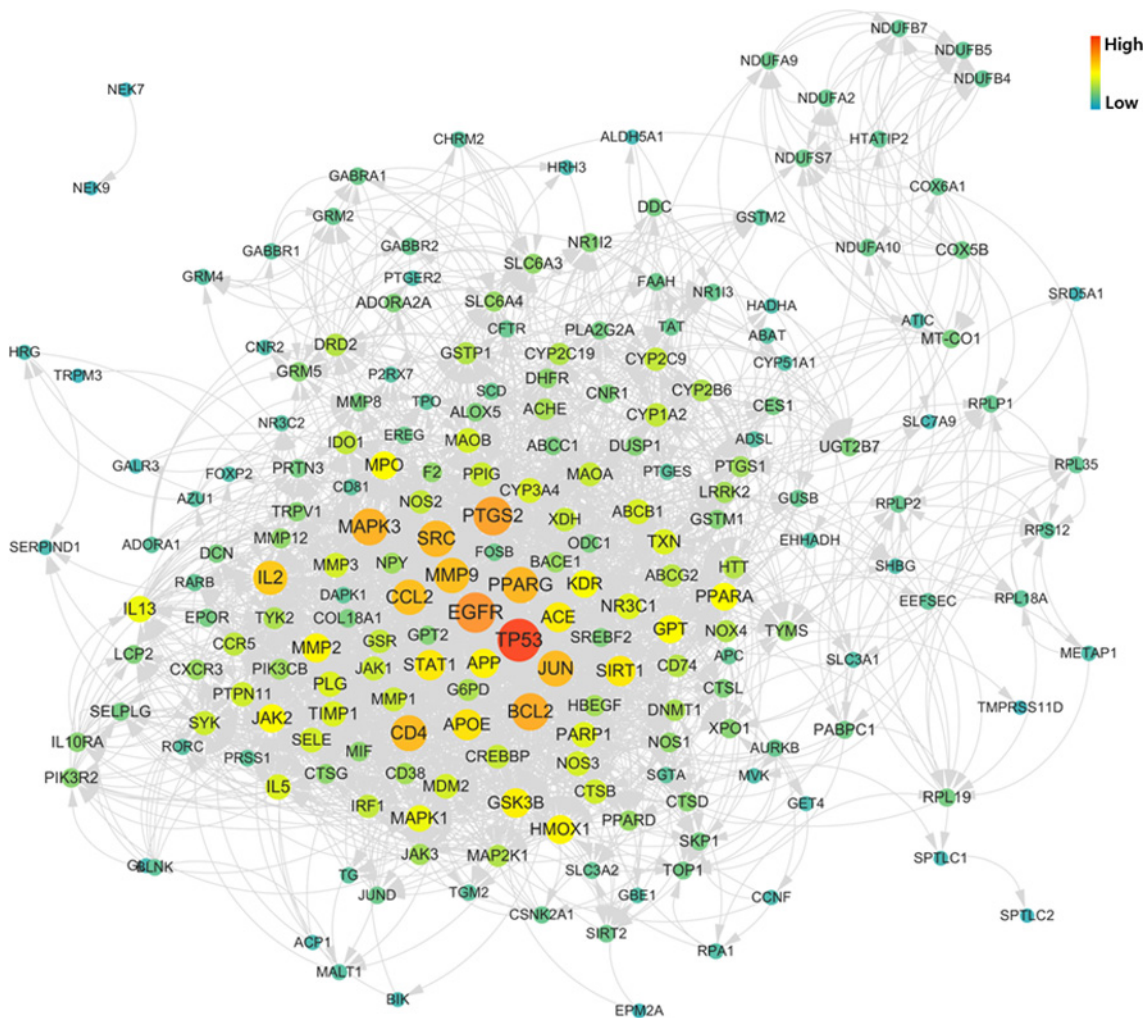
pulegone, and methyl eugenol). The monoterpenes had higher OB, DL, and BBB values than the flavonoids (Table 1).

### 5. Potential target genes and PPI

Based on STITCH and SwissTargetPrediction databases, 659 genes associated with the six active components of AR were identified, and 2,290 encephalitis-related proteins from the GeneCards database were retrieved. The overlap between the two datasets resulted in 161 common proteins (Fig. 4A). The network between 161 potential target proteins and six active components is shown in Fig. 4B. This network consisted of 167 nodes and 285 edges, of which ACHE, KDR, and PARP1 were the core proteins associated with five active components. ALOX5, CYP1A2, HMOX1, MAOB, MPO, NOS2, and SRD5A1 were related to four active components. CCR5,

which had a high relevance score with encephalitis in the GeneCards database, had links to limonene and pulegone.

We conducted the PPI analysis for analyzing the interaction of 161 target proteins using the frequently used STRING database (Wei *et al.*, 2021) and visualized the PPI network using Cytoscape 3.10.1. The topological characteristics are calculated by the “network analyzer” function of this software (Chen *et al.*, 2021; Gao *et al.*, 2021). The genes with a high confidence score were TP53, BCL2, CCL2, CD4, EGFR, IL2, JUN, MAPK3, MMP9, PPARG, PTGS2, and SRC (Fig. 5). A high confidence score indicates strong evidence for functional or physical relationship between target proteins. The top 10 proteins of edge count were TP53, EGFR, PTGS2, BCL2, MAPK3, SRC, PPARG, JUN, CD4, and CCL2. The top 10 proteins of betweenness centrality were EGFR, MAPK3, IL2,



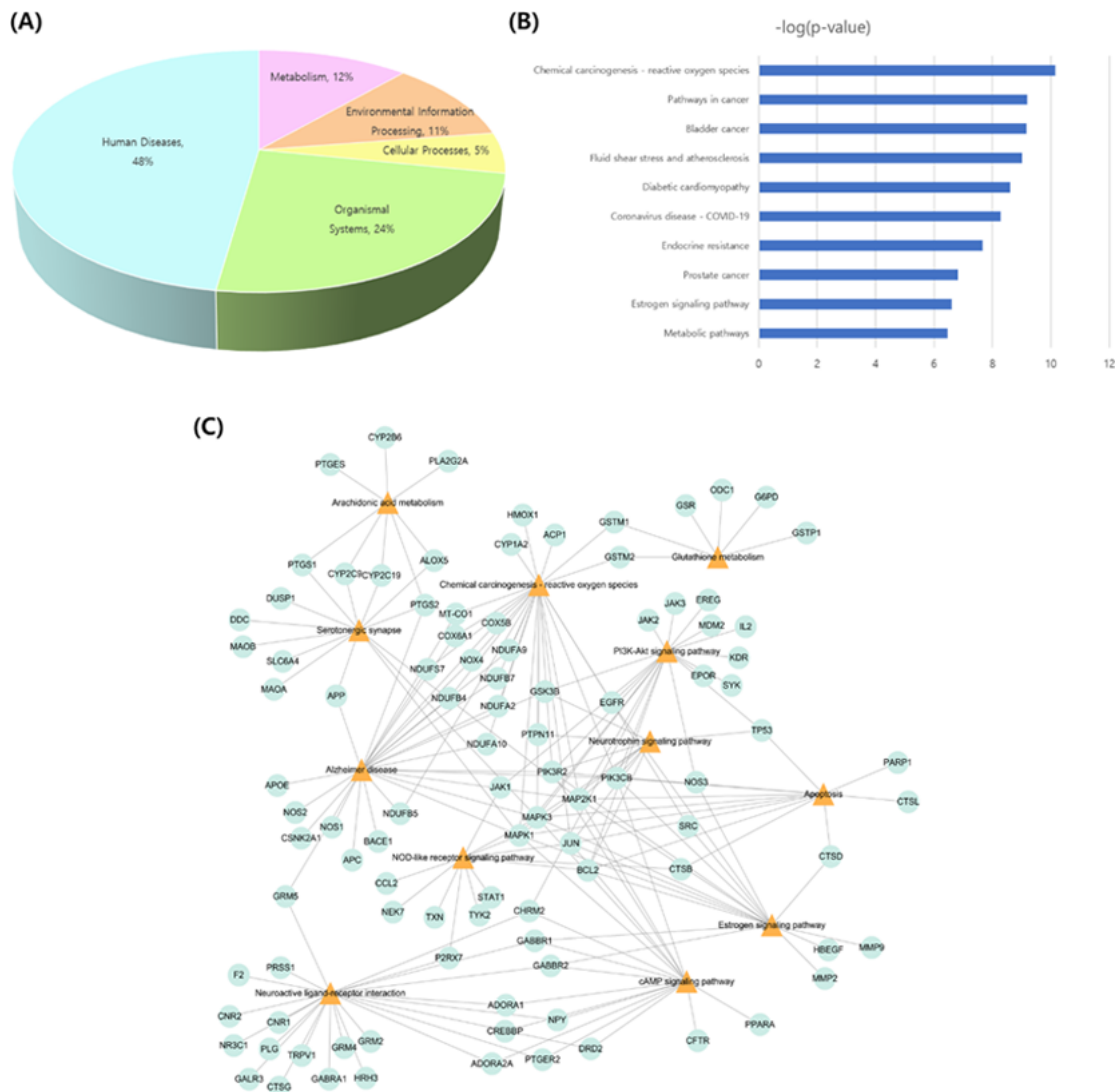
**Fig. 5. PPIs analysis of potential target proteins.** Large red circles mean high confidence score, and the number of arrows indicates primary related proteins.

PPARG, GPT, MMP9, JUN, BCL2, RPL19, and DRD2. The betweenness centrality indicates the frequency with which the shortest paths between any pair of nodes pass through that node; a larger value in the network indicates that the node has greater importance (Lee *et al.*, 2020).

### 6. Pathway analysis related to encephalitis

The signaling pathways were analyzed using the DAVID and KEGG databases with the p-value ( $p < 0.05$ ) correction algorithm (Fig. 6) (Xu *et al.*, 2022). A total of 128 pathways were searched in the DAVID database, 48% of which were pathways classified into the category of human diseases (Fig.

6A), and the top 10 pathways are shown in Fig. 6B). The 12 pathways related to encephalitis were serotonergic synapse, estrogen signaling pathway, neuroactive ligand-receptor interaction, chemical carcinogenesis-reactive oxygen species cAMP signaling pathway, arachidonic acid metabolism, apoptosis, PI3K-Akt signaling pathway, NOD-like receptor signaling pathway, neurotrophin signaling pathway, Alzheimer’s disease, and glutathione metabolism. A total of 86 potential target proteins were involved in the encephalitis-related pathways (Fig. 6C). The six proteins BCL2, JUN, MAP2K1, MAPK1, MAPK3, and PIK3CB were associated with more than five pathways.



**Fig. 6. Categories of KEGG pathways of AR (A), top 10 of KEGG pathway enrichment analysis of potential target genes of AR (B), and the network consisting of encephalitis-related proteins (mint oval) and KEGG pathways (orange triangles) with 112 nodes and 189 edges.**

## DISCUSSION

In this study, we demonstrated the neuroprotective effects of AR extract in a mouse model of CYP-induced neurodegeneration. We confirmed the anti-oxidant activity of AR extract. Additionally, pretreatment of AR extract significantly improved the cognitive impairment in contextual fear conditioning test and restored the reduced immunoreactivities of DCX and Ki67 in the hippocampus of CYP-treated mouse. Furthermore, by analyzing active ingredients of AR extract based on the published literature and conducting a network pharmacology analysis of AR-related target genes in CYP-induced neuroinflammation, we predicted several potential target genes that may be involved in the protective effects of AR extract against CYP-induced neurodegeneration.

Although chemotherapy is known to cause cognitive impairment in cancer patients, the underlying mechanisms remain poorly understood. Previous studies have shown a link between chemotherapy-induced hippocampal-dependent memory deficits and impairment in hippocampal neurogenesis (Sekeres *et al.*, 2021). Similarly, in the present study, we identified cognitive impairment and decrease of hippocampal neurogenesis in CYP-administered mice. Among several mechanisms underlying CYP-induced neurogenesis impairment, neuroinflammation and oxidative stress appear to play critical roles. Intraperitoneal injection of CYP induces inflammatory responses in the central nervous system, leading to the activation of glial cells (Hirshman *et al.*, 2020). Activated glial cells secrete inflammatory cytokines, which could inhibit the proliferation and differentiation of neural progenitor cells in the hippocampal DG and damage the neurogenic microenvironment (Monje *et al.*, 2003; Sato, 2015). In parallel, CYP metabolism generates reactive oxygen species and toxic aldehyde byproducts, which induce oxidative stress in neural stem cells, subsequently leading to the decline in neurogenesis (Hussein *et al.*, 2024). CYP-induced impairment in neurogenesis and synaptic plasticity, likely driven by neuroinflammation and oxidative stress, could ultimately be associated with cognitive impairment (Ibrahim *et al.*, 2024). Therefore, targeting neuroinflammation and oxidative stress may offer a promising strategy to ameliorate the chemotherapy-induced cognitive deficits.

AR has been traditionally used to treat digestive disorders, including diarrhea, abdominal discomfort, and vomiting (Ko *et al.*, 2013). Several studies have shown that AR extract and its compounds exhibit various therapeutic effects including anti-

oxidant (Desta *et al.*, 2016), anti-microbial (Shin, 2004), anti-inflammatory (Zielińska and Matkowski, 2014), and cardiovascular effects (Cao *et al.*, 2017). To explore the target genes potentially involved in the therapeutic effects of AR against CYP-induced neuroinflammation, we conducted a network pharmacology analysis based on AR compounds identified in our previous study (Nam *et al.*, 2020; Song *et al.*, 2024). 11 constituents of AR were classified as five flavonoids (acacetin, tilianin, acacetin 7-O-(6"-O-malonyl)-beta-D-glucopyranoside, isoagastachoside, acacetin 7-O-(2"-O-acetyl-6"-malony)-beta-D-glucopyranoside), one phenylpropanoid (rosmarinic acid) and five monoterpenes (limonene, menthone, estragole, pulegone, and methyl eugenol). Among the 11 components, five were excluded due to lack of information in the databases or failure to meet the ADME criteria. Subsequent network analysis showed that five small molecules including limonene, menthone, estragole, pulegone, and methyl eugenol had high values of OB, DL, and BBB permeability. These five compounds exhibit significant potential in improving cognitive function through various mechanisms including anti-inflammation, anti-oxidation and neuroprotection (de Sousa *et al.*, 2024; Eddin *et al.*, 2021; Gogoi *et al.*, 2020; Park *et al.*, 2022; Roy *et al.*, 2018; Wang and Heinbockel, 2018). These characteristics collectively indicate that the AR compounds could be promising candidates for the treatment of neurodegenerative diseases.

Next, to identify potential genes associated with the therapeutic effects of AR compounds in CYP-induced neuroinflammation, we performed a target prediction analysis followed by PPI network and functional enrichment analyses. The 659 proteins associated with AR and the 2,290 encephalitis-related proteins formed an intersection of 161 proteins. Analysis of the interactions between 161 proteins revealed that TP53, BCL2, CCL2, CD4, EGFR, IL2, JUN, MAPK3, MMP9, PPARG, PTGS2, and SRC were key nodes exerting significant influence within the PPI network. Among the proteins, BCL2, EGFR, JUN, MAPK3, and PPARG were identified as overlapping proteins with both the top 10 proteins ranked by edge count and the top 10 proteins ranked by betweenness centrality. Of the five proteins, BCL2, JUN, and MAPK3 were found to be involved in more than five of 12 KEGG pathways related with encephalitis. The KEGG pathways that included all three proteins were estrogen signaling pathway, apoptosis, and neurotrophin signaling pathway. Meanwhile, there are various studies about that BCL2, JUN, and MAPK3 are associated with neuroinflammation, neurodegeneration and cognitive deficits. BCL2,

generally recognized as anti-apoptotic protein, could mediate neuronal differentiation by regulating lipopolysaccharide-induced inflammation in cortical neural stem cells, suggesting a protective effect of BCL2 in neuroinflammation (Park and Han, 2022). c-Jun, a product of *JUN* gene, plays a complex role by regulating neurodegeneration, neuroinflammation, neuronal regeneration, and neuroplasticity (Raivich, 2008). Similarly, MAPK3, also known as ERK1, is essential for normal physiological brain function such as learning and memory, but it also involved in neuroinflammation and neurodegeneration (Impey *et al.*, 1999; Peng *et al.*, 2010; Sun and Nan, 2017). Therefore, dysregulating the three key genes, BCL2, JUN, and MAPK3, may induce alternation in neuronal microenvironment, ultimately leading to cognitive deficits.

In conclusion, we confirmed the protective effects of AR extract, which attenuated neuronal damage in a CYP-induced cognitive impairment mouse model. Furthermore, based on the network pharmacology analysis, AR compounds may exert their neuroprotective effects against CYP-induced neuroinflammation through key candidate target genes, BCL2, JUN, and MAPK3, although further validation is required to confirm these targets and their roles.

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