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Screening of monoamine oxidase inhibitory compounds from Tartary buckwheat through UPLC-MS/MS profiling, in silico analysis and other experimental investigations

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ABSTRACT: Central fatigue (CF) affects negatively cognition, emotion and physical performance. Monoamine oxidase (MAO), a key mitochondrial bound enzyme, modulates the levels of neurotransmitters, which is closely associated with the development of CF. This study follows recent attempts to discover natural MAO inhibitory compounds from foods, focusing on Tartary buckwheat. UPLC-MS/MS profiling and other experiments revealed 18 key polyphenols from Tartary buckwheat as MAO inhibitors, with six being reported for the first time. Network pharmacology and molecular docking studies indicated that quercetin, ellagic acid, emodin, galangin, kaempferol, morin and butein may be the MAO inhibitors. *In vitro* MAO inhibition assays confirmed that quercetin was the most potent inhibitor ($IC_{50}=3.982-4.386 \mu M$). Animal-friendly behavioral testing, neurotransmitter profiling, transcriptomic analysis and western blot experiments showed that quercetin significantly ameliorated behaviors of fatigue-dominant phenotypes, regulated the levels of neurotransmitters and alleviated CF via the PI3K/AKT signaling pathway. Accordingly, Tartary buckwheat provides anti-CF polyphenols.

Keywords: Tartary Buckwheat; Food-derived polyphenols; Quercetin; Anti-central fatigue effect; Monoamine oxidase; Enzyme inhibition assay.

1. Introduction

Central fatigue is a physiological state caused by impaired central nervous system function, primarily characterized as reduced voluntary motor ability, difficulty in concentrating, low mood and cognitive decline [1]. Unlike peripheral fatigue caused by muscular or metabolic factors, central fatigue manifests as impaired neural regulatory capacity, commonly observed in conditions such as intense exercise, chronic psychological stress or sleep deprivation [2-4]. Accumulating evidence suggests that central fatigue is closely associated with disruptions in neurotransmitter homeostasis, particularly involving serotonin, dopamine, and norepinephrine, which are essential for the regulation of mood, motivation, and cognitive performance [5].—With the

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accelerating pace of modern work and rising stress levels, the incidence of central fatigue has increased annually, seriously affecting physical and mental health in individuals [6]. Current interventions to central fatigue primarily encompass lifestyle adjustments, nutritional supplementation, psychological regulation and drug-based interventions [7-9]. Despite the demonstrated efficacy of certain pharmacological interventions in alleviating central fatigue, associated adverse effects including excessive stimulation, gastrointestinal discomfort, tachycardia, hypertension and potential dependency cannot be overlooked [10]. In this context, a natural active substance capable of alleviating central nervous system fatigue is urgently needed.

Monoamine oxidase (MAO) is a key enzyme that catalyzes the metabolism of monoaminergic neurotransmitters and is essential for maintaining central nervous system homeostasis [11]. In recent years, accumulating evidence has clarified the role of MAO in neuroregulation, establishing it as an important therapeutic target for neurological disorders such as Parkinson's disease and depression [12]. MAO comprises two isoforms, MAO-A and MAO-B. MAO-A is mainly involved in the degradation of serotonin (5-HT), norepinephrine (NE), while MAO-B primarily participates in the metabolism of dopamine (DA) and other monoamines [13]. The development of central fatigue is closely associated with disrupted neurotransmitter homeostasis in the brain, particularly changes in the levels and balance of monoamine neurotransmitters. Under stress or prolonged fatigue conditions, sustained MAO-mediated degradation could further aggravate this imbalance, thereby impairing neural signal transmission [14]. Therefore, inhibiting MAO activity to prevent excessive neurotransmitter degradation is considered a potential therapeutic strategy [15]. Previous studies indicate that polyphenolic compounds exhibit MAO inhibitory activity and modulate neurotransmitter metabolism by interacting with the enzyme active site, thereby contributing to neuroprotection and emotional regulation [16]. However, it remains largely unclear whether polyphenols can alleviate central fatigue by modulating MAO activity to restore neurotransmitter homeostasis. Therefore, identifying functional polyphenols with MAO inhibitory activity and elucidating their roles in central fatigue regulation is crucial for developing safe and effective natural anti-central fatigue agents.

Tartary buckwheat is a functional food with excellent nutritional value and health benefits, including abundant bioactive components such as high-quality proteins, polysaccharides, dietary fiber and particularly rich polyphenolic compounds [17, 18]. Among these, polyphenols are considered the main contributors to the neuroprotective and anti-central fatigue effects [19]. Our preliminary research found that tartary buckwheat polyphenols (TBP) can alleviate central fatigue by regulating gut microbiota and neurotransmitter levels [20]. However, due to the complex composition of TBP, the key bioactive compounds responsible for their anti-central fatigue effects remain unidentified, and the underlying mechanisms of action are still unclear.

In this study, we first characterized the chemical constituents of TBP using untargeted and targeted metabolomics and then identified key active compounds through an integrative approach combining network pharmacology, molecular docking, and *in vitro* enzyme activity assays. Subsequently, a mouse model of central fatigue was established, followed by behavioral assessments and the measurement of neurotransmitter expression. Furthermore, transcriptomic analysis was conducted to further validate the effect of key

compound and to explore its molecular mechanisms. This study aims to identify the key component in TBP responsible for alleviating central fatigue, offering insights into the potential of natural food-derived in alleviating central fatigue.

2. Materials and Methods

2.1. Materials

Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) grains were purchased as a commercial product sourced from the Daliangshan region of Sichuan Province, China. The sample identity was confirmed based on the supplier-provided product information and the characteristic morphological appearance of Tartary buckwheat grains. Gallic acid, (-)-Epigallocatechin, Catechin, Caffeic acid, Mangiferin, Eriocitrin, Liquiritin, Rutin, Hyperoside, Phlorizin, Ellagic acid, Quercitrin, Narcissin, Morin, Quercetin, Butein, Kaempferol, 6-Gingerol, Biochanin A, Galangin, Hypericin, and Emodin were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). The purity of all reference compounds was $\geq 98\%$, as specified by the manufacturer.

2.2. Extraction and purification of TBP

The Tartary buckwheat samples were pulverized and extracted using ultrasound-assisted extraction with 70% (v/v) ethanol at a solid-to-liquid ratio of 1:10 (w/v) for 50 min per cycle, repeated three times. The extract was purified using AB-8 macroporous adsorption resin, concentrated by rotary evaporation, and freeze-dried to obtain polyphenol powder. The lyophilized powder was stored at $-80\text{ }^{\circ}\text{C}$ in the dark for use.

2.3 UHPLC-Q-TOF-MS/MS identification of phenolic compounds in TBP

The chemical constituents of TBP were identified and analyzed using a high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) system (Triple TOF 6600+, AB Sciex, USA). Chromatographic separation was performed on a C18 column ($2.1 \times 100\text{ mm}$, $1.9\text{ }\mu\text{m}$) at $40\text{ }^{\circ}\text{C}$. Mobile phase A was 0.1% formic acid in water, and mobile phase B was acetonitrile, with a flow rate of 0.3 mL/min . The gradient elution program was as follows: 0–1.5 min, 5–13%; 1.5–5 min, 13–25%; 5–8 min, 25–40%; 8–13 min, 40–60%; 13–14 min, 60–97%; 14–16 min, 97%; 16.1–20 min, 5%. Mass spectrometry was conducted using an electrospray ionization (ESI) source at $550\text{ }^{\circ}\text{C}$, with a spray voltage of 4500 V in negative ion mode and a scan range of m/z 60–1500.

2.4. Quantitative Analysis of TBP

The detected TBP components were quantified using a liquid chromatography–triple quadrupole tandem mass spectrometry system (LCMS-8060, Shimadzu, Japan). Chromatographic separation was performed on a Shim-pack GIST C18-AQ HP column ($1.9\text{ }\mu\text{m}$, $2.1 \times 50\text{ mm}$, PN: 227-30807-01). Mobile phase A was water containing 0.05% formic acid, and mobile phase B was methanol containing 0.05% formic acid. The gradient elution program was as follows: 0–1 min, 1% B; 1–8 min, B increased to 99%; 8–9 min, 99% B; 9.1–12 min, B returned to 1%. The flow rate was 0.3 mL/min , column temperature was $30\text{ }^{\circ}\text{C}$, and injection volume was $2\text{ }\mu\text{L}$. Mass spectrometry detection was performed in multiple reaction monitoring (MRM) mode under negative

ionization. Standard curves of the corresponding monomeric compounds were used for calibration to ensure quantification accuracy.

2.5. Network Pharmacology Analysis

The target genes of polyphenolic compounds identified from metabolomic analysis were predicted using SwissTargetPrediction (<https://swisstargetprediction.ch>) and PubChem (<https://pubchem.ncbi.nlm.nih.gov>). Disease-related genes associated with “central fatigue” were retrieved from the OMIM (<https://www.omim.org>) and GeneCards (<https://www.genecards.org>) databases. Shared targets between the polyphenols and these disease genes were determined using Venn diagram analysis. Subsequently, core targets and polyphenol interactions were predicted using Cytoscape software (version 3.9.1) and the STRING database (<https://cn.string-db.org>). Finally, Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the DAVID database (<https://david.ncifcrf.gov>) and the results were visualized via a bioinformatics platform (<https://www.bioinformatics.com.cn>).

2.6. Molecular Docking Analysis

For molecular docking simulations, the receptor structures of 5-HT₄ (PDB: 7XTA), MAO-A (PDB: 2Z5X), MAO-B (PDB: 2V5Z), and NURR1 (PDB: 5Y41) were retrieved from the UniProt protein database (<https://www.uniprot.org>) and their conformations were evaluated using SAVES v6.0 (<https://saves.mbi.ucla.edu>). The ligand structure of compound was drawn using ChemDraw (PerkinElmer, MA, USA) and converted to a 3D ligand structure. Processed ligands including Quercetin, Ellagic acid, Emodin, Galangin, Kaempferol, Morin, Butein and Narcissin were docked to the protein receptors using AutoDock 4.2 (Scripps, New York, USA). The binding pocket center coordinates were set as follows: 5-HT₄, X: 111.044, Y: 90.282, Z: 100.879; MAO-A, X: 93.9, Y: 102.107, Z: 96.773; MAO-B, X: 39.036, Y: 119.918, Z: 17.976; NURR1, X: 17.266, Y: 37.038, Z: 16.638. The lowest binding energy, bonding interactions and intermolecular forces between ligands and receptors were analyzed and the docking results were visualized using PyMOL (Schrödinger & Co., CA, USA).

2.7. In Vitro Determination of MAO-A and MAO-B Enzyme Inhibition

The inhibitory activities of MAO-A and MAO-B were measured using a Monoamine Oxidase A (MAO-A) Inhibitor Screening Kit (E-BC-D012, Elabscience, China) and a Monoamine Oxidase B (MAO-B) Inhibitor Screening Kit (ab284511, Abcam, UK), respectively. Quercetin, Ellagic acid, Emodin, Galangin, Kaempferol, Morin and Butein were prepared at concentrations of 0.1, 20, 40, 60, 80 and 100 μ M, and the assays were performed following the manufacturers' instructions.

2.8. Animal experimental design

2.8.1. Animals and diet

32 6-week-old male BALB/c mice (Sibeifu Biotechnology Co., Ltd., Beijing, China) were used in this study and housed at the Beijing Yaoxuan Animal Center. Animals were maintained under standard conditions

(22 ± 1 °C, 40–70% relative humidity, 12/12 h light-dark cycle) and all experimental procedures were conducted in strict accordance with Chinese guidelines for the use and welfare of laboratory animals (BJYX-2024-YX-081). Epigallocatechin gallate (EGCG), a compound with well-established anti-fatigue effects^[21], was used as a positive control. After one week of acclimatization, the mice were randomly assigned to four groups (n=8): CON, MOD, POS (100 mg/kg EGCG) and QUE (100 mg/kg Quercetin). Following two weeks of dietary intervention, mice in the MOD, POS and QUE groups underwent a 4-week high-intensity exercise regimen, which included treadmill running in the morning (15–18 min at 15 m/min) and forced swimming in the afternoon (8–10 min at 25 ± 1 °C), followed by behavioral tests for evaluation.

2.8.2. Behavioral test

Behavioral assessments included the grip strength test, rotarod test and open field test. Forelimb grip strength was measured using a grip strength meter (DS2-5N-X, DAILIGU, Shenzhen, China), with each mouse tested three times and the average value recorded. Motor coordination and balance were evaluated using an automated rotarod apparatus (Ugo Basile Rota Rod, Shanghai, China) at a constant speed of 30 rpm for three consecutive trials and the average latency to fall was recorded. The open field test was conducted in a dark room using a $40 \times 40 \times 30$ cm arena. The arena was divided into a central area and four corner zones, with a digital camera positioned above the center. Mice were placed in the central area and allowed to explore freely for 5 minutes. The proportion of movement time, time spent along the edges and time spent in the central area were recorded to assess fatigue-related behavior and activity levels. Before each trial, the apparatus was cleaned with ethanol to eliminate olfactory cues.

2.8.3. Neurotransmitter Detection

After sacrifice, the brain tissues were rapidly collected and rinsed with ice-cold PBS to remove residual blood. The tissues were gently blotted dry, weighed, immediately frozen, and stored at -80 °C until analysis. For neurotransmitter detection, brain tissues (100 mg) were homogenized in 1 mL of PBS to prepare tissue homogenates. The homogenates were centrifuged at 6000 rpm for 20 min and the supernatants were collected for neurotransmitter analysis using commercial kits (Nanjing Boryan Biotechnology Co., Nanjing, China). The levels of serotonin (5-HT), dopamine (DA), γ -aminobutyric acid (GABA), glutamate (Glu) and norepinephrine (NE) were measured according to the manufacturer's instructions.

2.8.4. Intestinal transcriptome analysis

Total RNA was extracted from intestinal tissues using TRIzol® Reagent (Invitrogen, California, USA). mRNA was isolated by pairing the poly(A) tail at the 3' end of mRNA with magnetic beads containing Oligo(dT). The mRNA was then fragmented to approximately 300 bp using lysis buffer. Using mRNA as a template, reverse transcription was performed to synthesize stable double-stranded cDNA. The double-stranded cDNA, which had sticky ends, was repaired to blunt ends using End Repair Mix and an adenine (A) base was added at the 3' end to facilitate subsequent adapter ligation. The adapter-ligated products were purified and size-selected, followed by PCR amplification and the final library was purified.

High-throughput sequencing of the library was conducted on the NovaSeq X Plus platform (Shanghai Meiji Biopharmaceutical Technology Co., Ltd., China). To ensure accuracy in bioinformatic analyses, raw sequencing data were filtered using fastp software (<https://github.com/OpenGene/fastp>). Filtered reads were aligned to the reference genome to obtain mapped data for downstream transcriptome assembly and expression analysis.

Gene and transcript expression levels were quantified using RSEM software, and differential expression analysis was performed with DESeq2. Differentially expressed genes (DEGs) were identified based on the criteria of $FDR < 0.05$ and $|\log_2FC| \geq 1$. Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs were conducted using the GO database (<http://geneontology.org>) and KEGG database (<https://www.genome.jp/kegg>), followed by pathway enrichment analysis.

2.8.5. Western Blot

Intestinal tissues from mice were first homogenized and lysed using RIPA buffer, and protein concentrations were determined using the BCA assay. Equal amounts of protein from each sample were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk at room temperature for 2 h, followed by incubation with primary antibodies at 4 °C for 12 h. Membranes were then incubated with secondary antibodies (Jackson ImmunoResearch Inc., USA) at room temperature for 1 h. Protein signals were detected using an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, USA) and visualized with an Amersham Imager 600 (GE, Fairfield, Connecticut, USA). Band intensities were quantified using ImageJ software. Detailed antibody information is as follows: p-PI3K (HA721672, Anhui huabao biology co., ltd, 1:1000), Nrf2 (bsm-61093R, Bioss, 1:1000), p-AKT (66444-1-Ig, Mitaka, 1:2000), AKT (10176-2-AP, Mitaka, 1:2000), HO-1 (BF8020, Affinity, 1:1000), β -actin (AC026, Abclonal, 1:50000).

2.9. Statistical analysis

Experimental results are expressed as mean $\pm$ standard deviation ($n \geq 3$). Graphs were generated using Origin 8.5 software (OriginLab, Northampton, MA, USA). Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range test in SPSS version 25.0. Differences were considered statistically significant at $P < 0.05$.

3. Results and Discussion

3.1. Identification of polyphenolic constituents in TBP

Previous studies have shown that rutin, quercetin, gallic acid, catechin, kaempferol, and other phenolic compounds are the major polyphenolic constituents of tartary buckwheat^[22]. Building on these findings, we applied UHPLC-Q-TOF-MS/MS to further characterize the polyphenolic profile of TBP (Table 1). A total of 22 polyphenolic compounds were identified, among which six (phlorizin, gallic acid, galangin, biochanin A, 6-gingerol, and eriocitrin) were reported for the first time in tartary buckwheat. Based on chemical structure,

the polyphenols in TBP are mainly classified into three categories: flavonoids (68.2%), phenolic acids (13.6%) and other phenolics (18.2%). Flavonoids constitute the major active components of TBP, including quercetin, rutin, catechin and kaempferol. These compounds have been widely reported for their strong antioxidant, anti-inflammatory, neuroprotective and anti-fatigue activities [23-25]. Specifically, quercetin alleviates fatigue by enhancing muscle function and increasing antioxidant enzyme activity [26]. Rutin mitigates fatigue by upregulating antioxidant enzymes and modulating the MAPK signaling pathway [27]. Hypericin alleviates chronic fatigue by reducing oxidative stress and inflammation, as well as through the AMPK signaling pathway [28]. In addition, phenolic acids in TBP, including gallic acid, caffeic acid and ellagic acid, exhibit strong antioxidant properties. These compounds modulate inflammation and protect neural function. Notably, ellagic acid was reported to alleviate fatigue by inhibiting oxidative stress, regulating gut microbiota and modulating energy metabolism [29].

Table 1 Characterization of polyphenol compounds in the Tartary buckwheat through UHPLC-TOF-MS/MS analysis

No	Compound	Retention Time (min)	MS/MS(m/z)	Peak area (Mean value)	Formula
1	Narcissin	2.61	137.02	190912	C ₂₈ H ₃₂ O ₁₆
2	Butein	5.59	624.17	160355	C ₁₅ H ₁₂ O ₅
3	Rutin	15.22	277.22	1740.33	C ₂₇ H ₃₀ O ₁₆
4	Quercitrin	13.21	307.19	133944	C ₂₁ H ₂₀ O ₁₁
5	Quercetin	1.37	117.02	735456	C ₁₅ H ₁₀ O ₇
6	Phlorizin	5.01	223.06	308044	C ₂₁ H ₂₄ O ₁₀
7	Morin	1.50	610.15	16176	C ₁₅ H ₁₀ O ₇
8	Mangiferin	10.23	327.04	22262.8	C ₁₉ H ₁₈ O ₁₁
9	Kaempferol	4.71	463.09	112763	C ₁₅ H ₁₀ O ₆
10	Hypericin	4.34	771.20	544674	C ₃₀ H ₁₆ O ₈
11	Gallic acid	2.47	315.07	23485	C ₇ H ₆ O ₅
12	Galangin	6.02	180.07	38029.5	C ₁₅ H ₁₀ O ₅
13	Catechin	10.26	283.06	54038.6	C ₁₅ H ₁₄ O ₆
14	(-)-Epigallocatechin	14.70	452.28	20261.8	C ₁₅ H ₁₄ O ₇
15	Ellagic acid	5.75	567.17	9275.36	C ₁₄ H ₆ O ₈
16	Biochanin A	6.07	543.13	209391	C ₁₆ H ₁₂ O ₅
17	6-Gingerol	15.69	368.32	47025.3	C ₁₇ H ₂₆ O ₄
18	Caffeic acid	8.1049	179.1501	7724.1	C ₉ H ₈ O ₄
19	Eriocitrin	5.2113	619.1526	109623	C ₂₇ H ₃₂ O ₁₅
20	Liquiritin	7.553	419.132	8056.52	C ₂₁ H ₂₂ O ₉
21	Emodin	10.2308	327.0449	6824.58	C ₁₅ H ₁₀ O ₅
22	Hyperoside	4.777	951.1803	29053.4	C ₂₁ H ₂₀ O ₁₂

3.2. Quantitative analysis of polyphenols in TBP

Quantitative analysis of polyphenols in TBP was conducted using LCMS-8060 (Fig. 1). To ensure reliable quantification, standard curves were established for each compound (Fig. S1) and detection conditions were optimized to improve accuracy and precision. Among the analyzed compounds, mangiferin, liquiritin, biochanin A and hypericin were not detected, likely due to their low abundance in TBP or physicochemical properties that hindered detection under the current conditions. On the other hand, relatively high levels of gallic acid, catechin, narcissin, quercetin, kaempferol and emodin were observed in TBP. Various studies have shown that these compounds possess strong antioxidant, anti-inflammatory and neuroprotective activities, with notable potential in alleviating fatigue [30]. In particular, rutin, quercetin,

hypericin, and catechin have been demonstrated to be effective in enhancing physical endurance and promoting recovery [31, 32]. These findings support our assumption that TBP being a functional food, particularly for mediating central fatigue. Moreover, these active constituents provided evidence for screening key factors targeting central nervous system fatigue.

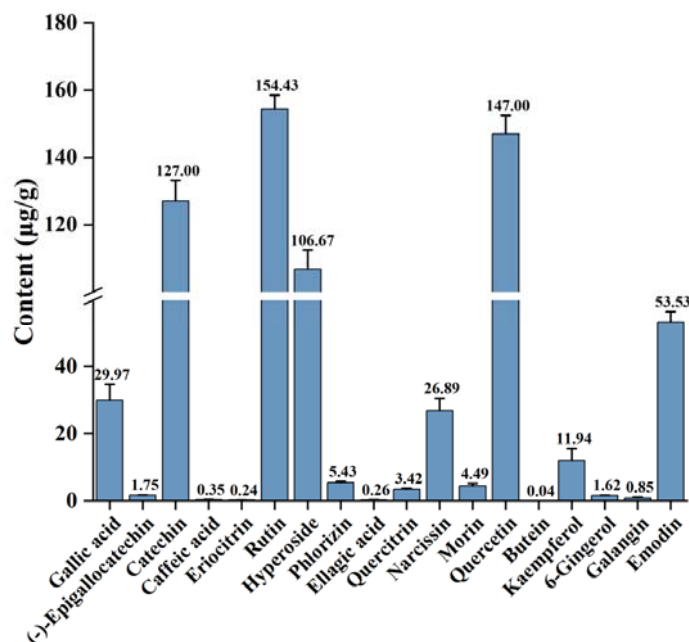


Fig.1. Quantitative analysis of polyphenol composition in tartary buckwheat.

3.3. Network pharmacology-based screening of key anti-central fatigue components in TBP

3.3.1. Screening of Anti-Central Fatigue Active Compounds and Associated Targets in TBP

Network pharmacology is a powerful multi-target analysis approach that can predict potential pharmacological targets and pathways of TBP, providing insights into the application of polyphenolic compounds in functional foods [33]. Polyphenolic compounds generally exert anti-fatigue effects through free radical scavenging, inhibiting inflammatory factor expression, or modulating key proteins in cellular signaling pathways. To identify potential TBP targets associated with central fatigue, we performed comprehensive screening of compound-related targets using SwissTargetPrediction and the TCMP database, based on both qualitative and quantitative analyses of TBP. Subsequently, target genes related to central fatigue were identified via Genecards and OMIM database searches, yielding a total of 1,400 genes. A total of 77 overlapping targets were identified between TBP and central fatigue (Fig. 2A). Furthermore, Cytoscape software was used to analyze the interactions among these targets, and a protein-protein interaction (PPI) network was constructed (Fig. 2B). The constructed network contains 77 nodes and 1005 edges. Key targets include AKT1 (AKT serine/threonine kinase 1), TNF (tumor necrosis factor), BCL2 (B-cell CLL/lymphoma 2), HSP90AA (heat shock protein 90 alpha), EGFR (epidermal growth factor receptor), ESR1 (estrogen receptor 1), SRC (SRC proto-oncogene, non-receptor tyrosine kinase), MAOA (monoamine oxidase A), MAOB (monoamine oxidase B) and HIF1A (hypoxia-inducible factor 1 alpha). It is worth noting that previous network pharmacology studies have shown that signaling nodes such as SRC, AKT1, ESR1 and TNF are closely linked to regulating fatigue states [34]. These findings suggested that these

targets might have played crucial roles in the pathophysiology of central fatigue by modulating key biological processes, including energy metabolism, inflammatory responses, and neuroprotection. Figure 2C illustrated the interaction network between TBP compounds and central fatigue-related targets, revealing the relationships between bioactive components and their molecular targets. In this network, the size and color intensity of the nodes represent the degree of intersection between polyphenolic compounds and central fatigue-related targets [35]. The results indicated that compounds such as morin, quercetin, emodin, galangin, narcissin, butein, kaempferol, and ellagic acid exhibited strong correlations with central fatigue-related targets, suggesting that these substances may be key components in TBP responsible for alleviating central fatigue. Rutin is generally recognized as one of the most abundant flavonoids in Tartary buckwheat; however, in the present network pharmacology analysis, it was not prioritized among the major candidate compounds associated with the core targets and key pathways of central fatigue. These results suggest that the potential contribution of a compound to the anti-central fatigue effect is not determined solely by its abundance, but also depends on its predicted biological relevance to disease-related targets. Therefore, compound abundance was considered as one aspect of the screening strategy, whereas the predicted associations with central fatigue-related targets and pathways were used as the primary criterion for selecting compounds for subsequent activity verification.

3.3.2. GO and KEGG pathway enrichment analysis

To further elucidate the functional roles of TBP anti-fatigue targets and their associated signaling pathways, we conducted the Gene Ontology (GO) enrichment analysis using the DAVID database. The analysis identified 449 biological processes (BP), 67 cellular components (CC) and 131 molecular functions (MF). Based on p-values, the top 10 most significantly enriched terms ($P < 0.01$) were selected for each category. In the BP category, target proteins were primarily related to signal transduction, positive regulation of gene expression and activation of the PI3K/AKT signaling pathway, etc. In the CC category, key targets were mainly distributed in the plasma membrane, cytosol, cytoplasm, nucleus and extracellular exosomes. In the MF category, most targets were involved in protein kinase activity, ATP binding and protein binding. Notably, these functions are closely related to energy metabolism, regulation of oxidative stress and cellular signal transduction, offering key mechanistic insights into the anti-fatigue effects of TBP.

KEGG pathway analysis identified 1143 pathways, of which the 10 most significantly enriched were selected (Fig. 2E). These pathways included the PI3K–AKT, MAPK and HIF-1 signaling pathways, among others. Central fatigue is often related to pathological processes including elevated oxidative stress, dysregulated inflammatory mediator expression and impaired energy metabolism. Among these, the PI3K–AKT pathway contributes critically to maintaining cell survival and modulating stress responses. This pathway activates the downstream transcription factor Nrf2, which in turn upregulates antioxidant enzymes such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1), thereby suppressing oxidative stress [36]. Given that oxidative stress is a key mechanism in the development of central fatigue, activation of this pathway may be crucial for fatigue alleviation [37]. The MAPK signaling pathway is

strongly implicated in fatigue. By modulating inflammatory mediator expression, mitochondrial function and energy metabolism, the MAPK pathway contributes to oxidative stress, inflammatory injury and neuromodulation, thereby affecting the physiological response to fatigue [38]. Furthermore, the MAPK pathway is essential for maintaining energy homeostasis [39]. Hypoxia-inducible factor-1 (HIF-1), a key transcription factor in cellular responses to hypoxia, promotes glucose uptake and upregulates glycolytic enzymes, thereby enhancing energy supply and improving endurance. HIF-1 signaling also modulates oxidative stress and inflammatory responses, thereby sustaining energy homeostasis and protective mechanisms in the central nervous system [40]. Previous studies have shown that HIF-1 signaling is pivotal in enhancing exercise endurance and mitigating fatigue [41].

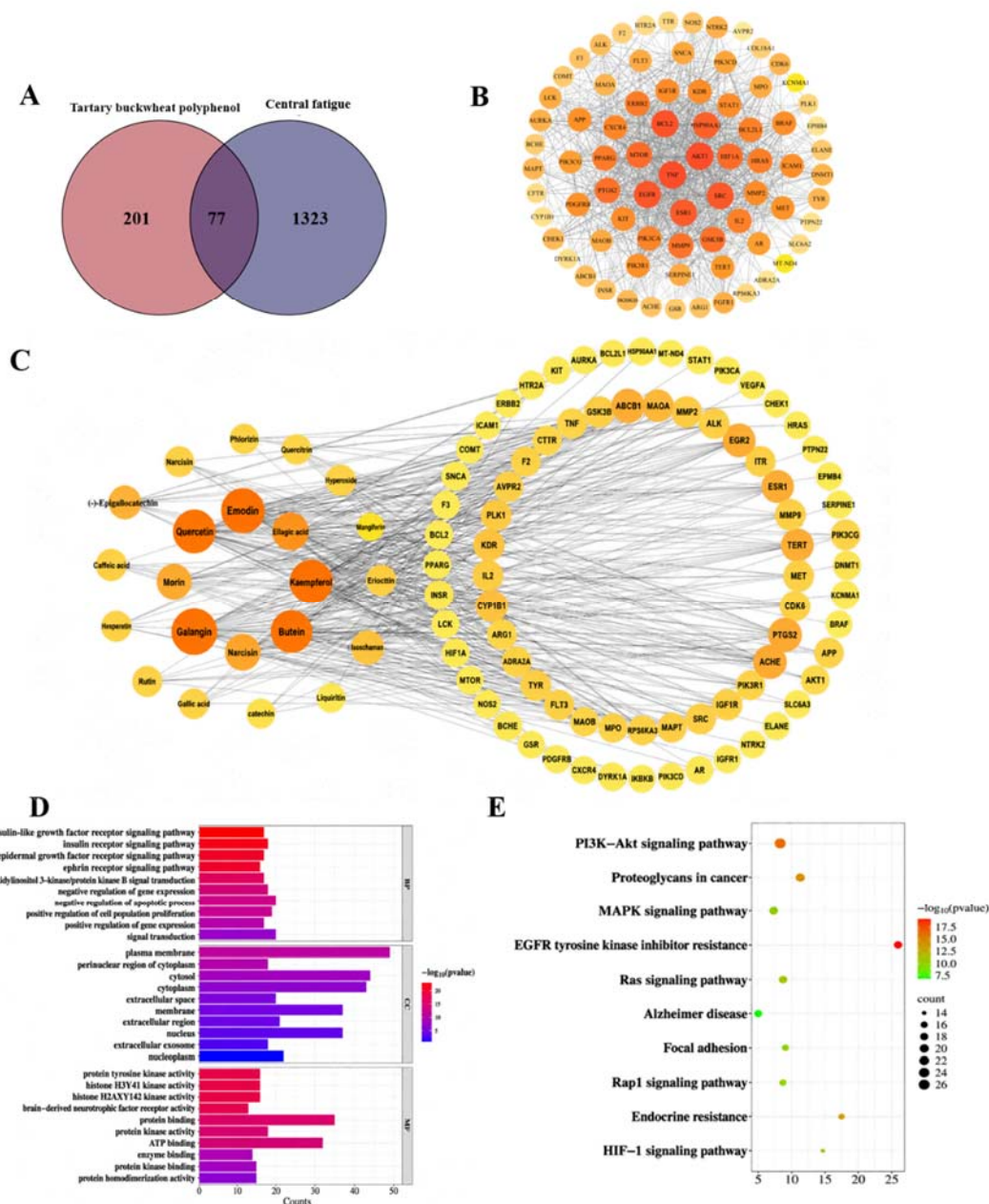


Fig.2. Network pharmacological analysis of key components of tartary buckwheat polyphenols (TBP) in alleviating central fatigue. A. Venn diagram of TBP and central fatigue targets. B. Protein-protein interaction network of central fatigue targets. C. TBP-central fatigue target interaction network. D. GO enrichment analysis. E. Bubble chart of KEGG pathways analysis.

3.4. Molecular Docking of TBP Key Components with Neurotransmitter-Related Enzymes

Central fatigue is often associated with dysregulated neurotransmitter expression. To identify TBP components with potential anti-central fatigue effects, we performed molecular docking of eight active compounds predicted by network pharmacology (morin, quercetin, emodin, galangin, narcissin, butein, kaempferol and ellagic acid) with neurotransmitter-related enzymes. Molecular docking was used to predict the binding modes and interactions between these phenolic compounds and target proteins, where lower binding energy indicates stronger interactions and more stable conformations [42]. Docking results showed that, except for narcissin, which exhibited binding energies above -5 kcal/mol for all four target proteins, the remaining seven compounds displayed relatively low binding energies, indicating strong binding affinity (Fig. 3A). Quercetin showed the highest binding affinity across the four proteins, followed by kaempferol, emodin, galangin, morin, butein, ellagic acid and narcissin. As quercetin exhibited the strongest binding affinity among all compounds, it was selected for molecular docking visualization to illustrate its binding mode and interaction sites with the target protein (Fig. 3B). Docking conformations of the other compounds are provided in the supplementary materials (Fig. S2).

Central fatigue is commonly associated with reduced levels of key neurotransmitters, including serotonin (5-HT) and dopamine (DA), a trend also confirmed in our previous research [43]. As a key component of the serotonin system, 5-HT₄ plays a pivotal role in regulating synaptic excitability. Activation of 5-HT₄ promotes serotonin release, thereby improving cognitive function, emotional regulation, and resistance to fatigue. The results of this study indicated that, except for narcissin, most components demonstrated strong binding potential to 5-HT₄ receptors, particularly quercetin and emodin. These compounds may mitigate central fatigue by upregulating 5-HT expression. Excessive activity of MAOA and MAOB, the primary enzymes responsible for degrading monoamine neurotransmitters such as DA and 5-HT, can reduce neurotransmitter levels, thereby exacerbating fatigue and cognitive impairment [44, 45]. TBP Key components exhibited strong binding affinity to MAOA and MAOB, suggesting they may increase neurotransmitter levels by inhibiting monoamine oxidation and thereby exerted anti-central fatigue effects. Notably, nuclear receptor related 1 protein (NURR1) is critical to the development and maintenance of the central nervous system, especially in DA neurons. Impaired NURR1 function leads to reduced dopamine synthesis, which is considered a critical mechanism contributing to neuropsychiatric disorders and central fatigue [46]. The above results demonstrated that TBP key compounds exhibit strong affinity for NURR1, suggesting they may mitigate central fatigue by enhancing dopaminergic neuron function and maintaining neurotransmitter homeostasis. In summary, molecular docking results indicated that narcissin exhibited relatively weak binding with neurotransmitter-related enzymes and was therefore excluded from further analysis. Subsequently, *in vitro* enzyme inhibition assays were performed to further validate and identify the key compounds among the remaining seven responsible for alleviating central fatigue.

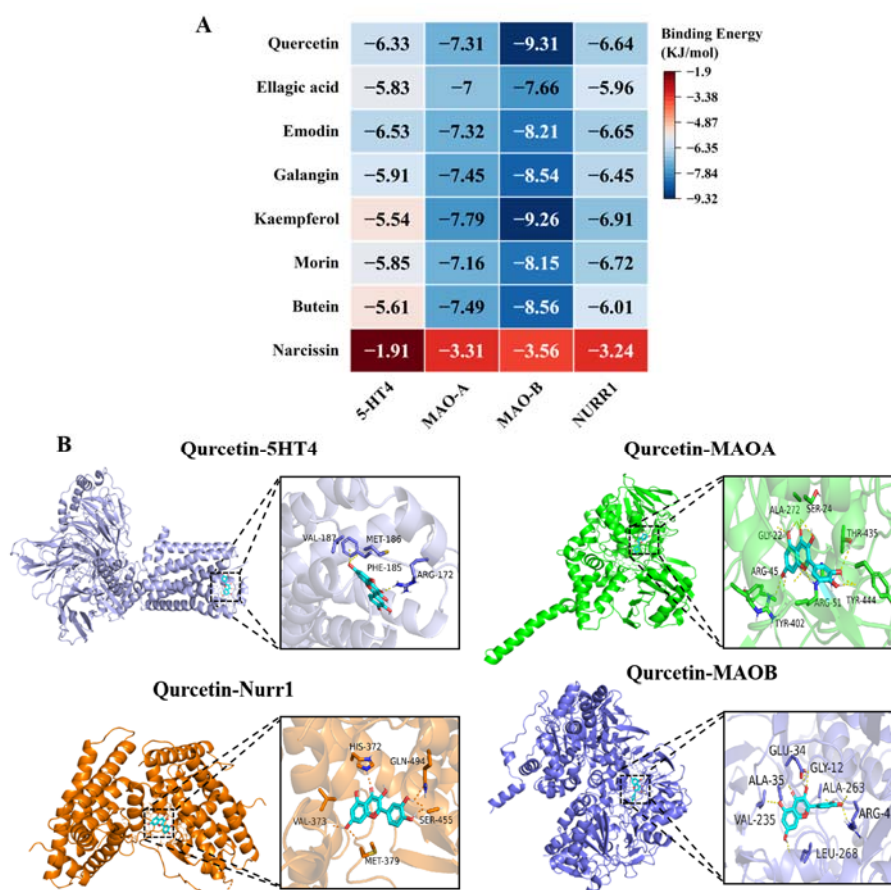


Fig.3. Molecular docking analysis of the binding affinity between TBP and central fatigue-related enzymes. A. Heatmap of binding scores between TBP and protein targets. B. Quercetin-protein target binding site.

3.5. Inhibitory Effects of TBP Key Components on MAO-A and MAO-B Enzyme Activities

Natural products with enzyme inhibitory activity have attracted considerable attention due to their potential roles in disease prevention and treatment [47]. To identify the key TBP components underlying anti-central fatigue effects, we evaluated their inhibitory activity against MAO-A and MAO-B, two neurotransmitter-metabolizing enzymes implicated in central fatigue (Fig.4). The results showed that TBP key components exert significant inhibitory effects on both MAO-A and MAO-B. Specifically, the MAO-A inhibitory activity ranked as follows: quercetin > emodin > kaempferol > morin > galangin > ellagic acid > butein. For MAO-B, the inhibitory activity ranked as follows: quercetin > emodin > kaempferol > galangin > ellagic acid > butein > morin. Comparative analysis showed that quercetin exhibited the strongest inhibitory activity (MAO-A: $IC_{50} = 4.386 \mu M$; MAO-B: $IC_{50} = 3.982 \mu M$), followed by emodin (MAO-A: $IC_{50} = 4.762 \mu M$; MAO-B: $IC_{50} = 4.612 \mu M$) and kaempferol (MAO-A: $IC_{50} = 5.761 \mu M$; MAO-B: $IC_{50} = 6.147 \mu M$). By contrast, morin, galangin, ellagic acid, and butein exhibited relatively weaker inhibition, suggesting they may contribute additionally or synergistically to the overall anti-fatigue effect.

Although eight compounds were identified as potential active constituents by network pharmacology analysis, quercetin was selected for subsequent validation based on an integrated evaluation combining network pharmacology, molecular docking, and in vitro MAO inhibitory activity assays. Among the candidate compounds, quercetin showed strong predicted associations with central fatigue-related targets,

favorable binding interactions with key proteins, and marked inhibitory activity against MAO, supporting its use as a representative active compound in TBP. Previous studies have also confirmed that quercetin, as a representative flavonoid, can inhibit both MAO-A and MAO-B [48, 49]. This effect is closely associated with the hydroxyl substitution pattern in its molecular structure and the stability of its conjugated system, which together provide the molecular basis for the regulatory function of quercetin in central fatigue. Notably, emodin, an anthraquinone compound, also exhibited MAO inhibitory activity comparable to that of quercetin, suggesting that non-flavonoid compounds in TBP may also contribute to the regulation of neurotransmitter homeostasis through MAO-targeted mechanisms. In contrast, although kaempferol is also a flavonoid, differences in the number and distribution of hydroxyl groups may account for its weaker MAO inhibitory activity compared with quercetin. Therefore, quercetin was selected as a representative compound for subsequent *in vivo* validation to further evaluate whether TBP-derived polyphenols alleviate central fatigue through MAO inhibition and neurotransmitter regulation. Nevertheless, the anti-central fatigue effect of TBP is unlikely to be attributable to a single compound. Other predicted active constituents, including less-studied candidates, could also contribute to the observed biological effects, and the potential additive or synergistic interactions among multiple polyphenolic compounds should be further investigated in future studies.

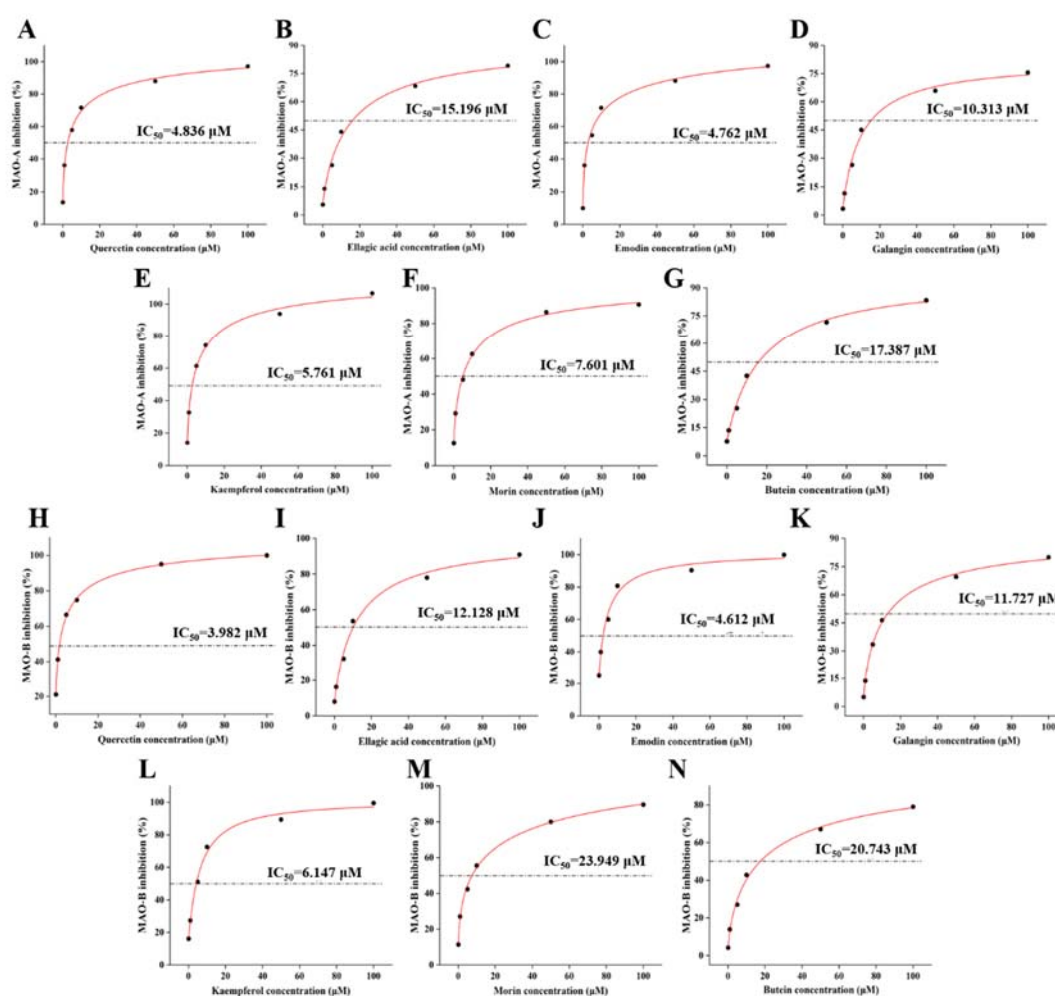


Fig.4. Inhibitory effects of TBP key bioactive compounds on central fatigue-related MAO enzymes. A-G: Inhibition of MAO-A activity by TBP key compounds. H-N: Inhibition of MAO-B activity by TBP key compounds.

3.6. TBP Key Component Alleviate Central Fatigue and Fatigue-Related Behaviors

To validate the anti-central fatigue effects of quercetin, identified as the most potent MAO inhibitor in previous analyses, we assessed its effects on motor performance and fatigue-related behaviors in mice with central fatigue using grip strength, rotarod, and open-field tests (Fig.5). Grip strength testing showed that the MOD group had significantly lower average grip strength than the CON group ($P < 0.05$), indicating that excessive exercise induced muscle weakness and fatigue. In contrast, grip strength was significantly higher in the POS and QUE groups ($P < 0.05$), suggesting that EGCG and quercetin alleviated exercise-induced muscle weakness. Rotarod testing further supported these findings, as mice in the MOD group showed significantly shorter dwell times at 30 rpm than mice in the CON group ($P < 0.05$), indicating that central fatigue impaired motor coordination and endurance. Both the POS and QUE groups displayed significantly longer dwell times than the MOD group ($P < 0.05$), with quercetin showing particularly strong effects, further confirming its role in alleviating fatigue-related motor impairments.

In the open-field test, we further evaluated spontaneous locomotor activity and emotion-related behaviors in mice. The MOD group spent significantly less total locomotor time than the CON group ($P < 0.05$), indicating that central fatigue reduced spontaneous locomotor activity. Meanwhile, the MOD group spent significantly less time in the central area and more time in the periphery ($P < 0.05$), suggesting that fatigue was associated with anxiety-like behavior. In contrast, the POS and QUE groups showed significantly active time and longer exploration in the central area ($P < 0.05$), indicating that quercetin effectively ameliorates the reduced spontaneous locomotor activity and anxiety-related behaviors caused by central fatigue. Previous studies have shown that quercetin alleviates depression-like behavior in the chronic unpredictable mild stress (CUMS) model and enhances fatigue resistance by increasing antioxidant capacity, promoting muscle glycogen storage, and improving muscle function [26, 50]. Furthermore, quercetin can mitigate muscle atrophy in mice, thereby improving grip strength [51].

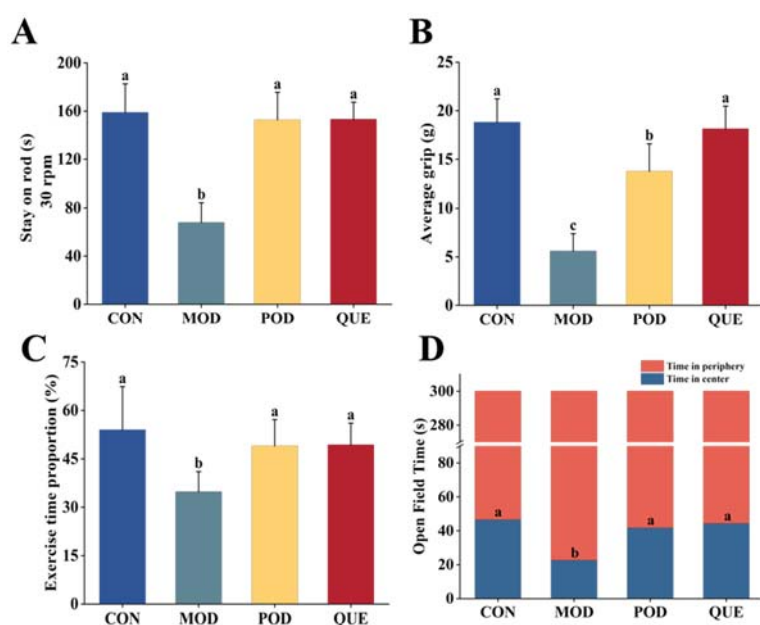


Fig.5. TBP key compounds alleviate central fatigue-like behaviors in a mouse model. A. Performance on the rotarod at 30 rpm. B. Average grip strength. C. Exercise time proportion. D. Open field time.

3.7. TBP Key Component Alleviate Central Fatigue by Modulating Neurotransmitters in Mice

To further investigate the role of TBP key components in alleviating central fatigue, we measured neurotransmitter levels in mouse brain tissue across all groups. Results showed that compared with the CON group, 5-hydroxytryptamine (5-HT), dopamine (DA), gamma-aminobutyric acid (GABA) and norepinephrine (NE) levels were significantly reduced in the MOD group ($P < 0.05$), indicating that central fatigue is strongly correlated with the downregulation of multiple neurotransmitters. In the POS and QUE groups, these neurotransmitters partially recovered, suggesting that quercetin mitigates neurotransmitter dysregulation induced by central fatigue. Furthermore, glutamate (Glu) levels were significantly elevated in the MOD group compared with the CON group ($P < 0.05$), whereas QUE treatment markedly reduced them to near-normal levels (Fig.6).

Neurotransmitters are essential chemical messengers that mediate signal transmission within the central nervous system, and their homeostasis is critical for normal central nervous system function [7]. Research indicates that central fatigue is closely correlated with neurotransmitter imbalance. Among these, 5-HT primarily regulates mood, sleep and appetite, playing a vital function in maintaining nervous system homeostasis and psychological well-being [43]. DA is involved in motivation, reward processing, pleasure and motor control, serving as a key regulator of normal central nervous system function [52]. GABA is the primary inhibitory neurotransmitter and its decrease weakens neural inhibition, thereby raising the risk of anxiety and hyperexcitability [53]. NE is key to cognitive function, and its depletion is frequently linked to cognitive deficits and increased fatigue. Glu is the main excitatory neurotransmitter and its abnormal elevation can induce synaptic excitotoxicity, thereby worsening neurological dysfunction [54]. This study demonstrates that central fatigue markedly decreases 5-HT, DA, GABA and NE levels in mouse brain tissue, while increasing Glu. These findings suggest a close association between central fatigue and neurotransmitter dysregulation. Importantly, quercetin intervention significantly restored the altered neurotransmitter levels, indicating its potential to alleviate central fatigue by restoring neurotransmitter homeostasis. Moreover, the *in vivo* findings corroborate results from *in vitro* enzyme inhibition assays, further supporting the contribution of quercetin in mitigating central fatigue.

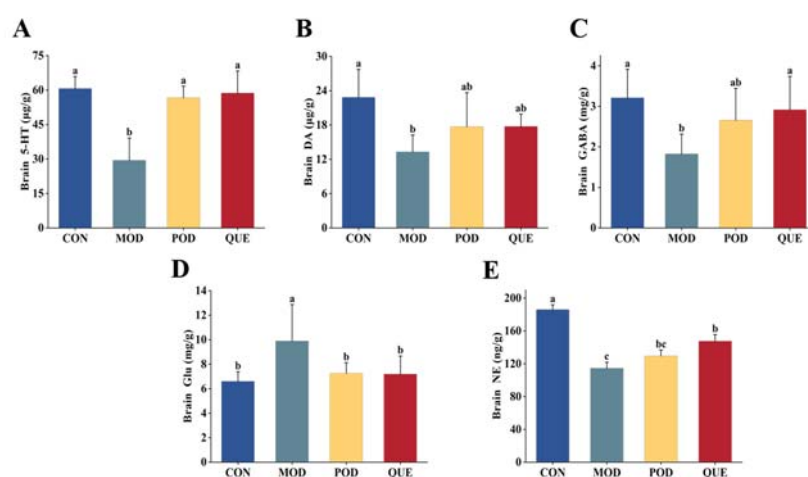


Fig.6. TBP modulates brain neurotransmitters in exercise-induced central fatigue. A. 5-hydroxytryptamine (5-HT). B. dopamine (DA). C. gamma-aminobutyric acid (GABA). D. glutamate (Glu). E. norepinephrine (NE).

3.8. TBP Key Component Regulate Gut Transcriptome in Central Fatigue Mice

Given the close link between central fatigue and intestinal injury, we analyzed the mouse gut transcriptome to assess the regulatory effects of TBP key components. As shown in the Fig.7A, a total of 2016 differentially expressed genes (DEGs) showed significant transcriptional differences between the CON and MOD groups (1238 upregulated and 778 downregulated), indicating that central fatigue induces gut transcriptome remodeling. Comparatively, 532 DEGs (306 upregulated, 226 downregulated) were detected between the MOD and POS groups, whereas 1,047 DEGs (580 upregulated, 467 downregulated) were identified between the MOD and QUE groups, suggesting that quercetin exerts a more extensive transcriptional effect. KEGG enrichment analysis showed that the DEGs between the CON and MOD groups were primarily enriched in pathways such as neuroactive ligand-receptor interactions, calcium signaling, serotonergic synapses, dopaminergic synapses, neurodegenerative disease pathways, MAPK signaling, and PI3K-AKT signaling (Fig.7B). In addition, significant pathways enriched in the MOD and POS groups included neuroactive ligand–receptor interaction, glycine/serine/threonine metabolism, IL-17 signaling, retrograde endocannabinoid signaling, mTOR signaling and GABAergic synapse. By contrast, DEGs between the MOD and QUE groups were predominantly enriched in oxidative phosphorylation, neuroactive ligand–receptor interaction, PI3K–AKT signaling, calcium signaling, glutamatergic synapse, tight junction, dopaminergic synapse, and serotonergic synapse.

These findings suggest that central fatigue activates multiple neurotransmitter-related pathways (e.g., neuroactive ligand–receptor interactions, serotonergic and dopaminergic synapses), consistent with the experimentally observed neurotransmitter imbalances. In parallel, enrichment of calcium signaling, MAPK and PI3K–AKT pathways indicates that central fatigue elicits stress responses, whereas activation of neurodegenerative disease associated pathways suggests a potential risk of neurological damage [55–57]. EGCG intervention (POS group) mainly affected pathways such as neuroactive ligand–receptor interactions, GABAergic synapses and IL-17 signaling, suggesting that it alleviates central fatigue by suppressing inflammatory responses and enhancing inhibitory neurotransmission. In contrast, quercetin intervention predominantly enriched pathways such as oxidative phosphorylation, PI3K–AKT signaling and calcium signaling. This demonstrates that quercetin can enhance mitochondrial function, promote cell survival signaling and maintain calcium homeostasis, thereby mitigating energy metabolism disorders and stress-induced damage [58]. Concurrently, the enrichment of glutamatergic, dopaminergic and serotonergic synapses indicates that quercetin extensively regulates multiple neurotransmitter synaptic transmissions. This finding is highly consistent with the restoration of 5-HT, DA and Glu levels observed in this study. Moreover, alterations in the tight junction pathway indicate that quercetin may protect the central nervous system by strengthening the intestinal barrier and thereby reducing the permeability of inflammatory mediators. Notably, the PI3K–AKT signaling pathway was significantly enriched in all three groups (CON, MOD and QUE), consistent with network pharmacology predictions. This suggested that the PI3K–AKT pathway may have played a central role in both the development of central fatigue and the effects of

polyphenol intervention. This study further investigated and validated the key molecular mechanisms of this pathway in following experiments.

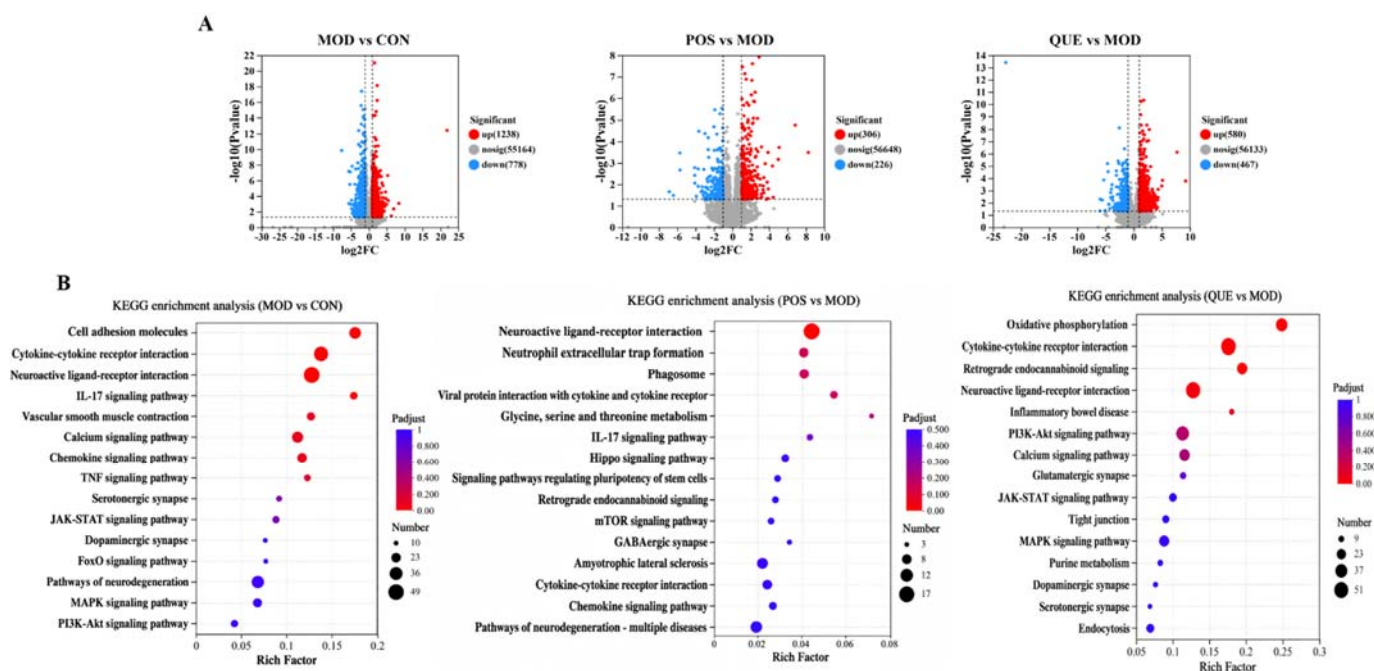


Fig.7. Transcriptomic analysis of TBP key components in alleviating central fatigue. A. Volcano plot of differentially expressed genes. B. KEGG pathway enrichment analysis.

3.9. TBP Key Component Alleviate Central Fatigue via PI3K/AKT–Nrf2 Pathway

The PI3K/AKT–Nrf2 signaling pathway is essential for regulating cellular metabolism and serves as a key regulator of cell proliferation, apoptosis and related processes [59]. Additionally, this pathway is central to oxidative stress defense and energy homeostasis during fatigue and is regarded as a key mechanism for fatigue alleviation [60]. To further clarify how quercetin regulates central fatigue, we evaluated its effects on activating the PI3K/AKT–Nrf2 signaling pathway (Fig.8). Western blot analysis showed that, compared with the CON group, the MOD group had significantly lower levels of p-PI3K, p-AKT/AKT, Nrf2 and the downstream antioxidant enzyme HO-1 ($P < 0.05$). These results indicated that central fatigue impaired cellular self-regulation by suppressing the PI3K/AKT–Nrf2 pathway. Compared with the MOD group, the POS and QUE groups showed significantly higher levels of p-PI3K, p-AKT/AKT, Nrf2 and HO-1 ($P < 0.05$), indicating that both EGCG and quercetin alleviated central fatigue by activating the PI3K/AKT–Nrf2 pathway. Notably, activation in the QUE group was stronger than in the POS group, suggesting that quercetin may had greater potential for alleviating central fatigue.

When fatigue occurs, the PI3K/AKT signaling pathway can be activated, providing protective effects to the body [61]. Nrf2 is a central regulator of cellular defense against stress-induced damage. Under normal conditions, it bound to its repressor protein Keap-1 in the cytoplasm [62]. Upon stimulation, Nrf2 dissociates from Keap-1 and translocates into the nucleus, where it binds to the ARE to induce expression of the target gene HO-1 [63]. HO-1 is a critical antioxidant enzyme that defends cells against oxidative stress, fatigue and other types of damage. Under stress conditions, HO-1 expression is upregulated, and it contains binding sites

resembling the ARE. Thus, Nrf2 can directly regulate HO-1 expression [64]. Activation of the PI3K/AKT signaling pathway facilitates Nrf2 nuclear translocation, thereby enhancing expression of its downstream HO-1. The results indicated that central fatigue inhibited the PI3K/AKT signaling pathway in mice, leading to decreased Nrf2 expression and diminished intracellular antioxidant enzyme activity. Conversely, quercetin intervention reactivated the PI3K/AKT pathway, promoted Nrf2 nuclear translocation and upregulated HO-1 expression, thereby enhancing antioxidant defense and mitigating central fatigue. Moreover, other natural polyphenols, including hawthorn polyphenols, dragon fruit polyphenols and curcumin, have been found to exert anti-fatigue effects through PI3K/AKT pathway modulation, suggesting that this pathway is a critical target for fatigue alleviation via polyphenolic compounds^[65-67].

In summary, this study identified and validated the key components of TBP that mitigate central fatigue as well as elucidated their mechanisms of action using *in vitro* assays and *in vivo* experiments. Qualitative and quantitative analyses combined with network pharmacology, molecular docking and *in vitro* enzyme assays pointed out quercetin as the primary TBP component responsible for alleviating central fatigue. Subsequently, the grip strength, rotarod and open-field tests confirmed that quercetin markedly improved fatigue-related behavioral phenotypes and effectively restored the neurotransmitter balance disrupted by central fatigue. Furthermore, transcriptomic analysis and western blot results suggested that quercetin enhanced antioxidant capacity and alleviated central fatigue by activating the PI3K/AKT pathway, promoting Nrf2 nuclear translocation and upregulating the downstream antioxidant protein HO-1. In summary, these findings indicate that natural food-derived, especially quercetin, have considerable potential for alleviating central fatigue.

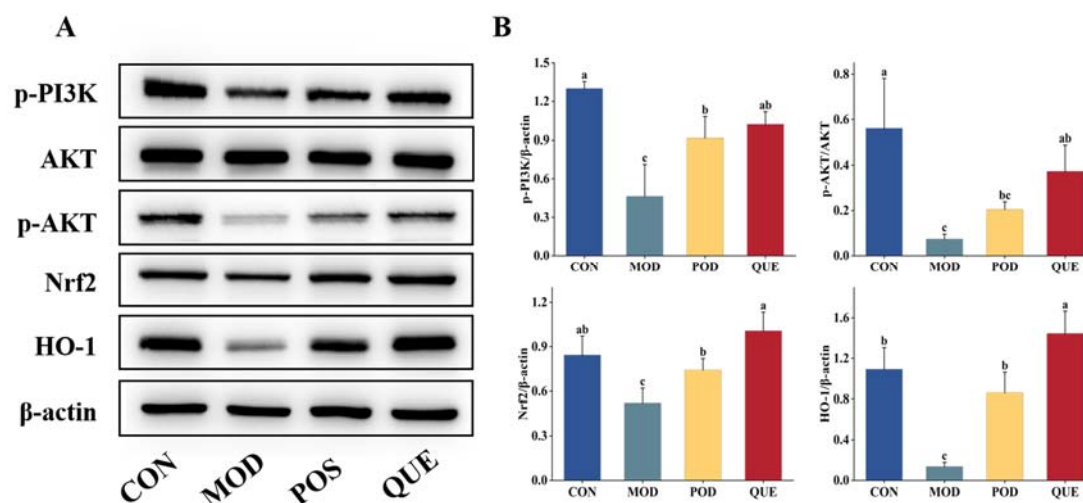


Fig.8. TBP Key Components Alleviate Central Fatigue by Regulating the PI3K/AKT-Nrf2 Signaling Pathway. A. Western blot analysis showing the protein expression levels of p-PI3K, AKT, p-AKT, Nrf2 and HO-1 in different treatment groups. B. Relative protein expression levels.

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Conflicts of Interest

Yanbo Wang is an editorial board member for Food Science and Human Wellness and was not involved in the editorial review or the decision to publish this article. 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 Statement

Data will be made available on request.

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