



The ameliorative effects of honeysuckle extract and its major component luteolin on autism-like behaviors in the NDE1 deficiency model

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ABSTRACT

Objective: To evaluate the effects of honeysuckle extract and its active component, luteolin, on the autistic-like behaviors and neuroinflammatory responses in NDE1-deficient autism spectrum disorder (ASD) zebrafish models. Also, to assess whether differences exist in their behavioral improvement effects and impacts on brain inflammatory factor expression levels.

Methods: Behavioral phenotyping (hyperactivity, stereotypic back-and-forth swimming, and 1VS6 social preference/grouping tests) and molecular analyses (quantification of NF- κ B, IL6, TNF α , and IL1 β) were performed on NDE1-deficient zebrafish treated with honeysuckle extract or luteolin.

Results: Honeysuckle extract improved two core symptoms of ASD, small circling repetitive stereotyped behavior and 1VS6 social preference behavior. While luteolin enhanced one core symptom, shoaling behavior, and one comorbid symptom, hyperactive locomotor activity. Molecularly, honeysuckle extract normalized IL6 levels, and luteolin reduced IL1 β overexpression; their effects on brain inflammation in the NDE1-deficient autism model differed.

Conclusion: Both honeysuckle extract and luteolin demonstrated behavioral rescue and anti-neuroinflammatory effects in NDE1-deficient ASD zebrafish models. The ameliorating effects of luteolin on ASD-related behaviors and neuroinflammation are supported by literature, while the beneficial effects of honeysuckle on ASD-related behaviors represent a novel finding of this study, highlighting medicinal plants and plant-derived compounds as potential ASD therapeutics. Given honeysuckle's traditional Chinese medicinal and food uses, established safety, and superior improvement of core ASD symptoms compared to luteolin, it may offer a safer autism treatment option than luteolin-based small-molecule medication.

1. Introduction

Autism Spectrum Disorder (ASD) is a pervasive neurodevelopmental condition with global implications, affecting an estimated 0.7%–2.3% of children, highlighting its significant public health impact and the urgency of research efforts.¹ The etiology of ASD is multifaceted, with genetic factors playing a predominant role, and environmental influences adding to the complexity.² Genetic studies have identified rare variants in hundreds of genes, yet the mechanisms remain unclear, and we still lack targeted pharmacological interventions.¹ Among the numerous potential mechanisms, neuroinflammation is one of the significant ones.³ Multiple clinical and basic research studies have suggested the important role of the neuroinflammation mechanism in the occurrence and development of autism.^{4–6}

NDE1, encoding a cytoskeletal protein, plays a pivotal role in cellular processes such as chromosome segregation, neural precursor differentiation, and neuronal migration, which are crucial for proper neurological development.^{7,8} Located within the 16p13.11 chromosome segment, associated with neuropsychiatric disorders, NDE1 has been implicated in conditions like autism due to its significant impact on brain function and structure.⁹ Studies have shown that deletions in the NDE1 region are strongly correlated with intellectual disability, epilepsy, and autism, highlighting the gene's importance in cognitive and social behavior.^{10–12}

Our previous work established the link between NDE1 deficiency and autism-like behaviors through zebrafish models, where *nde1* homozygous deletion leads to increased locomotor activity, repetitive behaviors, and impaired social interactions, which are characteristic of autism.¹³

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Moreover, these *nde1*-deficient zebrafish exhibit heightened neurological apoptotic responses and brain inflammatory reactions, indicating a molecular basis for the observed autism-related phenotypes.¹³ Minocycline treatment significantly improves NDE1 deletion mutations' social deficits and hyperactive locomotor activity, and didn't rescue the repetitive behaviors. These findings underscore the necessity of further research to unravel the intricate mechanisms by which *NDE1* mutations lead to neuroinflammation and autism-like behaviors, and to explore more effective interventions.

Honeysuckle, with Latin name as *Lonicera japonica* Thunb. and Chinese name as Jin Yin Hua, has been an integral part of traditional Chinese medicine for centuries. It is revered for its detoxifying and heat-clearing properties, which are particularly effective in treating conditions associated with exopathogenic wind-heat, such as epidemic febrile diseases and sores.¹⁴ Many key bioactive compounds have been identified in honeysuckle, including essential oils, organic acids, and flavones, which contribute to its anti-inflammatory, antibacterial, antiviral, antioxidative, and hepatoprotective activities.¹⁴ The anti-inflammatory properties of honeysuckle extracts have been of particular interest, as they have demonstrated the ability to inhibit the production of pro-inflammatory cytokines and modulate immune responses. This has positioned honeysuckle as a promising candidate for the development of novel therapeutics in modern medicine, especially in the context of inflammatory diseases, such as autism.

Luteolin, a natural flavonoid with the chemical structure of 3',4',5,7-tetrahydroxyflavone, is found in various plants including honeysuckle.¹⁴ This bioactive compound has garnered significant attention for its potential neuroprotective properties. Luteolin has been recognized for its anti-inflammatory and antioxidant activities, which are particularly relevant in the context of neuroinflammation and associated cognitive impairments. Existing research has demonstrated luteolin's ability to suppress the release of pro-inflammatory cytokines, such as TNF α and IL-1 β , which play pivotal roles in neuroinflammatory responses.^{15–18} Studies have shown that luteolin can inhibit the production of these cytokines in lipopolysaccharide (LPS)-stimulated BV2 microglia cells, a common *in vitro* model for neuroinflammation.¹⁹ Furthermore, luteolin has been reported to mitigate cognitive deficits and neuronal damage in LPS-induced neuroinflammatory mouse models, suggesting its potential in alleviating inflammation-induced cognitive impairments.^{19,20} The neuroprotective effects of luteolin are likely mediated through the inhibition of microglial activation and the subsequent reduction of pro-inflammatory mediators, highlighting its therapeutic potential in autism.

In this study, we investigated whether honeysuckle extract and luteolin could intervene in the abnormal behaviors and neuro-inflammatory responses associated with NDE1 deficiency. The results indicated that both substances were capable of rescue social deficits, or repetitive stereotypic behaviors, hyperactive locomotor activity caused by NDE1 deficiency. Additionally, they were able to differentially ameliorate the overexpression of inflammatory factors in the brain.

2. Materials and methods

2.1. Zebrafish maintenance

Zebrafish were sourced from the Zebrafish Research Platform affiliated with the Children's Hospital of Fudan University, and were raised following the same protocol as previously described.¹³ All procedures comply with the guidelines established by the institutional animal care committee of Children's Hospital of Fudan University, China. All procedures were approved by the Research Ethics Board of Children's Hospital of Fudan University, China (Approval number: 2022-311).

2.2. Medication treatment

Referring to published methods, an ethanol extraction method was

employed to obtain honeysuckle extract.²¹ Honeysuckle was ground into a fine particulate powder using a grinder and then soaked with 70% ethanol in a conical flask at a solid-to-liquid ratio of 1:15 (g:mL). The flask was sealed with aluminum foil and subjected to continuous extraction in a water bath at 70 °C for 6 h. During the extraction process, the conical flask was shaken several times to prevent the residue of herbal powder on the flask wall. After 6 h, the filtrate from the conical flask was collected into a 50 mL centrifuge tube and centrifuged at 5000 rpm for 10 min. The supernatant was retained after discarding the precipitate and transferred to a centrifuge tube. Using a concentrator plus concentrator, the extract was concentrated to a paste without an ethanol odor under vacuum concentration mode, V-AL mode, and room temperature. The extract was then dissolved in 0.4% DMSO to prepare a stock solution with a concentration of 50 mg/mL, which was diluted to the appropriate working concentration with system water before the experiment.

0.5 g Luteolin (ACMEC, 491-70-3, Shanghai, China) was dissolved in 10 mL of 2% ethanol to prepare a stock solution, which was diluted to a working concentration of 80 μ M before the experiment.

For chronic toxicity analysis, developmentally normal wild-type zebrafish were randomly placed in each experimental group for study, with daily observations of the survival status of the zebrafish. In the early developmental stage, we designed six concentration gradients: 0, 0.1, 1, 10, 100, and 1000 μ g/mL. In the mid-developmental stage, we used four concentration gradients: 0, 0.1, 1, and 10 μ g/mL.

For intervention analysis, 2 month post fertilization (mpf) zebrafish were used, and four groups were set up: wild type control group (*nde1*^{+/+}), wild type with medication treatment group (*nde1*^{+/+} + hs represents wild type with honeysuckle extract treatment, *nde1*^{+/+} + lut represents wild type with luteolin treatment), *nde1* deficient group (*nde1*^{-/-}), and *nde1* deficient with medication treatment group (*nde1*^{-/-} + hs represents *nde1* deficient with honeysuckle extract treatment, *nde1*^{-/-} + lut represents *nde1* deficient with luteolin treatment). The wild type control group and *nde1* deficient group were normally raised without medication treatment. The medication treatment groups received 1 μ g/mL of honeysuckle extract or 80 μ M luteolin treatment for four weeks, followed by relevant analyses. Fresh honeysuckle extract working solution or luteolin working solution was replaced every day.

2.3. Locomotor activity analysis

Assessments of zebrafish locomotor activity behavior were conducted at a temperature of 28.5 °C utilizing a novel tank (inner dimensions, 30 × 30 × 20 cm) (refer to Fig. 2). Quantification of the data was achieved using ZebraLab software (developed by ViewPoint Behavior Technology, France), with the video capture rate set at 25 frames per second (fps) and the data aggregated into 1-min intervals. The detection threshold was calibrated to 29, which was determined to be an optimal setting for precisely tracking the motion paths of the larval zebrafish.

2.4. Repetative behavior analysis

Upon investigating the locomotor activity of zebrafish, we use the same data to analyze small circling and back-and-forth stereotypic movements. Each of these behavioral assessments commenced following a brief acclimation phase (lasting 1–2 min) during which the zebrafish became accustomed to their new environment within the tank. A back-and-forth motion was characterized by a single traversal along one side or adjacent sides of the tank, followed by a return to the starting point. Small circling was identified as the zebrafish's repetitive actions confined to a limited space, with the total distance traveled from start to finish not exceeding 30 mm and the duration of continuous movement surpassing 5 s. Once these stereotypic behaviors were clearly delineated, the corresponding data were systematically collected via a computer algorithm throughout the course of the experiment.

2.5. 1VS6 social preference and shoaling behavior analysis

To assess shoaling behavior, six zebrafish were placed in a new tank of internal size $30 \times 30 \times 20$ cm (see Fig. 3A). After acclimating for 1–2 min, their behavior was recorded for 30 min, with the mean interspace within the shoal derived from camera footage serving as the shoaling metric.

For the social preference test, an isolated zebrafish was put in a standard breeding tank compartment (internal size $21 \times 10 \times 7.5$ cm), facing six wild type zebrafish separated by a transparent plastic barrier (Fig. 3C). Following acclimatization, the fish's behavior was recorded and quantified based on its proximity to the group, measured by distance or duration.

2.6. RT-qPCR analysis

RT-qPCR was performed utilizing a LightCycler® 480 system from Roche, Germany, in conjunction with Super-Real PreMix Plus supplied by Tiangen, China, following the protocol provided by the manufacturers. For each sample, quadruplicate measurements were conducted, with the β -actin serving as a reference gene for normalization. Relative quantification of RNA expression levels was determined employing the $2^{-\Delta\Delta Ct}$ formula. A tabulation of the RT-qPCR primer sequences can be found in the supplementary material, designated as Table S1.

2.7. Quantification and statistical analysis

In this research, data evaluation and graphical representation were executed utilizing GraphPad Prism version 9.0. For the survival curve, the Kaplan-Meier estimator and log-rank test for trend were utilized. For independent datasets, we first conduct normality and homogeneity of variance tests using the Shapiro-Wilk and Levene's tests, respectively. Datasets that meet the assumptions of normality and homogeneity of variance are analyzed with parametric ANOVA. The Kruskal-Wallis test is employed for datasets that fail to meet these assumptions. In the event of a significant interaction effect in two-way ANOVA, simple effects analyses are performed. For data without a significant interaction effect, post-hoc Tukey's test is used to conduct multiple comparisons among groups.

3. Results

3.1. Chronic toxicity analysis of honeysuckle extract in zebrafish during early and mid-development

Honeysuckle is considered highly safe for human consumption and is classified as both a food and a medicinal herb in China. However, due to the high permeability of zebrafish skin, which results in increased absorption of substances, it is essential to conduct a toxicity analysis of honeysuckle extract immersion treatment. After a three-day treatment from 5 days post fertilization (dpf) to 7 dpf, the results indicated that

concentrations below $10 \mu\text{g/mL}$ had no lethal effects on wild type early-development zebrafish (Fig. 1A). Log-rank (Mantel-Cox) test shows significant differences between survival curves ($P = 0.0185$), and log-rank test for trend shows the survival rate exhibited a significant decreasing trend in a dose-dependent manner with increasing drug concentration ($P = 0.0023$). In the mid-developmental stage, we used four concentration gradients: 0, 0.1, 1, and $10 \mu\text{g/mL}$. Starting from 1.5 mpf, the treatment lasted for three weeks, and the results showed that concentrations below $1 \mu\text{g/mL}$ had no lethal effects on mid-development zebrafish (Fig. 1B). Log-rank (Mantel-Cox) test shows significant differences between survival curves ($P = 0.0293$), and log-rank test for trend shows the survival rate exhibited a significant decreasing trend in a dose-dependent manner with increasing drug concentration ($P = 0.0201$). In subsequent intervention studies with honeysuckle extract, a concentration of $1 \mu\text{g/mL}$ was utilized.

3.2. Honeysuckle extract rescued small circling stereotypic behaviors in *nde1*^{-/-} zebrafish

Hyperactivity and stereotypic behaviors are two characteristic behaviors resulting from *nde1* deficiency. After a four-weeks treatment with $1 \mu\text{g/mL}$ of honeysuckle extract, we analyzed the two characteristic behavioral phenotypes of the animal model.

For the locomotor activity, two-way ANOVA analysis didn't show an interaction effect ($F(1,43) = 3.543$, $P = 0.0666$). However, post-hoc Tukey's test revealed a significant difference between control group *nde1*^{+/+} and model group *nde1*^{-/-} ($P = 0.0009$), suggesting hyperactivity in *nde1*^{-/-} zebrafish. Although the direct comparison between the *nde1*^{-/-} + hs group and the *nde1*^{-/-} group did not reach statistical significance ($P = 0.1963$), the lack of significant difference between the *nde1*^{-/-} + hs group and the group ($P = 0.1523$) indicates that the honeysuckle extract has the potential to rescue the hyperactivity induced by *nde1* deficiency (Fig. 2A and B).

For the small circling stereotypic behaviors, two-way ANOVA analysis showed a significant interaction effect ($F(1,44) = 6.752$, $P = 0.0127$) (Fig. 2C), and post-hoc Tukey's test revealed a significant difference between control group *nde1*^{+/+} and model group *nde1*^{-/-} ($P = 0.0376$), *nde1*^{-/-} + hs group and *nde1*^{-/-} group ($P = 0.0058$), and the lack of significant difference between the *nde1*^{-/-} + hs group and the *nde1*^{+/+} group ($P = 0.8932$) indicates that honeysuckle extract is effective in improving small circling stereotypic behaviors in *nde1*^{-/-} zebrafish.

For the back-and-forth stereotypic behaviors, two-way ANOVA analysis didn't show a significant interaction effect ($F(1,43) = 0.4330$, $P = 0.5140$) (Fig. 2D).

3.3. Honeysuckle extract improves 1VS6 social preference but not shoaling behavior in *nde1*^{-/-} zebrafish

Social deficits are a characteristic autistic behavior resulting from *nde1* deficiency. In this study, we used shoaling and 1VS6 social

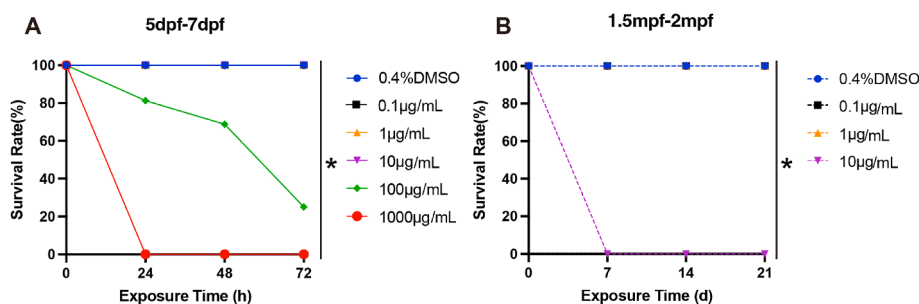


Fig. 1. A. Survival curves of wild type zebrafish embryos at different concentration gradients ($n = 16$). B. Survival curves of zebrafish at mid-development stage at different concentration gradients ($n = 6$). Data are analyzed using Log-rank (Mantel-Cox) test, $*P < 0.05$.

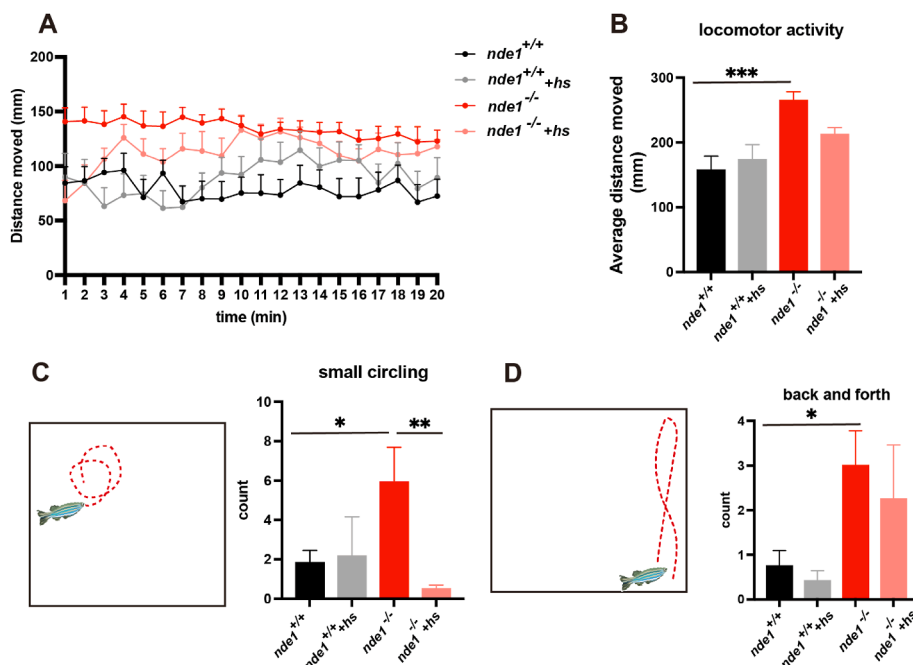


Fig. 2. A. Trend of locomotor activity distance of 3 mpf zebrafish every minute ($n = 12$). B. Average distance moved per minute ($n = 12$). C. Schematic diagram and count of small circling repetitive behavior ($n = 12$). D. Schematic diagram and count of back and forth repetitive behavior ($n = 12$). Data are analyzed using two-way ANOVA analysis and presented as the mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

preference tests to analyze whether honeysuckle extract could improve social deficit behaviors. The results showed that after four weeks of treatment, honeysuckle extract did not improve shoaling behavior (Fig. 3A and B), and two-way ANOVA analysis didn't show a significant interaction effect ($F(1,44) = 0.4773$, $P = 0.4933$). However, it significantly improved the time and distance spent in the social area by *nde1*-deficient zebrafish in the 1VS6 social preference test (Fig. 3C and D), and two-way ANOVA analysis showed significant interaction effect ($F(1,44) = 5.736$, $P = 0.0209$, and $F(1,44) = 5.201$, $P = 0.0275$, respectively), and post-hoc Tukey's test revealed a significant difference between control group *nde1*^{+/+} and model group *nde1*^{-/-} ($P = 0.0026$, $P = 0.0027$, respectively), *nde1*^{-/-} + hs group and *nde1*^{-/-} group ($P = 0.0025$, $P = 0.0047$, respectively), and the lack of significant difference between the *nde1*^{-/-} + hs group and the *nde1*^{+/+} group ($P = 0.8807$, $P = 0.9974$, respectively) indicates that the honeysuckle extract rescued the 1VS6 Social Preference deficit induced by *nde1* deficiency.

3.4. Luteolin inhibits hyperactive locomotor activity in *nde1*^{-/-} zebrafish

Luteolin is one of the main components of honeysuckle, and existing studies have suggested its potential in treating autism.²² To investigate the effects of luteolin on the abnormal behaviors of *nde1*-deficient zebrafish, we conducted further pharmacological treatments and behavioral analyses. Previous studies have indicated that 80 μ M is the maximum tolerated dose for 48 h post-fertilization (hpf) zebrafish larvae; hence, this concentration was utilized in our research.²³ The results showed that 80 μ M luteolin improved the hyperactive locomotor activity in *nde1*^{-/-} zebrafish (Fig. 4A and B), and two-way ANOVA analysis showed significant interaction effect ($F(1,44) = 13.37$, $P = 0.0007$), post-hoc Tukey's test revealed a significant difference between control group *nde1*^{+/+} and model group *nde1*^{-/-} ($P = 0.0437$), *nde1*^{-/-} + lut group and *nde1*^{-/-} group ($P = 0.0001$), and the lack of significant difference between the *nde1*^{-/-} + lut group and the *nde1*^{+/+} group ($P = 0.2263$) indicates that the luteolin rescued the hyperactivity induced by *nde1* deficiency.

For the small circling and back-and-forth stereotypic behaviors, two-way ANOVA analysis didn't show significant interaction effect

(compared *nde1*^{-/-} + lut group with *nde1*^{-/-} group, $F(1,44) = 1.307$, $P = 0.2592$ and $F(1,44) = 0.3414$, $P = 0.5620$, respectively) (Fig. 4C and D).

3.5. Luteolin improves shoaling behavior but not 1VS6 social preference in *nde1*^{-/-} zebrafish

After four weeks of luteolin treatment, the shoaling behavior of *nde1* zebrafish was significantly improved (Fig. 5A), and two-way ANOVA analysis showed a significant interaction effect ($F(1,44) = 10.23$, $P = 0.0026$), post-hoc Tukey's test revealed a significant difference between control group *nde1*^{+/+} and model group *nde1*^{-/-} ($P < 0.0001$), *nde1*^{-/-} + lut group and *nde1*^{-/-} group ($P = 0.0003$), and the lack of significant difference between the *nde1*^{-/-} + lut group and the *nde1*^{+/+} group ($P = 0.8112$) indicates that the luteolin rescued the shoaling behavior of *nde1*^{-/-} zebrafish.

For the 1VS6 social preference behavior, two-way ANOVA analysis didn't show a significant interaction effect of time and distance ratio (compared *nde1*^{-/-} + lut group with *nde1*^{-/-} group, $F(1,44) = 0.05400$, $P = 0.8173$ and $F(1,44) = 0.001421$, $P = 0.9701$, respectively) (Fig. 5B and C).

3.6. Honeysuckle extract and luteolin differentially improve inflammatory factor levels in *nde1*^{-/-} zebrafish

nde1 Deficiency leads to neuroinflammatory responses in the zebrafish brain, with significant increases in inflammatory factors such as NF- κ B, IL1 β , IL6, and TNF α .¹³ We used semi-quantitative PCR to detect the effects of honeysuckle extract and luteolin on these inflammatory factors.

The results showed that honeysuckle extract significantly inhibited IL6 levels in the brain of *nde1*^{-/-} zebrafish. Two-way ANOVA analysis showed a significant interaction effect ($F(1,12) = 13.81$, $P = 0.0029$), and post-hoc Tukey's test revealed a significant difference between control group *nde1*^{+/+} and model group *nde1*^{-/-} ($P = 0.0002$), *nde1*^{-/-} + hs group and *nde1*^{-/-} group ($P < 0.0001$), *nde1*^{-/-} + hs group and *nde1*^{+/+} group ($P = 0.0077$), and also *nde1*^{+/+} + hs group and *nde1*^{+/+}

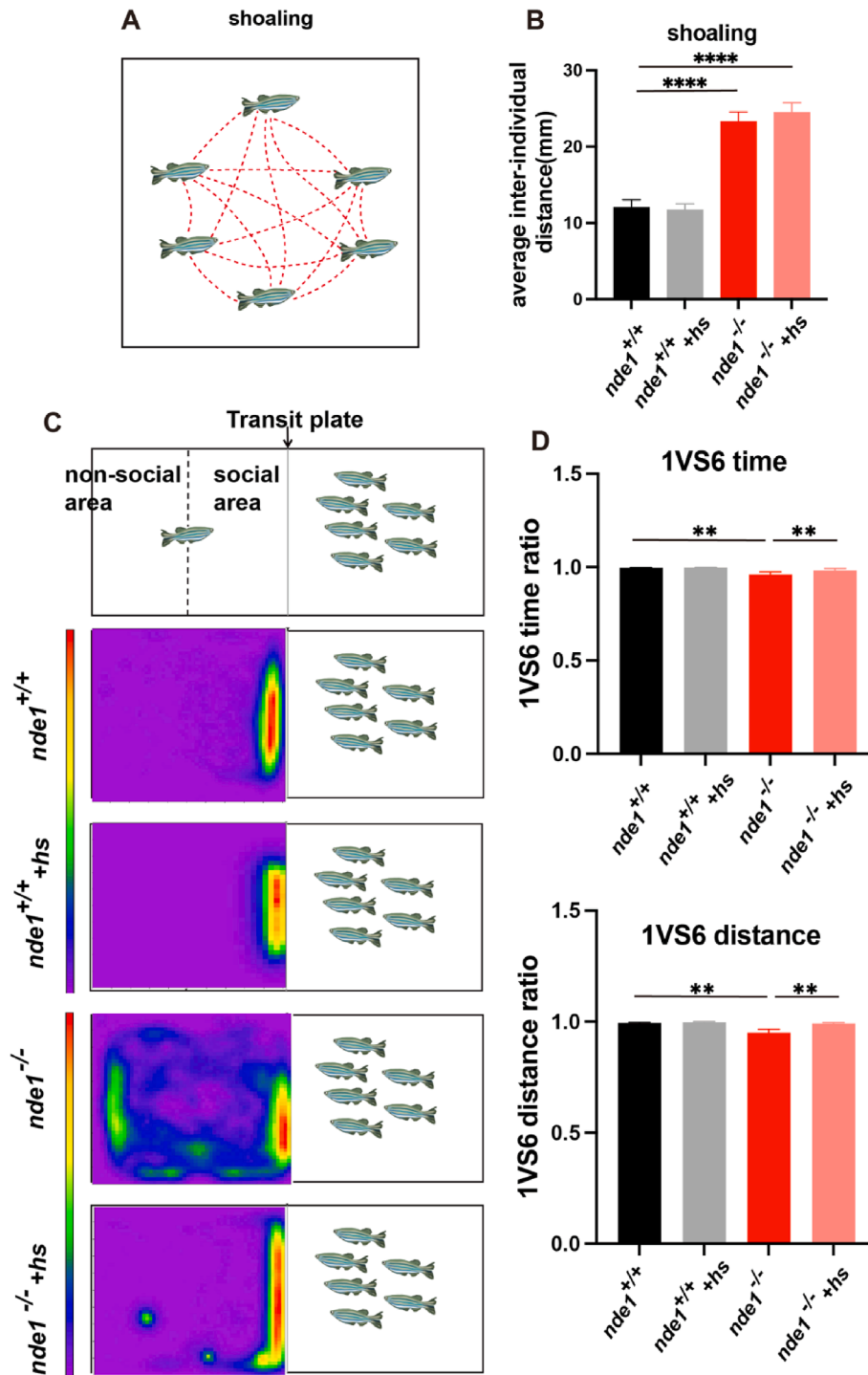


Fig. 3. A. Schematic diagram of shoaling behavior. B. Average inter-individual distance moved of zebrafish ($n = 12$). C. Schematic diagram and heat map of the 1VS6 social behavior experiment. D. The ratio of distance and time spent in the social area was improved by honeysuckle extract treatment in $nde1^{-/-}$ zebrafish ($n = 12$). Data are analyzed using two-way ANOVA analysis and presented as the mean $\pm$ SEM, $**P < 0.01$, $****P < 0.0001$.

group ($P = 0.0016$). For NF- κ B, two-way ANOVA analysis didn't show a significant interaction effect ($F(1,12) = 2.626$, $P = 0.1311$), and post-hoc Tukey's test revealed a significant difference between control group $nde1^{+/+}$ and model group $nde1^{-/-}$ ($P = 0.0065$), $nde1^{-/-} + hs$ group and $nde1^{-/-}$ group ($P = 0.0155$), and the lack of significant difference between the $nde1^{-/-} + lut$ group and the $nde1^{+/+}$ group ($P = 0.8305$) indicates that the honeysuckle extract has the potential to restore the NF- κ B level to normal. For IL1 β and TNF α , two-way ANOVA analysis didn't show a significant interaction effect ($F(1,12) = 0.4729$, P

$= 0.5047$ and $F(1,12) = 1.385$, $P = 0.2621$, respectively) (Fig. 6A).

Luteolin significantly inhibited IL1 β levels in the brain of $nde1^{-/-}$ zebrafish, two-way ANOVA analysis showed a significant interaction effect ($F(1,12) = 19.94$, $P = 0.0008$), and post-hoc Tukey's test revealed a significant difference between control group $nde1^{+/+}$ and model group $nde1^{-/-}$ ($P < 0.0001$), $nde1^{-/-} + lut$ group and $nde1^{-/-}$ group ($P < 0.0001$), and the lack of significant difference between $nde1^{-/-} + lut$ group and $nde1^{+/+}$ group ($P = 0.6517$). For IL6, two-way ANOVA analysis didn't show a significant interaction effect ($F(1,12) = 2.527$, P

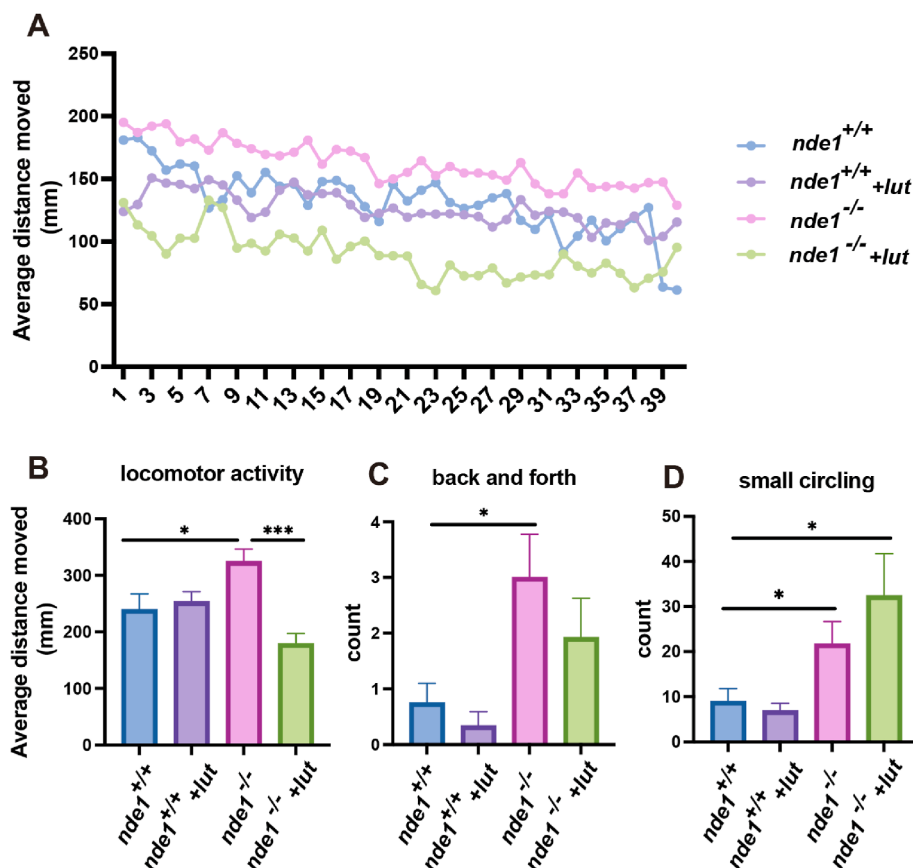


Fig. 4. A. Trend of locomotor activity distance of 3 mpf zebrafish every minute ($n = 12$). B. Average distance moved per minute ($n = 12$). C. Count of back-and-forth Repetitive behavior ($n = 12$). D. Count of small circling repetitive behavior ($n = 12$). Data are analyzed using two-way ANOVA analysis and presented as the mean $\pm$ SEM, * $P < 0.05$, *** $P < 0.001$.

= 0.1379), and post-hoc Tukey's test revealed a significant difference between control group *nde1*^{+/+} and model group *nde1*^{-/-} ($P = 0.0430$), *nde1*^{-/-} + lut group and *nde1*^{-/-} group ($P = 0.0009$), and the lack of significant difference between the *nde1*^{-/-} + lut group and the *nde1*^{+/+} group ($P = 0.9735$) indicates that the luteolin has the potential to restore the IL6 level to normal. For NF- κ B and TNF α , two-way ANOVA analysis didn't show a significant interaction effect ($F(1,12) = 4.333e-005$, $P = 0.9949$ and $F(1,12) = 0.8359$, $P = 0.3786$, respectively) (Fig. 6B).

4. Discussion

The results of this study indicate that honeysuckle extract and luteolin demonstrate considerable therapeutic potential for autism-related behaviors caused by NDE1 deficiency. A concentration of 1 μ g/mL honeysuckle extract is capable of ameliorating small circling repetitive stereotypic behaviors and 1VS6 social preference. Honeysuckle extract also significantly inhibits the higher level of pro-inflammatory factors IL6. Besides, honeysuckle extract also exhibits potential to rescue the hyperactivity and inhibit higher NF- κ B expression induced by *nde1* deficiency. Luteolin, one of the main components of honeysuckle, at a concentration of 80 μ M, can rescue hyperactive locomotor activity and shoaling behavior. It also significantly inhibits the higher level of pro-inflammatory factors IL1 β . Besides, luteolin also exhibits potential to inhibit higher IL6 expression induced by *nde1* deficiency.

The neuroinflammatory effects of honeysuckle extract and luteolin are highly likely the molecular basis for the improvement of autism-related behaviors. NDE1 deficiency leads to increased apoptosis in the brain and elevated expression of various pro-inflammatory cytokines.¹³ Such immune stress responses, particularly the pro-inflammatory

cytokines, can affect the surrounding microenvironment of cells, leading to the aggregation of more inflammatory factors, thereby triggering a cascade amplification reaction.^{24,25} In the persistent presence of NDE1 deficiency, the neuroinflammatory response is progressively amplified, eventually affecting the entire brain and inducing abnormal behaviors. Honeysuckle extract and luteolin, as well as minocycline that we previously published,¹³ all inhibited the progressively amplified neuroinflammatory response to varying degrees, thereby improving autism-related behaviors.

Although both possess neuroinflammatory capabilities, honeysuckle and luteolin differ in the types of behaviors they rescue. This may be related to the complex cellular networks and molecular mechanisms of neuroimmune stress. At the cellular level, neuroimmune stress involves different states and responses of microglia, oligodendrocytes, and astrocytes.^{26,27} At the molecular level, pathways involved in stress include cGAS-STING, NF- κ B, MAPK, mTOR, and others.^{3,25,28} Luteolin, a flavonoid monomer, has been extensively studied for its anti-inflammatory mechanisms. Existing research indicates that luteolin exhibits neuroprotective potential by upregulating the ER/ERK, PI3K/AKT, and Nrf2 pathways and downregulating the MAPK/JAK2/STAT pathway.^{19,29} Luteolin also reduces IL6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1.¹⁸ Meanwhile, over 140 compounds have been isolated from honeysuckle, primarily composed of essential oils, organic acids, and flavonoids, which collectively possess a wide range of pharmacological effects, including anti-inflammatory, antibacterial, antiviral, antioxidant, and hepatoprotective actions.¹⁴ Xu et al. evaluated the anti-inflammatory properties of honeysuckle extract in A549 cells, showing that it directly inhibits COX-1 and COX-2 activity, as well as COX-2 protein expression induced by IL1 β .³⁰ Yip et al.'s research indicates that honeysuckle extract can induce

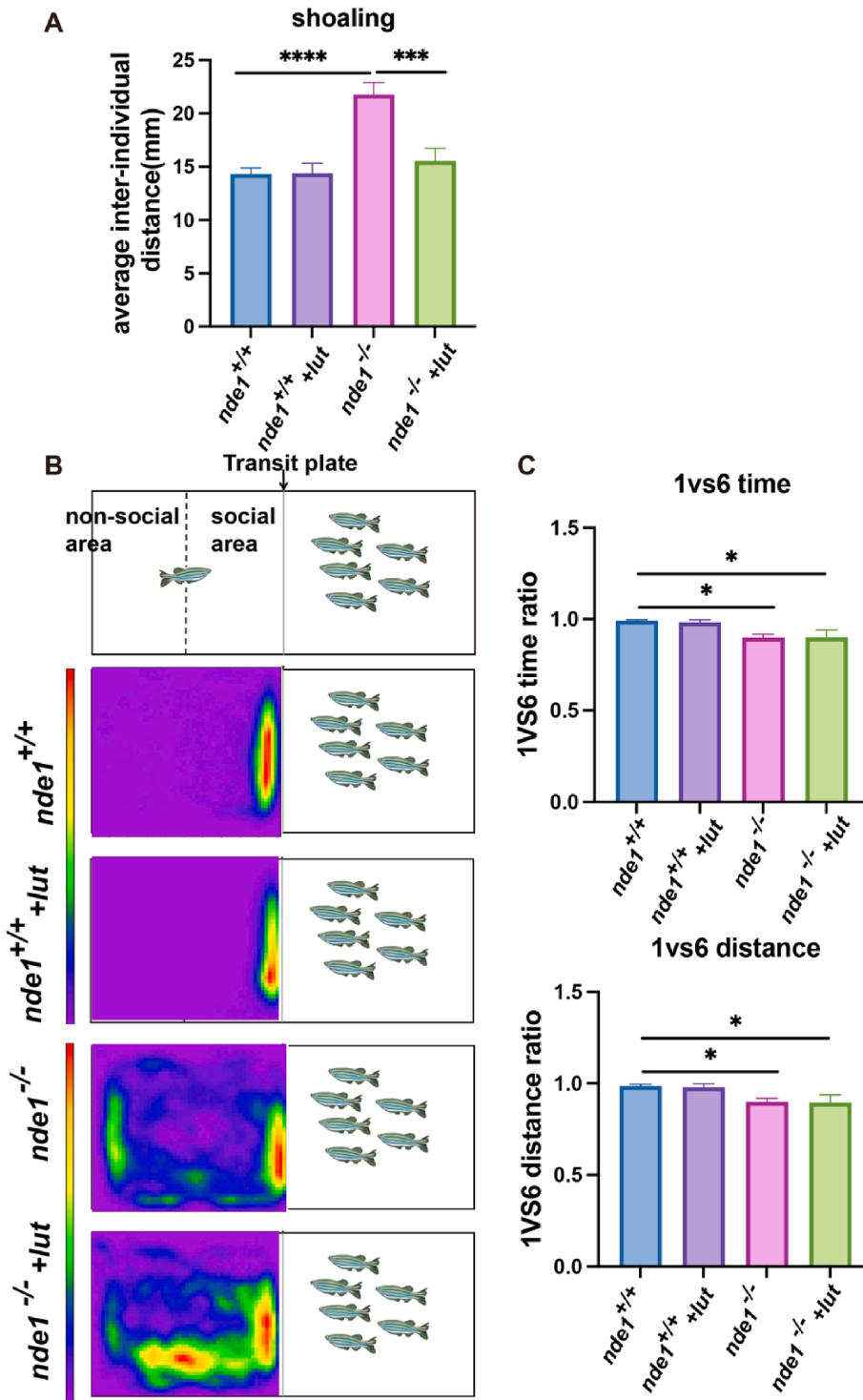


Fig. 5. A. Average inter-individual distance moved of zebrafish ($n = 12$). B. Schematic diagram and heat map of the 1vs6 social behavior experiment. C. The ratio of distance and time spent in the social area after luteolin treatment ($n = 12$). Data are analyzed using two-way ANOVA analysis and presented as the mean $\pm$ SEM, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

caspase-3-independent apoptosis by activating AIF.³¹ Additionally, the aqueous extract of honeysuckle acts on the JNK and p38 pathways, thereby exhibiting anti-cancer effects by inducing HepG2 cell death.³¹ Therefore, luteolin and honeysuckle extract influence both overlapping and unique signaling pathways. Within the overlapping pathways, the extent of their influence varies. For example, both luteolin and honeysuckle extract inhibit the JNK pathway, thereby reducing IL6 production. In this study, honeysuckle extract demonstrated a rescuing effect in

inhibiting IL6 levels, while luteolin only showed potential for rescuing IL6 levels (Fig. 6A and B). Due to these differences in pathway mechanisms, they may lead to different effects on neurons and, consequently, result in different behavioral interventions and restorative outcomes.

Neuroinflammatory stress plays a significant role in the pathogenesis of autism. Hundreds of risk factors associated with autism have been identified, including genetic and environmental factors.³² Currently, neuroinflammatory stress has been found in the molecular mechanisms

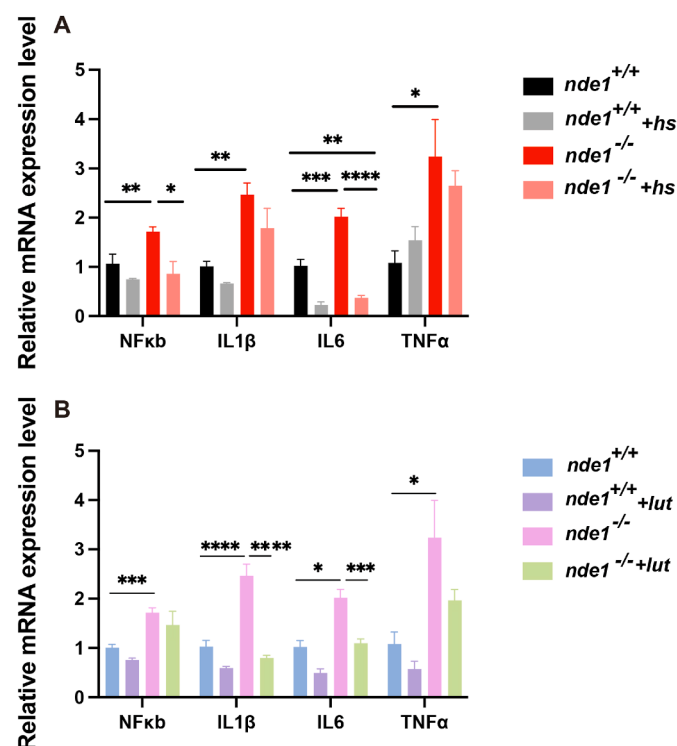


Fig. 6. The relative expression level of neuroinflammation related genes in the brain after honeysuckle (A) or luteolin treatment (B) ($n = 4$). Data are analyzed using two-way ANOVA analysis and presented as the means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

related to many risk factors of autism, such as SHANK3, CDKL5, CNTNAP2, NOMO1, and VPA exposure.^{33–37} Moreover, the suppression of corresponding immune stress can, to varying degrees, improve autism-related behaviors.^{36–38} This suggests that the application of immune stress inhibitors has the potential to intervene in many risk factors. In the recently published comment article by the researchers,³⁹ it is elaborated that these neuroimmune stress responses are part of Brain Damage caused by both internal and external risk factors. The accumulation and cascade amplification of brain damage are significant pathogenic mechanisms of autism. Therefore, as one of the means of Damage Control, honeysuckle extract and luteolin can naturally exhibit effects in the intervention of autistic behavioral characteristics.

Honeysuckle is a commonly used, easily cultivated, and highly safe traditional Chinese medicine. Luteolin is a flavonoid monomer with a high content in plants, and both have anti-inflammatory effects. Therefore, they are likely to not only have intervention potential for autism behaviors caused by NDE1 deficiency but also for many autism risk factors, which is worth further in-depth research.

5. Conclusion

Honeysuckle extract and luteolin both demonstrate behavioral rescue and anti-neuroinflammatory efficacy in NDE1-deficient ASD zebrafish models. Behavioral analyses showed that honeysuckle extract significantly improved small circling stereotypic behaviors and 1VS6 social preference behaviors, while luteolin improved hyperactive locomotor activity and shoaling behavior. Molecularly, honeysuckle extract restored IL6 to control levels, while luteolin rescued over-expression of IL1β. The ameliorating effects of luteolin on ASD-related behaviors and neuroinflammation are supported by literature, while the beneficial effects of honeysuckle on ASD-related behaviors represent a novel finding of this study, highlighting medicinal plants and plant-derived compounds as potential ASD therapeutics. Given honeysuckle's

traditional Chinese medicinal and food uses, established safety, and superior improvement of core ASD symptoms compared to luteolin, it may offer a safer autism treatment option than luteolin-based small-molecule medications.

CRediT authorship contribution statement

Qi Zhang: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Shenglan Gou:** Visualization, Methodology, Investigation. **Jia Lin:** Investigation. **Yinglan Zhang:** Methodology. **Qiang Li:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhip.2025.06.007>.

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