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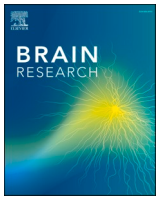

Gentisic acid exerts neuroprotective effects in neurotoxin-induced Parkinson's disease model in zebrafish: Cross-talk between pathways related with neurodegeneration in the gut-brain axis

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ABSTRACT

Given that global prevalence of Parkinson's disease (PD) is expected to rise over the next few decades, understanding the mechanisms and causes of PD is critical. With emphasis on gut-brain axis, we sought to assess the impact of gentisic acid (GA), a diphenolic compound generated from benzoic acid, in rotenone (Rot) induced PD model in zebrafish. For thirty days, adult zebrafish were exposed to GA and rotenone. Tox-Track program was used to analyze locomotor behaviors in the control, GA, Rot, and Rot + GA groups. LC-MS/MS was performed in brain and intestinal tissues. Proteome Discoverer 2.4 was used to analyze raw files, peptide lists were searched against *Danio rerio* proteins. Protein interactions or annotations were obtained from STRING database. Tyrosine hydroxylase (Th) staining was performed immunohistochemically in the brain. PD-related gene expressions were determined by RT-PCR. Lipid peroxidation, nitric oxide, superoxide dismutase, glutathione S-transferase, and acetylcholinesterase were measured spectrophotometrically. Improved locomotor behaviors were observed by GA treatment in Rot group as evidenced by increased average speed, exploration rate, and total distance. 5214 proteins were identified in intestinal tissues, 4114 proteins were identified in brain by LC-MS/MS. Rotenone exposure altered protein expressions related to oxidative phosphorylation in brain and intestines. Protein expressions involved in ferroptis and actin cytoskeleton changed in brain and intestines. Altered protein expressions were improved by GA. GA ameliorated Th-immunoreactivity in brain, improved *park2*, *park7*, *pink1*, and *lrrk2* expressions. Our results show that GA may be a candidate agent to be evaluated for its potential protective effect for PD.

1. Introduction

The second most prevalent neurological condition that affects the motor system is Parkinson's disease (PD). Cardinal motor impairment and synucleinopathy are PD's defining features. It is a chronic, progressive illness. Recently, it was shown that the gut microbiota profoundly affects the gut-brain axis through immunological, neuroendocrine, and neurological pathways. It's interesting to note that PD pathogenesis may begin with gastrointestinal symptoms brought on by disturbance of the gut-microbiome-brain axis. These symptoms may subsequently affect the motor system (Dogra et al., 2022). For this reason, the importance of the gastrointestinal tract and microbiota in PD continues to attract more attention day by day.

Examining neurodegenerative diseases with proteomic approaches allows revealing the affected metabolic pathways and the interactions between them. Neurodegenerative diseases, including PD, frequently exhibit protein deposits, cell death involving mitochondria, and oxidative stress. Patients get treatment to reduce symptoms, but because the fundamental causes are not addressed, the disease continues to worsen. In addition to well-known therapeutic alternatives, it's exciting to think

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about how nutrition may be able to delay the development or progress of the condition (Kasap et al., 2017;Wang, 2018).

The pharmacological activities of natural products have been the subject of scientific research in recent decades, leading to the generation and synthesis of novel treatments for the management and treatment of PD. Phenolic acids are well-known phytochemical elements that have been shown to have positive therapeutic benefits. They are produced as secondary plant products from tyrosine or phenylalanine via the shikimate pathway and are typically found in plants like fruits and vegetables (Russell and Duthie, 2011). Gentisic acid (2,5-dihydroxybenzoic acid) is a diphenolic chemical that is derived from benzoic acid. It comes from the root of the Gentiana species and is both a natural product and an aspirin metabolite, which is a common nonsteroidal anti-inflammatory medication (Abedi et al., 2020). Gentisic acid has been reported to have antiatherogenic, anticancer, antioxidant, and skeletal muscle relaxant properties (Ashidate et al., 2005; Sharma et al., 2004). The concentration-dependent inhibition of LDL oxidation and cholesterol ester hydro peroxides production by gentisic acid have been shwon. Gentisic acid has a strong ability to scavenge free radicals while having no chelating effect (Exner et al., 2000).

The inhibition of the generation of reactive oxygen species has been suggested to be the cause of the antiperoxide activity seen in animals treated with gentisic acid. Anti-Parkinson benefits of gentisic acid have been suggested in PD induced animals and the inhibition of oxidative stress, and lowering lipid peroxidation have been suggested as the possible mechanism (Kabra et al., 2014).

Based on the available data, we aimed to reveal in detail the molecular mechanism of the effects of gentisic acid in PD and, in particular, to examine the effects of gentisic acid on the gut-brain axis through the use of proteomic, biochemical, immunohistochemical, gene expression, and behavioral analyses.

2. Methods

2.1. Animals and treatment

The AB/AB strain of wild-type male and female zebrafish, aged 8 to 10 months, were kept in an aquarium rack system called ZebTEC (Tecniplast, Italy). Zebrafish were housed in disease-free conditions at 27 ± 1C under a 14/10 h light/dark cycle. The Directive 86/609/EEC of the European Communities Council, dated November 24, 1986, governed the use of animals in investigations. Experimental technique used in this work was authorized by Marmara University's Institutional Animal Care and Use Committee. Fish were randomly assigned to four groups, with fifteen fish in each group. Healthy fish treated with a vehicle were part of the control group. Exposure of fish in the rotenone group (R) to 5 μg/L rotenone (Sigma, USA) diluted in 0.1 % dimethyl sulfoxide (DMSO) (Sigma, USA) caused mortality. Fish in the gentisic acid group (GA) and R + GA were exposed to 4 mg/L gentisic acid. The dose of gentisic acid was modified and adapted to zebrafish from previous study of Fabinyi-Szebehely et al. (1953).

To guarantee consistent zebrafish ingestion, the water in the tanks of the control and rotenone-treated groups was replaced every 48 h. Twice a day, zebrafish were given Tetramine, a commercial fish meal. Tetramine composition is as follows: minimum 11 % crude oil, maximum 15 % ash, 51 % crude protein, 2.3 % calcium, 1.5 % phosphorus, and 6.5 % moisture. 3.39 kcal/g of energy and 0.36–0.65 mm in size make up each granule. Rotenone dosages were given according to our earlier research (Ünal et al., 2023; Unal et al., 2020; Yurtsever et al., 2020; Cansız et al., 2021).

After 4 weeks, the fish's locomotor activities were assessed, and they were euthanized by decapitation after which the brain and intestinal tissues were quickly removed for RNA extraction and biochemical testing. Data analysts were blinded to the experimental condition for the analysis of locomotor activity, biochemical parameters, and RT PCR.

2.2. Behavior assays

A rectangular transparent glass tank with dimensions of 21 cm by 9 cm by 11 cm and a volume of 1.5 l was utilized to measure the locomotor activities. Before being moved from their home tanks to the testing tank, fish were brought in their housing tanks to the behavioral room and allowed to acclimate to the surroundings for at least an hour. The examinations were conducted from 1 to 4 p.m. The tank's water temperature and lighting settings were meticulously matched to those of the home tank. The experiment video was started and monitored by a camera above the arena, which captured each fish's swimming pattern for five minutes.

The Tox-Track program, which finds and tracks animals inside enclosed arenas, was used to analyze the fish's average speed and total distance traveled (Rodriguez et al., 2018). Additionally, the exploration rate was established. Using the Tox-Track tracking software, a partition with 25 frames on the y-axis and 60 frames on the x-axis was created. The ratio of zebrafish activity in the vertical and horizontal sections of the tank was then measured and examined (Nadig et al., 2020).

2.3. LC-MS/MS proteomic assays

Three biological replicates and three technical replicates were prepared for each group in order to conduct proteomic analysis. The proteins were extracted and digested using the same procedure as in our earlier study (Sürmen et al., 2021), which was first published by Wiśniewski et al (2009). To put it briefly, 8 M urea was combined with 100 μg of protein extract and then added to the filter device. The proteins were then rinsed with urea and 50 mM ammonium bicarbonate, respectively, following a 5-minute reduction with DTT 95∘C and a 20-minute alkylation with iodoacetamide at room temperature. Trypsin (enzyme-protein ratio: 1:100) was then used to digest proteins for eighteen hours at 37 °C. Before being used, the obtained peptides were lyophilized and stored at −80 °C.

The methodology for the LC-MS/MS analysis followed that of our earlier work (Sürmen et al., 2021). In every group, a minimum of three technical replicates and three biological replicates were analyzed. The C18-reversed-phase analytical column (Thermo Scientific Acclaim PepMap100, C18, 2 μm particle size, 75 μm diameter) was connected to a reverse phase trap column (Thermo Scientific Acclaim PepMap100, C18 5 μm × 0.3 mm, nano Viper C18) that was loaded with the resuspended peptide yields in 0.1 % FA. The gradient of mobile phases A (0.1 % formic acid) and B (0.1 % formic acid in 85 % acetonitrile) was used to separate the peptide mixture at a flow rate of 300 nl/min, which was managed by Thermo Scientific Xcalibur software. For data-dependent acquisition, the Q Exactive Plus Mass Spectrometer (Thermo Fisher Scientific) was connected to the nanoLC system and run in the positive ion mode. At 200 m/z, peptides were found in the survey scan between m/z 400 and 2000, using an Orbitrap resolution of 70 K.

Thermo Scientific's Proteome Discoverer software (Version 2.3) was used to process the raw data that was obtained from the MS. The reference proteome of Danio rerio, which is accessible in the UniProtKB database (UniProt Consortium, 2019) and contains 62,031 sequences that were downloaded on December 25, 2021, was compared to the MS/MS spectra using the Sequest search engine. The peptide validator filter false discovery rate (FDR) was set to 0.01 for protein identification, and the molecular weight limits for the precursor and fragment tolerances were 10 ppm and 0.02 Da, respectively. The Proteome Discoverer platform computed P values for group comparisons using paired t-tests. Proteins with a statistically significant abundance ratio of ≥ 2 (up-regulation, $p < 0.05$) or ≤ 0.5 (down-regulation, $p < 0.05$) were deemed to be differentially expressed. Only proteins identified by two or more tryptic peptides (with protein FDR ≤ 0.01) were considered for further analysis following label-free protein quantitation.

Data sets were compared using the web-based InteractiVenn tool (Heberle et al., 2015). The network was visualized using the Cytoscape

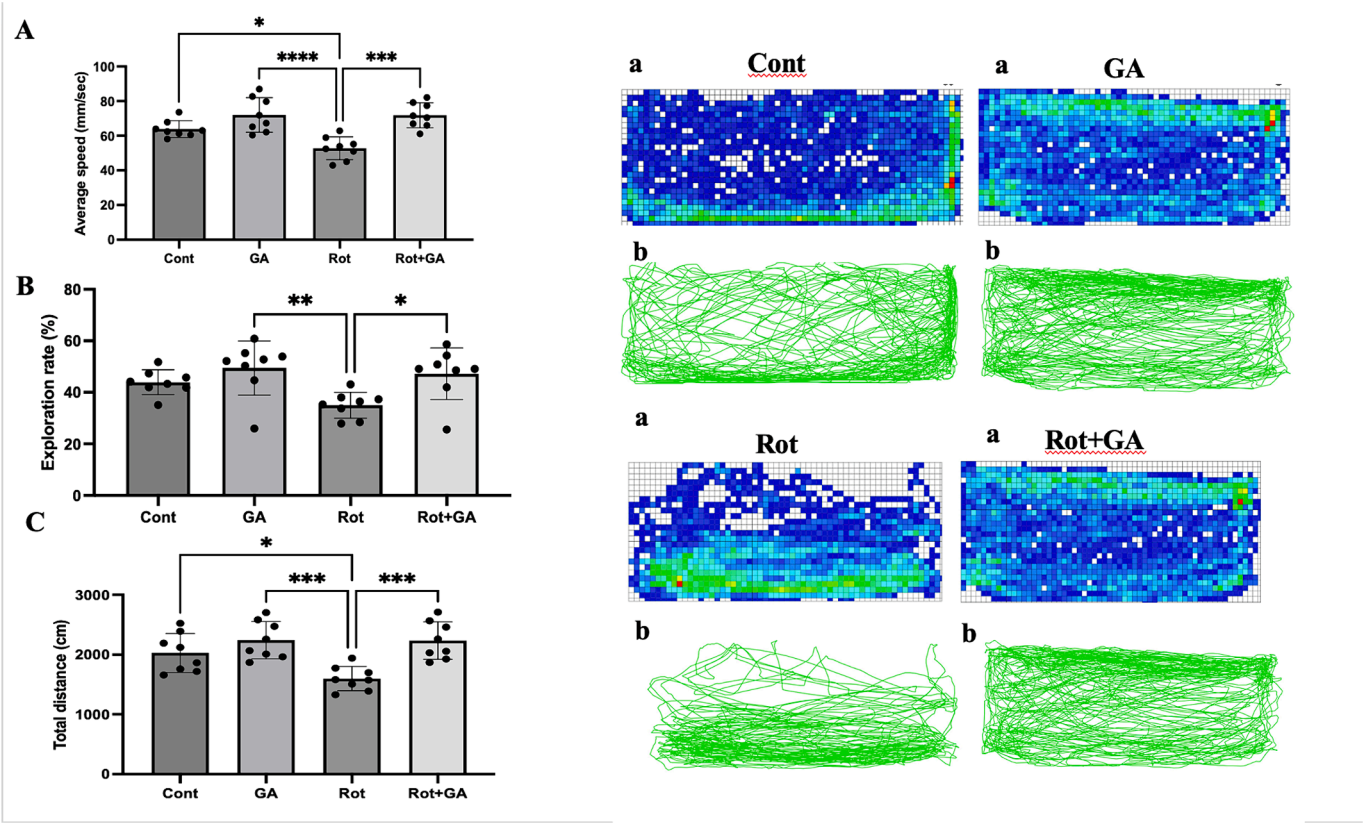

Fig. 1. Effects of rotenone and gentisic acid treatments on locomotor activities. (A) average speed; (B) Exploration rate, and (C) Total distance travelled (n = 8). Data is given as mean ± SD. Significant difference is indicated by an asterisk. *** $p < 0.001$, ** $p < 0.01$, ** $p < 0.05$. Cont: Control Group; GA: Gentisic Acid Group; Rot: Rotenone Group; Rot + GA: Rotenone + Gentisic Acid Group.

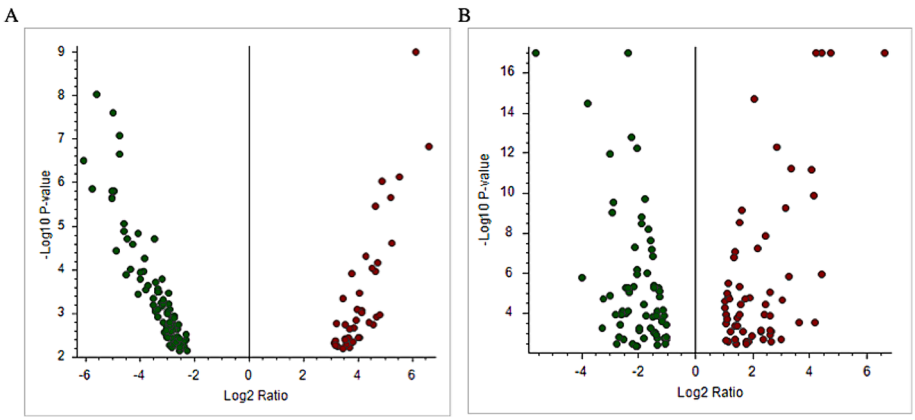

Fig. 2. Volcano plot of differentially expressed proteins between rotenone and control groups. Green spots indicate a ≤ 0.5-fold change and red spots indicate a ≥ 2-fold change. The list of significant proteins, their p values, and fold change (FC) are shown in Supplementary Table 2 and 5. a) intestinal tissue b) brain tissue.

program v3.8, and the STRING 11.0 software (https://string-db.org/) was used to conduct the protein interaction enrichment analysis. Disconnected nodes were hidden from the network and the minimum required interaction score was set at 0.7 (high confidence). The Cytoscape plugin BINGO tool was utilized to search for terms related to gene ontology (GO) (Maere et al., 2005). The enrichment analysis results were assessed using Fisher's Exact test ($p < 0.05$), and then the Benjamini-Hochberg False Discovery Rate correction was applied.

2.4. Biochemical assays

Fish were anesthetized before their brains and intestines were removed. For each group, tissues from four fish were combined to create a biological replicate, and three technical replicates were carried out for every biological replicate. Using physiological saline, tissues were homogenized to create 10 % (w/v) homogenates. The supernatant was separated and used for the biochemical parameters following a brief centrifugation. The total protein levels were assessed using Lowry's (1951) method, and the biochemical parameter results were expressed for each protein. As an end product of lipid peroxidation (LPO), malondialdehyde (MDA) was measured using the Yagi (1984) method. The LPO results were expressed as mg protein/nmol MDA.

Nitric oxide (NO) concentrations were calculated using the Miranda (2001) method, which involved reducing nitrate to nitrite with

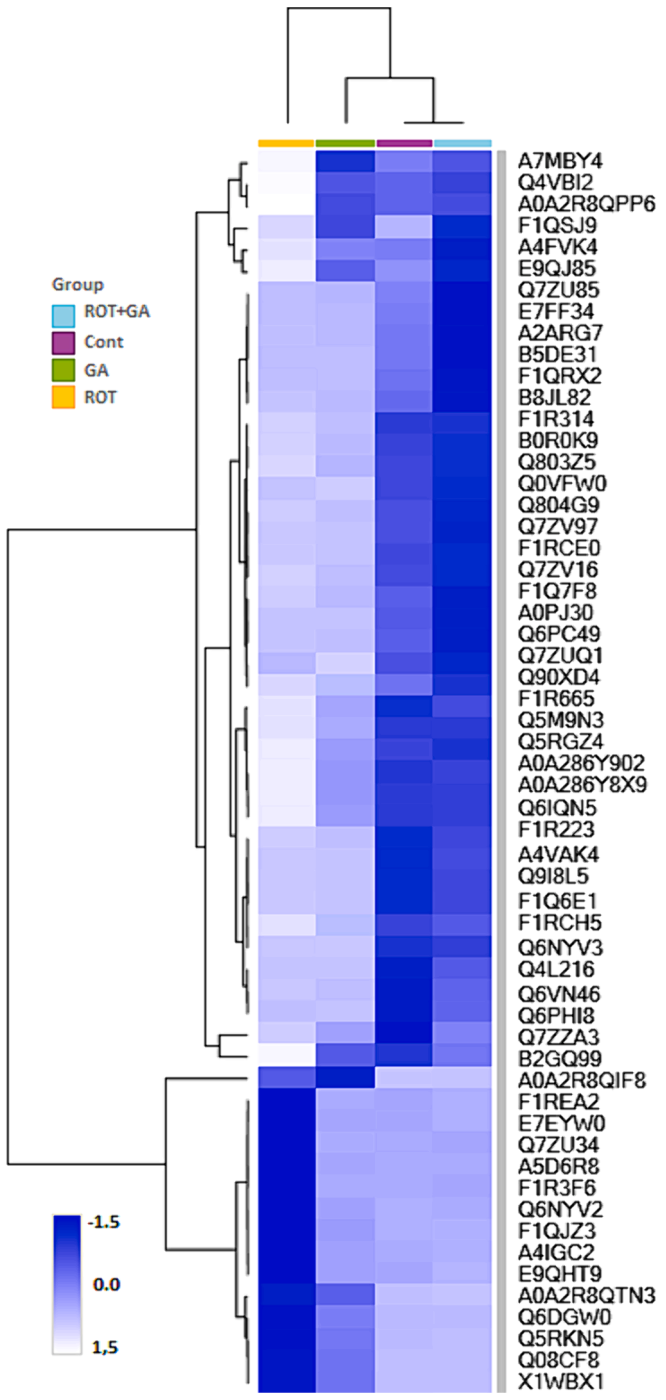

Fig. 3. Heatmap of 57 proteins in intestinal tissues responsive to gentisic treatment after exposure to rotenone.

vanadium (III) chloride. The results were expressed as nmol NO/mg protein.

The riboflavin-sensitized photo-oxidation reaction of o-dianisidine was used to assess the activities of superoxide dismutase (SOD), and the results were expressed as U/mg protein (Mylorie et al., 1986). Using a spectrophotometer set at 340 nm, the activity of glutathione-S-transferase (GST), which catalyzes the conjugation of glutathione, was determined (Habig and Jacoby, 1981). The method of Ellman (1961) was used to measure acetylcholinesterase (AChE) activity.

2.5. Reverse transcription (cDNA synthesis) and quantitative real-time PCR

Three fish's brain and intestinal tissues were pooled for each biological replicate in each group for the purpose of real-time PCR analysis. Three technical replicates were carried out for every biological replicate. The RNA from each pooled brain and intestinal tissue was isolated using the Rneasy Mini Kit and Qiacube (Qiagen, Germany). Aqiagen, Germany's RT2 Profiler PCR Arrays were utilized to create single-stranded cDNA. For the PCRs, Qiagen's DNA Master SYBR Green kit (Germany) was utilized. Using the Qiagen Rotor Gene-Q Light Cycler instrument, the expression levels of the PD-related genes park2, park7, pink1, and lrrk2 were measured. Using the Delta-Delta CT (DDCT) method, relative transcript levels were assessed by normalizing the levels to the housekeeping gene, β-actin (Livak and Schmittgen, 2001).

2.6. Immunohistochemical assays

To investigate the TH expression level in the dopaminergic neurons of the zebrafish brain, immunostaining of TH, a selected marker of dopaminergic neurons, was performed. Five zebrafish in each group (n = 5) were used for IHC. The brain was fixed overnight in 2 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for up to 24 h at 4 °C. The brains were incubated in 25 % sucrose solution (0.1 M phosphate buffer) for 2.5 h and in 35 % sucrose solution for 12 h at 4 °C until they sank. Then, the brains were embedded in sucrose (20 % w/v) – Gelatin (7.5 % w/v, Sigma) horizontally and flash-frozen in liquid nitrogen. Serial sections (~5 μm) were taken using a cryostat, and IHC was applied blindly to 11th sections taken equally from each tissue.

Serial sections (~5 μm) were taken using a cryostat, and IHC was applied blindly to 11th sections taken equally from each tissue. First of all, the slides were stored at − 20 °C until further use. Brain sections were post-fixed in ice-cold 4 % PFA (0.1 M phosphate buffer, pH 7.4) for 8 min at 4oC. The tissue sections were blocked and incubated with mouse monoclonal anti-zebrafish Tyrosine Hydroxylase (TH) antibody (1:4000, #22941; Immunostar, Hudson, USA) for labeling dopaminergic cells overnight at 4oC. For negative controls, antibody diluent (PBS) was used at the same concentration. The sections were washed three times for 5 min with TPBS (0.1 % Tween) and then, biotinylated secondary antibody (SHP125; ScyTek, West Logan, Utah) was applied for 20 min.

Next, the slides were treated with streptavidin-peroxidase solution (SHP125; ScyTek, West Logan, Utah), and a chromogen, 3,3′-Diaminobenzidine (DAB) (ACJ500; ScyTek, West Logan, Utah), was added. To identify the nuclei, the sections were then counterstained with Mayer's hematoxylin. Under an Olympus BX61 digital microscope (Olympus, Tokyo, Japan) connected to a computerized digital camera and CellSens software (DP72; Olympus, Tokyo, Japan), the sections were examined and photographed. The diencephalon images in each group were taken with identical adjustments and magnification. The zebrafish brain's diencephalon region was identified in accordance with Kaslin and Panula (2001) and Bartel et al. (2020).

Qupath software (Version 0.3.2; 2021) was used to assess the dopaminergic neurons in the diencephalon region and count the number of TH-positive cells in equivalent sections (11th) from each group (Hein et al., 2021; Mysona et al., 2020; Bankhead et al., 2017). The same threshold and modification were used for positive cell detection.

2.7. Statistics

GraphPad Prism 9.0 (GraphPad Software, San Diego, USA) was used for all statistical analyses, and the data were presented as mean ± standard deviation. Tukey's multiple comparison tests were conducted after the one-way ANOVA test was used to compare the data. The acquired data were presented as mean ± standard deviation. A p-value of less than 0.05 was considered significant.

Table 1
Some important pathways in which proteins differentially expressed in intestinal tissues are involved according to the "KEGG Pathway Database".

	Accession number	KEGG IDs	Gene name	Description
dre 00190 Oxidative phosphorylation	Q6PC49*	dre:393687	ndufa13	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13
	F1QQ42	dre:415243	ndufa1	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1
	F1RCH5*	dre:415253	cox5ba	Cytochrome c oxidase polypeptide Vb
	B0S5Q3	dre:445129	sdhc	succinate dehydrogenase cytochrome b560 subunit, mitochondrial
	Q68FN7	dre:445500	sdhdb	succinate dehydrogenase [ubiquinone] cytochrome b small subunit B, mitochondrial
	Q568N9	dre:550531	ppa1b	inorganic pyrophosphatase
	A2ARG7*	dre:767750	atp5meb	ATP synthase membrane subunit e, mitochondrial
dre 04810 Regulation of actin cytoskeleton	E7FG54	dre:100537244	myl2a	Myosin regulatory light chain 2a
	F1QSJ9*	dre:101882585	itgam.1	Si:dkey-32n7.4, integrin alpha-M-like
	A4IGC2*	dre:336623	gsnb	Gelsolin
	Q6IQN5*	dre:415151	rac2	Rac family small GTPase 2
	Q7ZUQ1*	dre:793158	Cdc42	Cell division control protein 42 homolog
	Q45QT2	dre:793949	tmsb4x	Thymosin beta
dre04370 VEGF signaling pathway	A4VAK4*	dre:368243	hspb1	HSP27
	Q6IQN5*	dre:415151	rac2	Rac family small GTPase 2
	Q7ZUQ1*	dre:793158	Cdc42l	Cell division control protein 42

Proteins with decreased and increased expression changes in the rotenone group compared to the control are indicated in blue and red, respectively. Among these proteins, those in which the effect of rotenone was reversed after gentisic acid treatment are marked with an asterisk. The list of significant proteins and the fold changes (FC) is shown in Supplementary Table S2.

3. Results

3.1. Results of Behavior assays

We used locomotor activity assessments to see if the fish's movements were impacted by rotenone, the inhibitor of mitochondrial complex 1 that raise ROS levels and destroy dopaminergic neurons. Fig. 1 shows the locomotor behaviors of the groups as average speed (A) exploration rate (B), and total distance (C). Rotenone treatment led to significant decreases in the average speed, exploration rate and total distance in the rotenone treated group ($p < 0.05$). Improved locomotor behaviors were observed due to gentisic acid treatment in the rotenone group as evidenced by increased average speed, exploration rate, and total distance ($p < 0.001$, $p < 0.01$, and $p < 0.001$).

3.2. Results of proteomic assays

Label-free quantitative proteomic analysis allowed the identification of 5214 proteins in intestinal tissues from zebrafish. All proteins are listed in Supplementary Table S1. The results showed that 128 proteins affected by rotenone had significant expression changes compared to control group, and 57 of these proteins responded to gentisic acid treatment (p value < 0.05). (Fig. 2A & 3, Supplementary Table S2). Differentially expressed proteins were searched in the KEGG PATHWAY database to make a more detailed examination, and as a result of the search, these proteins were found to be involved in many different pathways, especially in oxidative phosphorylation, regulation of actin cytoskeleton and VEGF signaling pathway (Supplementary Table S3, Supplementary Figure S1). Proteins whose expression is decreased by rotenone in oxidative phosphorylation responded to gentisic acid treatment, while gentisic acid did not have a significant effect on proteins with increased expression by rotenone (Table 1, Supplementary Fig. S1A, Table S2). There was also an increase in the expression of gelsolin (also known as actin-depolymerizing factor: 15.5-fold, $p = 0.01$) associated with actin stabilization, while a decrease in the expression of Rac protein (0.11-fold, $p < 0.01$), which is an upstream inhibitor of gelsolin, and integrin alpha-M−like protein (0.13-fold, $p = 0.01$). Interestingly, these changes were significantly reversed with gentisic acid treatment Table 1, Supplementary Fig. S1B. Another remarkable finding is that the decreased expression change in Cdc42 (0.11-fold, $p < 0.01$), HSP27 (0.13-fold, $p = 0.01$) and Rac proteins, which use VEGF-mediated signaling pathway and play a role in migration and permeability due to actin reorganization, increased significantly as a result of gentisic acid treatment Table 1, Supplementary Fig. S1C.

In the results of LC-MS/MS analysis performed on zebrafish brain tissues, a total of 4114 proteins were identified, 3831 of which were in the high confidence interval (Supplementary Table S4). In the rotenone-exposed group, 69 of the 132 proteins with significant expression changes compared to the control group had a decrease in expression and an increase in the expression of 62 (Fig. 2B & 4, Supplementary Table S5). Gentisic acid treatment provided an improvement the negative effect of rotenone in a significant part of these proteins (Fig. 3). KEGG pathway search results showed that proteins involved in the oxidative phosphorylation mechanism are more affected by rotenone in

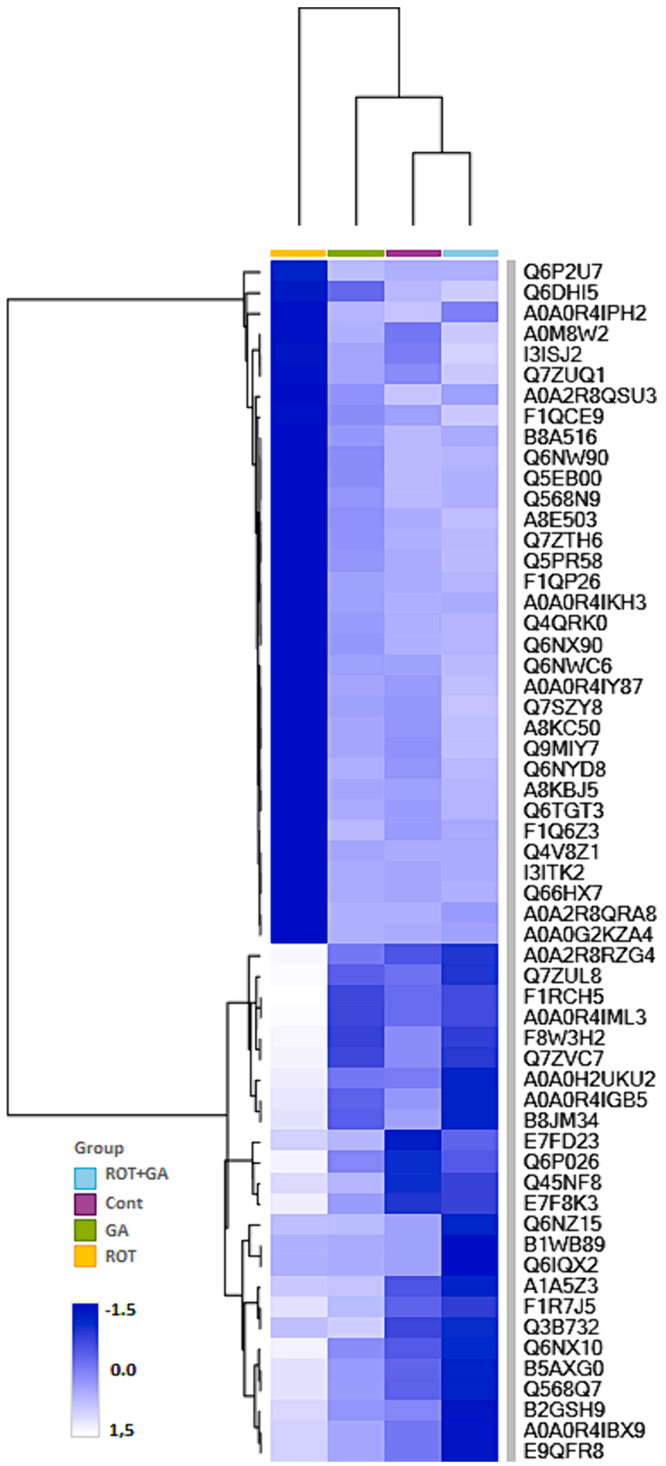

Fig. 4. Heatmap of 58 proteins in brain tissues responsive to gentisic treatment after exposure to rotenone.

brain tissues compared to intestinal tissues. As given in Table 2, 7 out of 11 matched proteins responded to gentisic acid treatment (Table 2, Supplementary Fig. S2A). Moreover, rotenone caused a change in the expression of proteins involved in ferroptis, a new type of cell death known to be associated with neurodegenerative diseases. It was found that tfa (serotransferrin; 0.43-fold, $p < 0.001$) and vdac3 (voltage-dependent anion-selective channel protein 3; 0.24-fold, $p < 0.0001$) proteins decreased in the rotenone-exposed group compared to the control group, and fth1b (ferritin; 5.5-fold, $p < 0.001$) and map1lc3a (microtubule-associated protein 1 light chain 3 alpha; 2.2-fold, $p < 0.01$) proteins increased. Gentisic acid treatment worked, despite the increase in proteins due to the rotenone (Table 2, Supplementary Fig. S2B).

3.3. Results of biochemical assays

In PD, oxidative stress is a major factor in the death of dopaminergic neurons, which causes nitrative and oxidant damage to vital components of the cell. Levels of NO, lipid peroxidation (LPO), and the activity of the antioxidant enzymes superoxide dismutase (Sod) and glutathione S-transferase (Gst) were measured to assess the oxidant/antioxidant balance in the brain and intestines. Significant increases in brain and intestine LPO were seen in the rotenone groups ($p < 0.0001$ and $p < 0.001$ respectively). On the other hand, gentisic acid treatment caused significant decreases in LPO both in the brain and intestines ($p < 0.001$) (Fig. 5A and 5B). Similarly gentisic acid treatment reduced NO levels both in the brain and intestines ($p < 0.01$ and $p < 0.05$ respectively), which increased due to rotenone exposure (Fig. 5C and 5D).

Rotenone led to a significant increase in the activity of Superoxide dismutase (Sod) in brain which is an antioxidant enzyme that turns superoxide into H_2O_2 (Saravanan et al., 2006) ($p < 0.0001$). In the rotenone groups, gentisic acid treatment improved Sod activities both in the brain and in the intestines ($p < 0.0001$ and $p < 0.05$ respectively) (Fig. 6A and 6B).

When we examined the GST activity to examine the effect of gentisic acid, known for its antioxidant effects, on GST-dependent detoxification pathways through the conjugation of GSH with reactive electrophiles (Ozdamar and Can-Eke, 2013), gentisic acid was found to improve Gst activity both in the brain and intestines ($p < 0.0001$ and $p < 0.01$ respectively) (Fig. 6C and 6D).

AChE is needed for the proper functions of the basal ganglia as it maintains the balance in cholinergic and dopaminergic systems and in our study rotenone treatment led to significantly decreased AChE activity both in the brain and in the intestines (Zhou et al., 2003) ($p < 0.05$ and $p < 0.0001$ respectively). On the other hand, in the rotenone group gentisic acid treatment caused a significant increase in intestinal AChE activity ($p < 0.05$) (Fig. 7).

3.4. Results of gene expression assays

The novel E3 ligase complex has been proposed to include Park7, Park2, and Pink1. The fact that Park7 (Dj-1) is H_2O_2-responsive suggests that it has antioxidant and oxidative stress sensing capabilities (Zhang et al., 2005; Xiong et al., 2009). Lrrk2 participates in several cellular processes that trigger mitophagy in a way that is PINK1/parkin-dependent (Wauters et al., 2020). Accordingly in our study *pink1*, *park7*, *park2*, and *lrrk2* expressions were selected because they are connected to mitochondrial activities in PD. Under physiological circumstances, *Park2* improves mitochondrial maintenance and may trigger autophagy in faulty mitochondria (Weihofen et al., 2009; Narendra et al., 2008). It has been suggested that the serine/threonine kinase Pink1 contributes to the cellular and oxidative stress mediated mitochondrial defense (Valente et al., 2004). It is also a crucial part of the Pink1/Parkin pathway, which controls the structure and functioning of mitochondria in stress situations (Nuytemans et al., 2010).

In our study rotenone exposure caused significant increases in the expressions of *pink1, park7, park2,* and *lrrk2* ($p < 0.0001$, $p < 0.0001$, $p < 0.05$, and $p < 0.0001$ respectively). Gentisic acid treatment increased the expressions of *pink1, park7,* and *lrrk2* ($p < 0.0001$, $p < 0.05$, and $p < 0.05$ respectively). However, in the rotenone group while increasing the expression of *park2* ($p < 0.0001$) gentisic acid treatment decreased the expressions of *pink1, park7,* and *lrrk2* ($p < 0.0001$, $p < 0.0001$, and $p < 0.001$ respectively) (Fig. 8).

Table 2
Some important pathways in which proteins differentially expressed in brain tissues are involved according to the "KEGG Pathway Database".

	Acession number	KEGG IDs	Gene name	Description
dre 00190 Oxidative phosphorylation	A0A0U2NKY1	dre:140539	cox1	Cytochrome c oxidase subunit 1
	Q9MIY7*	dre:140540	cox2	Cytochrome c oxidase subunit 2
	F1QCE9*	dre:325712	ndufab1b	NADH:ubiquinone oxidoreductase subunit AB1b
	Q6P1J5	dre:335709	atp5f1d	ATP synthase F1 subunit delta
	Q6PC48	dre:406359	uqcrfs1	Cytochrome b-c1 complex subunit Rieske, mitochondrial
	B8JM34*	dre:406599	atp5pf	ATPase subunit F6
	F1RCH5*	dre:415253	cox5ba	Cytochrome c oxidase polypeptide Vb
	Q5RKM2	dre:493605	ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial
	Q568N9*	dre:550531	ppa1b	Inorganic diphosphatase
	I3ISJ2*	dre:566723	uqcrh	Cytochrome b-c1 complex subunit 6
	F8W3H2*	dre:798291	atp5f1e	ATP synthase F1 subunit epsilon
dre 04216 Ferroptosis	B8JL43	dre:30255	tfa	Serotransferrin
	Q6NX90*	dre:406466	map1lc3a	Microtubule-associated protein 1 light chain 3 alpha
	Q6P0S2	dre:406529	vdac3	Voltage-dependent anion-selective channel protein 3
	Q66HX7*	dre:447823	fth1b	Ferritin

Proteins with decreased and increased expression changes in the rotenone group compared to the control are indicated in blue and red, respectively. Among these proteins, those in which the effect of rotenone was reversed after gentisic acid treatment are marked with an asterisk. The list of significant proteins and the fold changes (FC) is shown in Supplementary Table S5.

3.5. Results of tyrosine hydroxylase (TH) immunostaining

TH has numerous roles in the onset of PD as the rate-limiting step in the synthesis of dopamine, the production of L-DOPA, is catalyzed by TH (Haavik and Toska, 1998). In order to determine the effect of gentisic acid in the rotenone induced PD in zebrafish, the dopaminergic neurons in the diencephalon and the tectum were assessed using immunostaining of TH expression (Fig. 8 and Fig. 9). Regions considered as the diencephalon and the tectum in the zebrafish brain are illustrated in Fig. 9A and Fig. 10A, respectively. In the ROT group, TH expression was lower than in the control group in the diencephalon and tectum regions of the zebrafish brain (Fig. 9B and 9D, Fig. 10B and 10D). TH expression revealed very weak immunoreactivity in the ROT group (Fig. 9D and 10D), while in the ROT + GA group dopaminergic neurons demonstrated a remarkably strong TH immunoreactivity (Fig. 9E and 10E).

4. Discussion

Based on the critical interaction in the brain-gut line in PD, we found that gentisic acid changed the expression of 57 of 128 rotenon-affected proteins in the intestine. Rotenone changed the expression of 132 proteins in the brain, while the expression of 62 of them increased and the expression of 69 of them decreased with the application of gentisic acid in the rotenone given group. According to the KEGG pathway search results, proteins involved in the oxidative phosphorylation mechanism were more affected by rotenone in brain tissues compared to intestinal tissues. Moreover, rotenone caused a change in the expression of brain proteins involved in ferroptis, a novel type of programmed cell death brought on by the build-up of lipid reactive oxygen species that are dependent on iron. The main mechanism is the Fenton reaction, which produces excess lipid reactive oxygen species and lipid peroxidation through the catalysis with iron ions and ultimately results in cell death (Hirschhorn and Stockwell, 2019). Ferroptosis was reported to be an important pathway for the death of dopaminergic neurons related with

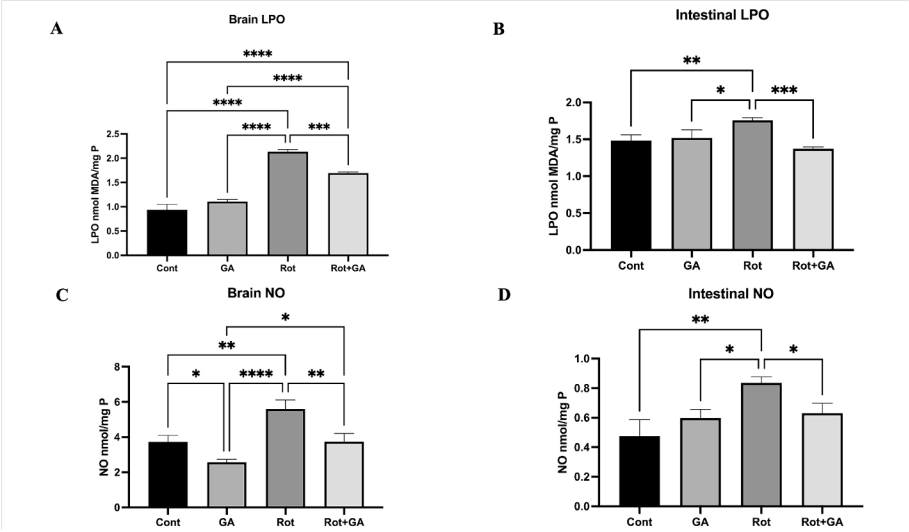

Fig. 5. Effects of rotenone and gentisic acid treatments on lipid peroxidation and nitric oxide levels. Brain (A) and intestinal (B) lipid peroxidation (LPO) levels (nmol MDA/mg P); nitric oxide (NO) levels (C,D) (nmol/mg P). Data is given as mean ± SD. Significant difference is indicated by an asterisk. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Cont: Control Group; GA: Gentisic Acid Group; Rot:Rotenone Group; Rot + GA: Rotenone + Gentisic Acid Group.

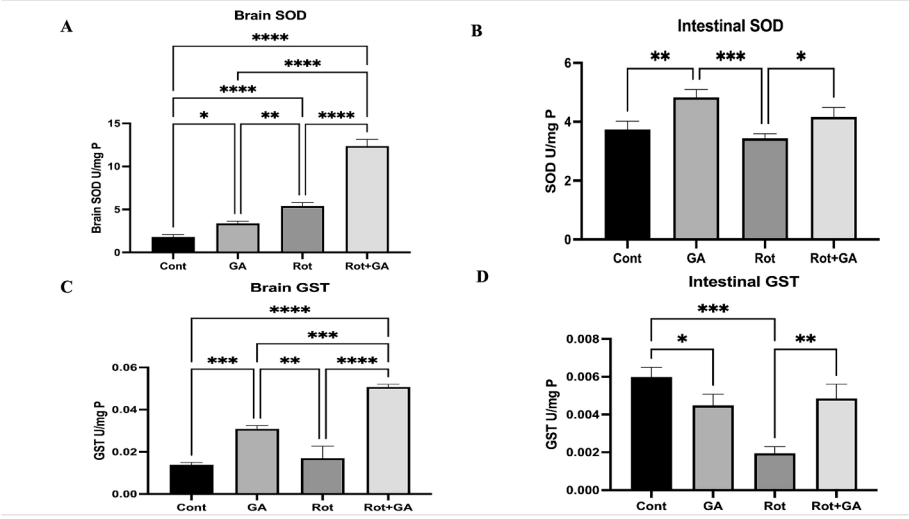

Fig. 6. Effects of rotenone and gentisic acid treatments on antioxidant enzyme activities. Brain (A) and intestinal (B) superoxide dismutase (Sod) activities (U/ mg P); glutathione-S- transferase (GST) activities (C,D) (U/ mg P) of the groups. Data is given as mean ± SD. Significant difference is indicated by an asterisk. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ Cont: Control Group; GA: Gentisic Acid Group; Rot:Rotenone Group; Rot + GA: Rotenone + Gentisic Acid Group.

PD progression and neurotoxins that are used to generate PD models can also induce ferroptosis (Van Do et al., 2016). This finding we obtained from proteomic analyzes was found to be compatible with the increased lipid peroxidation and decreased antioxidant enzyme activities we determined in brain tissue due to rotenone exposure. Ferritin functions as an iron storage protein complex and we determined increased fth1b (ferritin) expression in the rotenone group and this finding supported the view that rotenone application stimulated the development of ferroptosis. Gentisic acid application normalized the altered protein expressions and the possible mechanism of action may be due to gentisic acid reducing lipid peroxidation in the brain tissue.

Although rotenone has beneficial uses in agriculture and animal husbandry, long-term exposure to it has a negative impact on the health of those who work in these fields. Rotenone may cross the blood–brain barrier, and has been shown to inhibit the mitochondrial ETC complex I and lead to an increase in ROS production. After being exposed to rotenone, cofilin, a crucial component of the actin cytoskeleton, had changes in its transcription and expression pattern, which contributed to the breakdown of the cytoskeleton (Roy et al., 2023). In our study, consistent with these findings, an increase in lipid peroxidation and a decrease in antioxidant enzymes were observed in the rotenone-administered group, which are indicators of oxidative stress. Significant increases observed in the expressions of *pink1, park7, park2,* and *lrrk2* genes in the rotenone group indicating the activation of the mitochondrial activities due to oxidative stress mediated mitochondrial defense. Moreover increased NO levels in the rotenone group points to the involvement of inflammation in rotenone exposure as suggested by the increased expression and activity of matrix metalloproteinase9 (MMP9) in rotenone exposed N2A cells (Roy et al., 2023).

Decreased expressions of dopaminergic neurons in the diencephalon and the tectum were observed through TH immunostaining indicated neurodegeneration due to rotenone treatment. This finding was in consistent with decreased average speed, exploration rate, and total distance travelled which were ameliorated by gentisic acid treatment. The ameliorative properties of gentisic acid may be due to its ability to ameliorate mitochondrial damage due to oxidative stress caused by

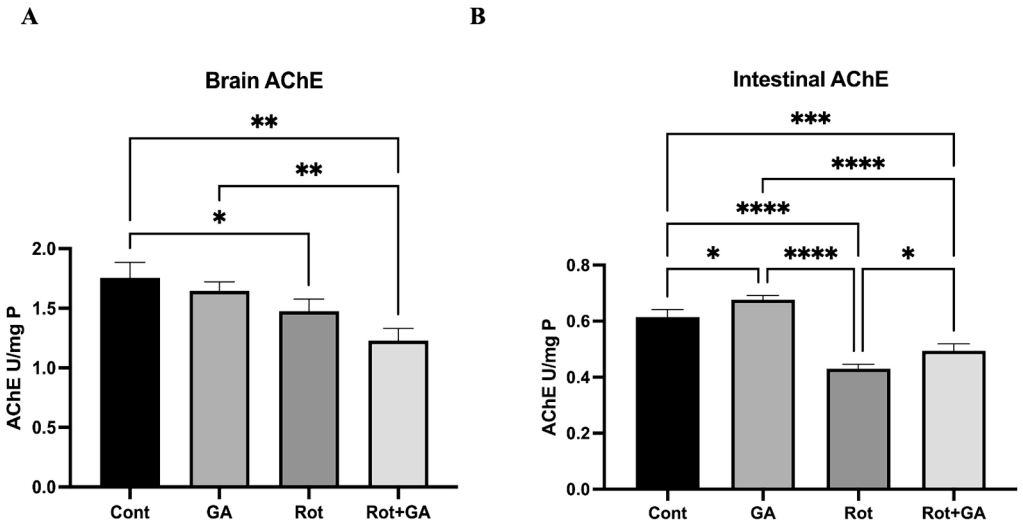

Fig. 7. Effects of rotenone and gentisic acid treatments on brain (A) and intestinal (B) acetylcholinesterase (AChE) activities (U/ mg P). Data presented are mean ± SD. Significant difference is indicated by an asterisk. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Cont: Control Group; GA: Gentisic Acid Group; Rot: Rotenone Group; Rot + GA: Rotenone + Gentisic Acid Group.

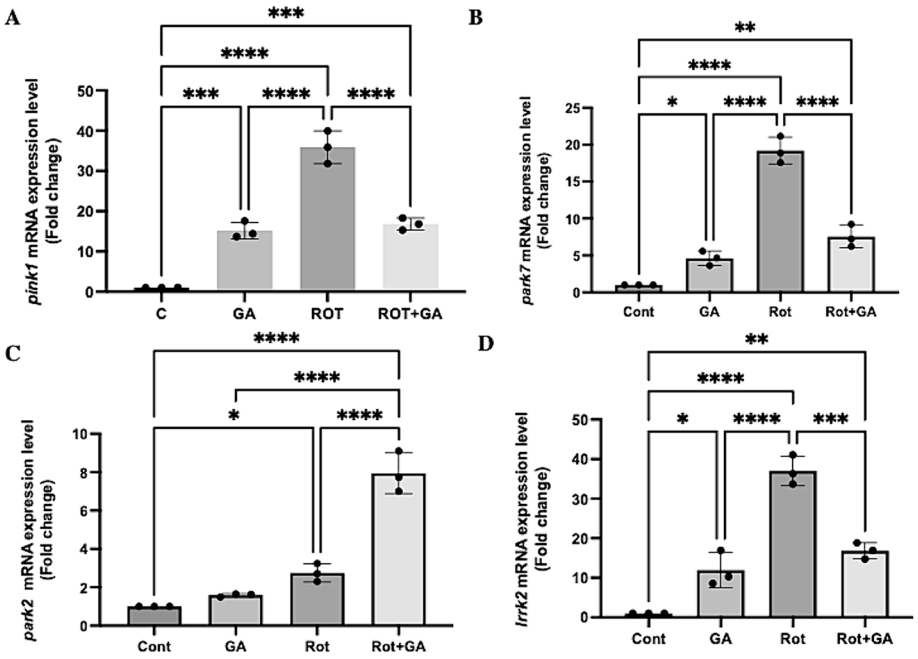

Fig. 8. Effects of rotenone and gentisic acid treatments on gene expressions. Bar graph presentation of the fold change of brain *pink1*(A), *park7* (B), *park2* (C), and *lrrk2* (D) transcripts determined by RT-PCR. All RT-PCR results are normalized to β-actin, the house keeping gene and expressed as change from their respective controls. The average values were obtained from three experiments. Data presented are mean ± SD. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Cont: Control Group; GA: Gentisic Acid Group; Rot:Rotenone Group; Rot + GA: Rotenone + Gentisic Acid Group.

rotenone as evidenced by improved activities of the antioxidant enzymes SOD, GST and *pink1, park7, park2,* and *lrrk2* expressions. Accordingly, gentisic acid provided an improvement in TH expressions that indicating protection of dopaminergic neurons and locomotor activity due to its preventive effects on oxidative stress and thus mitochondrial damage.

On the other hand, we should point out that the ameliorative effects of gentisic acid, especially on oxidative stress, was evident not only in the brain but also in the intestines. Gentisic acid therapy also restored normal AChE activity in the intestines of the rotenone group. AChE is a high-affinity ligand for donepezil and validation of the 5-[^{11}C]-methoxy-donepezil positron emission tomography has allowed for the monitoring of AChE density in the brain and other peripheral organs (Hiraoka et al.,2009). Decreased ^{11}C-donepezil binding was reported in the small intestines of PD patients (Gjerløff et al., 2015). The ameliorative effect of gentisic acid in response to decreased intestinal AChE may be effective in the treatment parasympathetic denervation in PD.

In the intestines, differentially expressed proteins were found to be involved in mainly oxidative phosphorylation, regulation of actin cytoskeleton and VEGF signaling pathway. In particular in the rotenone group, the increased expression of gelsolin (actin-depolymerizing factor) is an indicator of cytoskeleton change as gelsolin is related with actin stabilization. Together with decreased expression of Rac protein, an upstream inhibitor of gelsolin, and integrin alpha-M-like protein were reversed by gentisic acid treatment. This finding is quite remarkable as an actin binding protein gelsolin, has been reported to co-occure with

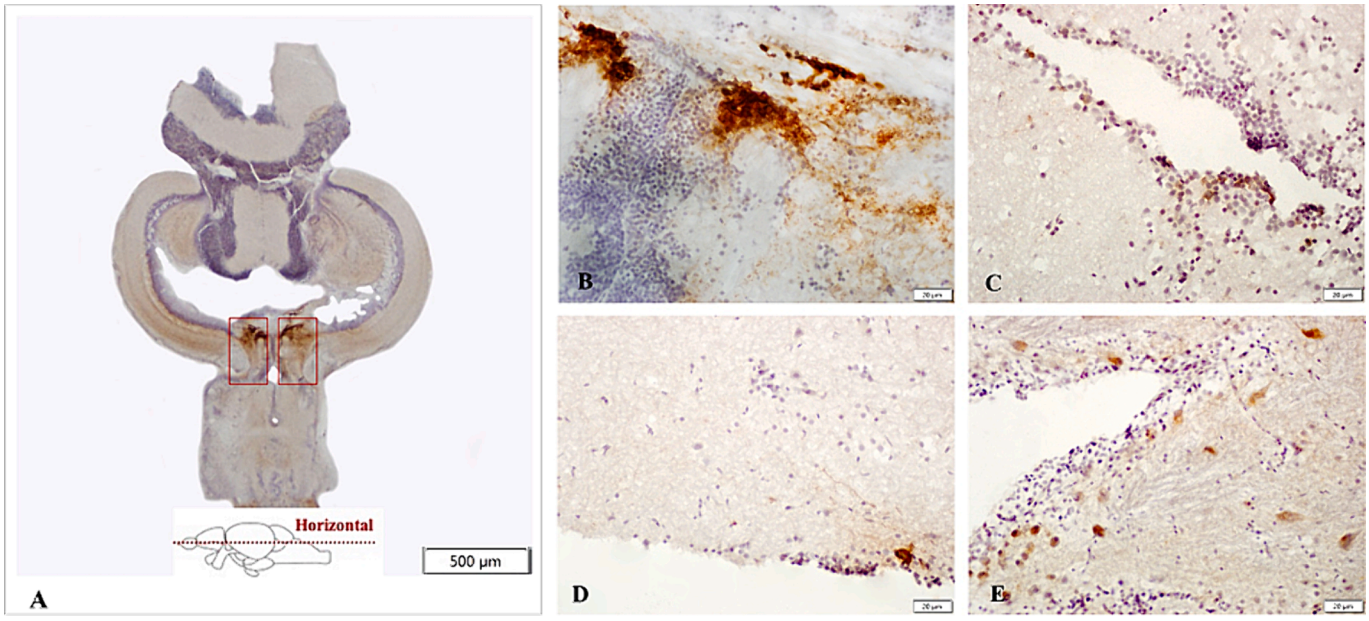

Fig. 9. Representative photomicrographs of TH immunoreactivity in the diencephelon region (in-squares; A) of zebrafish brain in control (B), GA (C), Rot (D), Rot + GA (E) A: 2X, B-E: 40X.

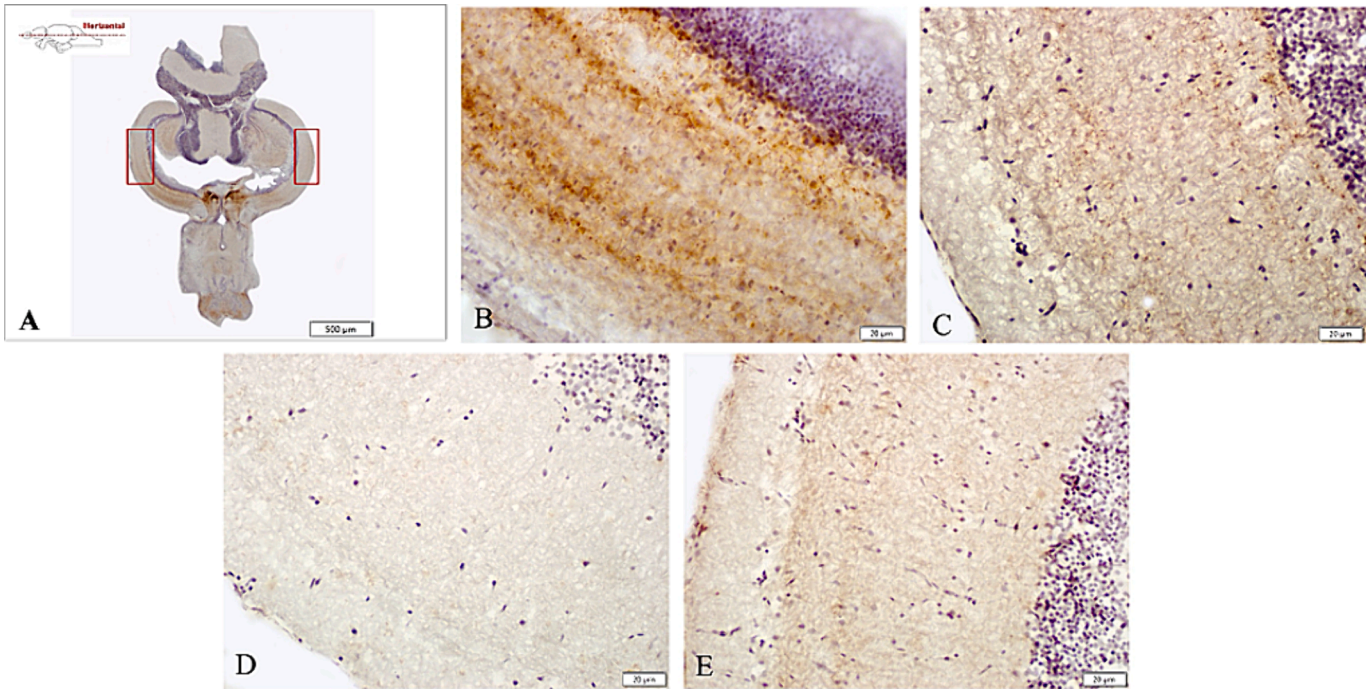

Fig. 10. Representative photomicrographs of TH immunoreactivity in the tectum region (in rectangles; A) of zebrafish brain in control (B), GA (C), Rot (D), Rot + GA (E) A: 2X, B-E: 40X.

α-synuclein in the Lewy bodies obtained from PD affected human brains as well as in α-synuclein over expressing cells with Lewy body-like inclusions (Welander et al., 2011).

Recent years the possibility that PD may have its origin in the gut because of the high incidence of gastrointestinal dysfunction in the early stages of the disease has gained significant attraction with the recognition of PD gut microbiome dysbiosis, and numerous reports of α-synuclein pathology related with enteric nervous system in PD patients (Fasano et al., 2015; Chen and Mor, 2023). The gut-brain axis is a sophisticated two-way signaling network that allows the brain to transfer motor and sensory information, neuroactive chemicals originating from bacteria, and cytokines triggered by the microbiota to the gastrointestinal system through the enteric nervous system (Bullich et al., 2019). A variety of conditions, including functional gastrointestinal disorders and irritable bowel syndrome, as well as mental problems and chronic pain, can result from disruption of the gut-brain axis (Mayer et al., 2015).

RhoA, Rac1, and Cdc42 are the classical proteins in the Rho GTPases subfamily that belongs to the Ras superfamily. Rho GTPases control morphogenesis, proliferation, survival, transcriptional activation, membrane trafficking, and adhesion in addition to essential nervous system functions (Arrazola et al., 2020). Neuronal actin cytoskeleton regulation, synaptic plasticity, dendritic spine interactions, and

neurotransmitter receptor organization are all influenced by Rho GTPases which are crucial mechanisms related with the development of neurodegenerative diseases (Zamboni et al., 2018; Guiler et al., 2021). Rho GTPases have a significant role in the pathophysiology of PD. LRRK2 has been shown by Chan et al. (2011) to activate Rac. On the other hand, activation is not induced by mutant LRRK2. Neurite retraction results from this absence of Rac activation. We found decreased intestinal expression of Cdc42 and Rac protein which were reversed by gentisic acid treatment. Moreover, the increase in *lrrk* expression in the rotenone treated group may have occurred in response to the decrease in Rac.

There is growing evidence that digestive issues are also present in PD, in addition to brain disorders. Digestive conditions like irritable bowel syndrome and inflammatory bowel disease have been frequently linked to functional and structural changes in the intestinal epithelial barrier (Clairembault et al., 2015). There are two ways in which this barrier can be penetrated: either by the transcellular pathway, which passes through epithelial cells, or between them via the paracellular pathway (Marchiando et al.,2010). The epithelial tight junctions, which link neighboring enterocytes to control paracellular permeability through the lateral intercellular gap, are among the most crucial components of the intestinal barrier (Suzuki, 2013). In the light of this information, the improvement of Cdc42 and Rac expressions, which decreased in the intestines with rotenone exposure determined in our study, by gentisic acid treatment is a very important finding for the regulation of intestinal morphology in PD. In addition, in our study, the ameliorative effects of gentisic acid on the oxidant-antioxidant balance as observed through the improved LPO, NO, SOD and GST activities were determined in the intestinal tissue in parallel with the brain tissue, indicates that gentisic acid may be an effective agent in the gut-brain axis. Elevated levels of AChE inhibit acetylcholine (ACh) to preserve the equilibrium between the cholinergic and dopaminergic systems, an essential component for proper basal ganglia function (Bohnen and Albin, 2011).

As a result, the proteomic analysis results showed that rotenone exposure caused alterations in the expression of proteins related to oxidative phosphorylation in both the brain and intestines, although the proteins in the brain were more affected. Changes were observed in the expression of proteins involved in ferroptis in the brain and actin cytoskeleton-related proteins in the intestines, and the altered protein expressions were improved with the treatment of gentisic acid. Gentisic acid ameliorated Th-immunoreactivity in the brain, improved the expression of PD-related genes and locomotor activity. On the other hand, although an increase in Th-immunoreactivity was observed in the gentisic acid treated rotenone group, this effect was not observed in the control group. GA positively affected the oxidant-antioxidant balance and the expression of PD-related genes in the brain tissue, but the Th-immunoreactivity was observed to be lower than the Control group, suggesting that the positive effect of gentisic acid may occur under stress conditions. The beneficial effects of gentisic acid may be attributed to the prevention of oxidative stress as the oxidant-antioxidant balance, which was disrupted by rotenone exposure, was improved with gentisic acid treatment both in the brain and intestines. The results of our study show that gentisic acid is a candidate agent to be evaluated for its potential protective effect for neurodegenerative diseases.

CRediT authorship contribution statement

Derya Cansız: Methodology, Investigation. **İsmail Ünal:** Methodology, Investigation. **Mustafa Gani Sürmen:** Methodology. **Saime Sürmen:** Methodology. **Zehra Sezer:** Methodology. **Merih Beler:** Methodology. **Elif Güzel:** Methodology. **A.Ata Alturfan:** Writing – review & editing. **Ebru Emekli-Alturfan:** Writing – review & editing, Supervision, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2024.148952.

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