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Original Research Article

Fermented *Pueraria lobata* alleviates H_2O_2-induced oxidative stress in zebrafish by regulating the Nrf2/ARE signaling pathway

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Abstract

This study aimed to investigate the changes in the composition of *Pueraria lobata* after fermentation by *Pichia kudriavzevii* and its impact on the antioxidant capacity of zebrafish. The LC-MS was utilized to analyze the constituents of the aqueous extract of *P. lobata* (non-fermented, AEP) and the fermented broth of *P. lobata* (fermented, FBP). An H_2O_2-induced oxidative stress model (OS) in zebrafish was constructed, and antioxidant enzyme activities and expression levels of antioxidant-related genes were compared after zebrafish were exposed to AEP and FBP. It was found that the composite species of FBP was reduced by 6.45% compared with AEP after fermentation by *P. kudriavzevii*. The main component of *P. lobata* is isoflavones, which increase by 1.27% after fermentation. Moreover, the FBP significantly reduced the increase in MDA caused by H_2O_2 in zebrafish and significantly increased T-AOC activity in vivo. In addition, the relative expression of genes connected to the antioxidant Nrf2/ARE signaling pathway was markedly reduced in the FBP group, approaching the level of normal control samples. Interestingly, among these effects, the FBP group was more significant than the AEP group. FBP promotes the expression of antioxidant factors and alleviates the oxidative stress response of Zebrafish. These findings provide a reference for the development and application of the antioxidant properties of fermented *P. lobata* in the food industry.

Keywords: *Pueraria lobata*, Antioxidant, *Pichia kudriavzevii*, Nrf2/ARE, Zebrafish

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1. Introduction

The dried root of Kudzu, a perennial vine belonging to the leguminous family, is known as *Pueraria lobata*. *P. lobata* is regarded as a traditional Chinese edible and medicinal herb in China (Chen et al., 2020). In addition to being nutrient-rich, *P. lobata* also has a wide range of pharmacological qualities, making it popular for treating conditions including fever, diarrhea, inflammation, and other related illnesses as well as for disease prevention (Wang et al., 2021, Zha et al., 2020). Scientists have found over 70 phytochemicals from *P. lobata*, which can be categorized into four categories based on their chemical composition: Alkaloids, coumarins, triterpenes, and isoflavones (Xi et al., 2023, Wong et al., 2011). The primary active ingredients in *P. lobata* are isoflavones, which also include genistein flavonoids, soybean glycosides, and puerarin (Wong et al., 2011, Xi et al., 2023). Additionally, the therapeutic potential of *P. lobata* extract and its chemical components have been proven due to their antioxidant, anti-ischemic, anti-cancer, anti-inflammatory, and antifatigue properties (Sook Kim et al., 2014, Wong et al., 2011, Kim et al., 2014, Zhou et al., 2014). Currently, *P. lobata* has a lot of potential in the international market and research sectors since it is extensively utilized in the pharmaceutical, health products, and food industries (Xi et al., 2023).

Pichia Kudriavzevii is a facultative anaerobic bacterium that evolved from *Candida krusei*. Its cell morphology is characterized by spherical ascospores, and its colony is light cream-colored. It is prone to film production on the surface of the fermentation broth. *P. Kudriavzevii* has a strong tolerance for low oxygen, low pH, high temperature, high -concentration ethanol, and other environments (Park et al., 2018). It is usually distributed in various natural fermentations, fruits, juice, and widely distributed in nature as a potential probiotic. *P. Kudriavzevii* plays an important role in the fermentation industry and has huge application prospects for liquor (Zheng et al., 2012), wine (Ruiz-Muñoz et al., 2020), dairy products (Geronikou et al., 2023), coffee, fermented cocoa bean (Maura et al., 2016), acid dough (De Vuyst et al., 2016), etc. WU discovered *P. Kudriavzevii* can promote the formation of pyrene compounds in fragrant liquor (Wu et al., 2015). *P. Kudriavzevii* has the ability to degrade L-apple acid, and low-yield ethanol, improve glycerol and acetic acid, and improve the sensory characteristics of wine (Scansani et al., 2022). By applying *P. Kudriavzevii*, an acetic acid interface is added to give the coffee a fruitier (banana) flavor. *P. Kudriavzevii* can also be used as an effective antagonistic yeast, which can significantly suppress rot, and weightlessness and delay color changes in tomatoes (Liu et al., 2020b). However, its use is rare in the application of Chinese medicine.

Free oxygen radicals are a type of reactive oxygen species (ROS) that are mainly produced within the cell during mitochondrial respiratory metabolism and in neutrophils during the cellular response to pathogenic threats (Thannickal and Fanburg, 2000, Shashni et al., 2023). Antioxidant enzymes, namely superoxide dismutase, glutathione, peroxiredoxins, and catalases, are responsible for neutralizing the ROS generated in cells. However, if there is an excess production of ROS, the antioxidant system becomes overwhelmed, causing severe oxidative damage to cellular components such as DNA, proteins, and lipids that cannot be repaired (Shashni et al., 2023). This imbalance in redox homeostasis is called oxidative stress (Thannickal and Fanburg, 2000, Freeman and Crapo, 1982). Many studies have linked oxidative stress to several pathological causes, including neurodegenerative diseases, cancer, rheumatoid arthritis, cardiovascular diseases, diabetes, and non-alcoholic steatohepatitis (Forman and Zhang, 2021, Sies et al., 2017).

Zebrafish have the advantages of easy observation, transparent embryos, small size, high reproductive capacity of adult fish, and *In vitro* fertilization, and the advantages of easy availability of materials, easy operation, short cycle time, high efficiency, and low cost to conduct related studies using zebrafish model (Nishimura et al., 2016). As well as similar physiological and biochemical characteristics to mammals: its genes are more than 87% similar to human genes (Woo et al., 1995), and the cardiovascular system, liver and other digestive systems, and the nervous system have similar developmental mechanisms and characteristics to those of human tissues, which has made zebrafish an internationally recognized

model animal for new vertebrates (de Jong and Zon, 2005). Currently, zebrafish have been used for the evaluation of biological activities such as antioxidant, immunomodulatory, and cardioprotective in a variety of plant extracts and products (Yang et al., 2018, Kang et al., 2015).

This study aimed to investigate the changes in the composition of *P. lobata* after fermentation and its impact on the antioxidant capacity of zebrafish. The liquid fermentation of *P. lobata* was carried out by *P. kudriavzevii*, and the components of *P. lobata* fermentation broth and *P. lobata* water extract was detected by LC-MS. The oxidative stress model of zebrafish was induced by H_2O_2, and the expression level of oxidative stress-related genes was determined. It provides a theoretical basis for the deep development and utilization of *P. lobata* and the development of antioxidants.

2. Materials and Methods

2.1. Reagents

Pueraria lobata （Lot No.191200219）were purchased from Kangmei (Haozhou) Century Chinese Medicine Co., Ltd., (Shandong, China). *P. kudriavzevii* from the laboratory of Yibin Campus of Sichuan University of Science & Engineering. MDA (Lot No.2304001) and T-AOC (Lot No.2303001) assay kits were purchased from Solarbio (Beijing, China). RNA Extraction (Lot No. R6834020000F07U028) and RT-PCR Kits were purchased from Andy Biotechnology (Shanghai, China). SYBR qPCR Master Mix (Lot No.027E2211BA) and cDNA transcriptase kits were purchased from Nanjing Vazyme Biotech Co., Ltd., (Nanjing, China, Lot No.027E2211BA).

2.2. Zebrafish husbandry and embryo collection

Wild-type AB zebrafish were procured from the China Zebrafish Resource Center based in Wuhan, China. The fish were kept in circulating aquaculture systems with a photoperiod of 14 h: 10 h light/dark cycle and were fed with *Artemia salina* at a constant temperature of 28 ±1°C. These fish were placed in a mating box with a male-to-female ratio of 2:1 and mate the next morning. Embryos were taken out around 10 o'clock and incubated in embryo culture medium for 6 hours. All experiments were conducted in accordance with the guidelines of the Experimental Animal Welfare and Ethics Committee of Gannan Normal University (protocol number: gnnu2022-0628) after obtaining relevant approval. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

2.3. Preparation of *P. lobata* aqueous extract and *P. lobata* fermentation broth

Ten grams of *P. lobata* was crushed and passed through an 80-mesh sieve. *P. lobata* was extracted with boiling distilled water (1:15, w/v) for 30 min. The powder was decocted twice for 30 minutes in 15 volumes of boiling water. Distilled water was used to fix the volume of the filtered extract to 200 mL and divided into two equal parts. After the extract was autoclaved at 121°C for 20 minutes, it was inoculated with 3% (v/v) *P. kudriavzevii* (fermented broth of *P. lobata*) and sterile water (aqueous extract of *P. lobata*). Fermentation occurred at 37°C and 180 r/min for 72 h followed by supernatant collection at 10,000 r/min for 5 min to obtain *P. lobata* water extract (AEP) and fermented broth of *P. lo bata* (FBP) respectively.

2.4. LC-MS analysis

The LC-MS analysis was performed using a Waters ACQUITY UPLC BEHC18 (250 mm×4.6 mm, 5 μm) chromatographic column with 0.1% acetonitrile in phase A and water in phase B. The temperature of the column and the sample chamber were set at 40°C and 10°C, respectively. The flow rate was set at 1 mL/min and the temperature of the autosampler was set at 4℃. The ionization source temperature was set to 120 ℃ with a cone gas flow rate of 50 L/h and desolvation gas temperature of 400 ℃ at a flow rate of 800 L/h. The capillary voltage was 3.0 and 2.5 kV, while the cone pore voltage was 40 V in the positive and negative ion modes, respectively. An extraction cone voltage of 80 V was used in the positive mode and a compensation voltage of 80 V in the negative ion mode. The range of mass numbers collected was 50-1000 kDa. Tandem mass spectrometry used argon as the collision gas, with a low collision energy of 4 eV and a high collision energy of 20–40 eV.

2.5. Developmental toxicity of FBP

The developmental toxicity of FBP was assessed by exposing Zebrafish embryos (aged 6-9 hpf, 20

embryos per well) to different FBP concentrations (v/v) (10.00, 8.00, 6.00, 4.00, 2.00, 1.33, 1.00, 0.67, 0.50 and 0.33%). The mortality and hatching rate of embryos were observed and recorded at 72 hpf. During the experiment, the solution was changed every 24 h and the dead embryos were aspirated and discarded.

2.6. Establishment of oxidative stress model and animal treatment groups

Treatment with H_2O_2 was used to induce oxidative stress in zebrafish. The zebrafish embryos at 6-9 hpf were treated with increasing (0.5, 1, 2, 3, 3.5,4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0, 15.0 and 16.0 mM) concentrations of H_2O_2, and the mortality of embryos was recorded at 72 hpf. The optimal concentration of H_2O_2 needed to induce oxidative stress in zebrafish was calculated

The standards of the Animal Ethics Committee of the Laboratory Animal Center, Gannan Normal University, Jiangxi, were followed in all zebrafish research. Twenty cultured zebrafish embryos were added to each well of a six-well plate. The zebrafish embryos were evenly and randomly divided into four groups (3 replicates per group): Blank control (C) group, H_2O_2-treated oxidative stress (M) group, Fermented broth of *P. lobata* + H_2O_2-treated (FBP) group, Aqueous extract of *P. lobata,* + H_2O_2-treated (AEP) group. For the treatment group, embryos were incubated in the optimal concentration of AEP and FBP from 24 hpf, while zebrafish in groups C and M were treated with ddH_2O as a control (shown in **Fig. 1A**) until samples were collected for measurement. Starting at 48 hpf, the optimal concentration of H_2O_2 was used to induce oxidative stress (shown in **Fig. 1A**) and samples were collected for measurement at 72 hpf.

2.7. Measurement of antioxidant activity

Zebrafish embryos (n=10) treated for 72 hours were washed with PBS three times for 5 minutes. Then 300 μL of 0.9% physiological saline was added to each group to homogenize the tissue. The homogenate was centrifuged at 8000 g for 10 min at 4 0C. MDA and T-AOC were measured in the supernatant using MDA and T-AOC assay kits (purchased from Solarbio, Beijing, China).

2.8. RNA extraction and real-time quantitative PCR

Thirty embryos from each group were used for RNA extraction and qRT-PCR and washed 5 times with phosphate buffer. Embryos were homogenized using TriZol reagent, RNA was extracted, and RNA was reverse transcribed into cDNA using the Prime Script® RT kit. Each sample was sequenced in triplicate, and the relative expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Using β-actin gene (Actb) as internal control. The primer sequences were shown in **Table 1**.

2.9. Statistical analyses

All results were analyzed using GraphPad Prism 9 software, and values were displayed as mean ± SD. One-way ANOVA and the Dunnett test were used to determine statistical differences between the control and treatment groups.

Table 1. List of primer sequences used for real-time quantitative PCR.

Gene	FP sequence（5'→3'）	RP sequence (5′→ 3′)
β-actin	GTCCACCTTCCAGCAGATGT	GAAAGGGTGTAAAACGCAGC
SOD1	GAAAGGGTGTAAAACGCAGC	ATGCAGCCGTTTGTGTTGTC
GPx	GACACGCCCACAAAAACTT	CTTTGAACAGTCCTGCTCGT
GST	TCTGGACTCTTTCCCGTCTCTCAA	ATTCACTGTTGCCGTTGCCGT
GADD45αa	TGGCTTGTTTGTGGGACTT	TGGAAAACAGTCCACTGAGA
Nrf2	CATGGCCCTCATCTTGACTT	GACAAAATCGGCGACAAAAT
C3	GTATTACTCACCCGATGCCCG	AGATGGGGTTCACAGGCTTTAAT

3. Results

3.1. Effect of fermentation on changes in composition *of P. lobata*

Fermentation of *P. lobata* by *P. kudriavzevii* was conducted as described under methods and its effect on changes in FBP and AEP composition was measured by LC-MS. The results showed that a total of 93 substances such as flavonoids, glycosides, coumarins, triterpenoids, sterols, organic acids, esters, aldehydes, and fatty acids were detected. Among them, 66 compounds were detected in the AEP accounting for 70.96 % of the total number of substances. 60 species were detected in the FBP, accounting for 64.51 % of the total number of species. The FBP group was 6.45 % lower than the AEP group. The content showed flavonoids as the main component. In the AEP group and FBP group, the proportion of flavonoids was 96.8 % and 98.07 % respectively. After fermentation, the total content of flavonoids showed an increasing trend (**Table 2**), and the complete content can be found in Supplementary Table 1.

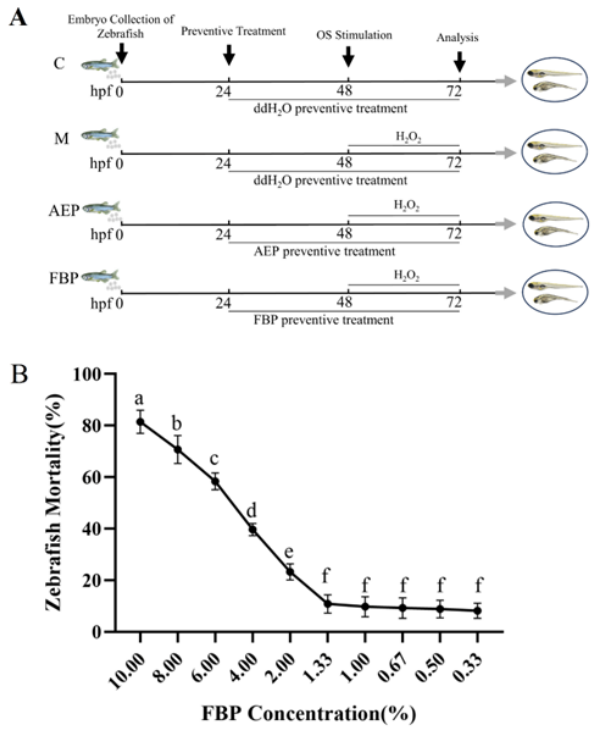

Fig. 1. Effects of different concentrations of FBP on zebrafish development. (A) Schema of the experimental design. (B) The mortality rate of zebrafish under different FBP treatment concentrations.

3.2. Effect of FBP on mortality rate of Zebrafish

Zebrafish embryos grown for hours post-fertilization were treated with increasing concentration of FBP for 72 h and mortality rate was measured. The results summarized in Table 2 show that there was a dose-dependent decrease in mortality with decreasing FBP concentration between 10% and 1.33% FBP. While, between the FBP concentration of 1.33% and 0.33%, the mortality rate was stable at about 10%. Therefore, an FBP concentration of 1.33% was chosen for subsequent studies (**Fig. 1**).

3.3. Effects of H_2O_2 on mortality rate of Zebrafish

Zebrafish were cultured in the presence of increasing concentrations of H_2O_2 and mortality resulting from oxidative stress was evaluated at 72 hpf. The results showed that the survival rate of zebrafish embryos was seriously affected when H_2O_2 concentration was ≥ 5 mM, and the oxidative damage effect was not obvious when H_2O_2 concentration was ≤ 3.5 mM. Therefore, 4 mM H_2O_2 was finally selected for subsequent experimental treatment (shown in **Fig. 2).**

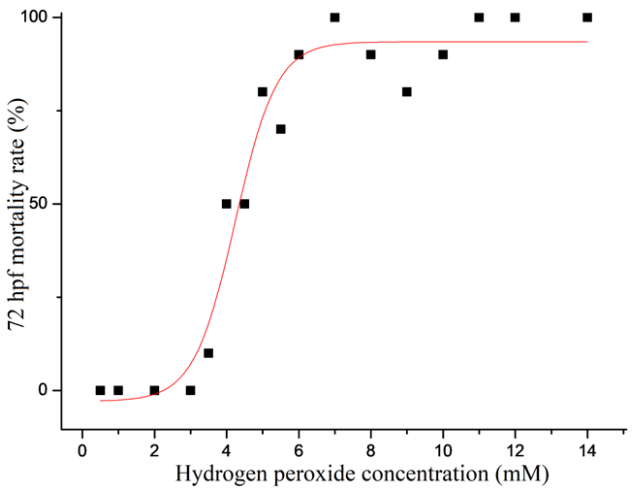

Fig. 2. The mortality rate of zebrafish under different H_2O_2 treatment concentrations.

Table 2 Chemical constituents and their relative contents in AEP and FBP.

Serial Number	Compound name	Formula	Molecular weight	Relative content (%)	
				AEP	FBP
3	Daidzein 7,4'-O-diglucoside	$C_{27}H_{30}O_{14}$	578	4.14±0.0033	4.51±0.00333
4	Puerarin-4'-O-glucoside	$C_{27}H_{30}O_{14}$	578	4.14±0.0033	4.51±0.00333
5	4',7-Dihydroxy-3 -methoxyisoflavone 8-C-[B-D-apiofuranosyl-(1→6)]-β -D-glucopyranoside	$C_{27}H_{30}O_{14}$	578	4.14±0.0033	4.51±0.00333
6	Puerarin-7-O-glucoside	$C_{27}H_{30}O_{14}$	578	4.14±0.0033	4.51±0.00333
7	6"-O-a-D-Glucopyranosylpuerarin	$C_{27}H_{30}O_{14}$	578	4.14±0.0033	4.51±0.00333
8	4',5,7-Trihydroxyisoflavone-6-methylether-7-O-B-D-xylopyranosyI-(1→6)-β-D-glucopyranoside	$C_{27}H_{30}O_{14}$	578	4.14±0.0033	4.51±0.00333
9	3'Hydroxy-daidzein 8-C-apiosyl (1→6) glucoside	$C_{26}H_{28}O_{14}$	564	0.70±0.0002	0.17±0.00024
10	3'-Hydroxy-6"-O-xylosylpuerarin	$C_{26}H_{28}O_{14}$	564	0.70±0.0002	0.17±0.00024
11	Genistein 8-C-apiofuranosyl (1→6) glucoside	$C_{26}H_{28}O_{14}$	564	0.70±0.0002	0.17±0.00024
17	Tectoridin	$C_{22}H_{22}O_{11}$	462	5.61±0.0051	3.38±0.00510
21	3 '-Methoxypuerarin	$C_{22}H_{22}O_{10}$	446	8.86±0.0328	10.11±0.03282
22	5-Hydroxy ononin	$C_{22}H_{22}O_{10}$	446	8.86±0.0328	10.11±0.03282
23	Glycitin	$C_{22}H_{22}O_{10}$	446	8.86±0.0328	10.11±0.03282
24	3'-Hydroxypuerarin	$C_{21}H_{20}O_{10}$	432	6.84±0.3980	10.11±0.39799
27	Genistein 8-C-glucoside	$C_{22}H_{22}O_{10}$	446	8.86±0.0328	10.11±0.03282
30	Puerarin	$C_{21}H_{20}O_{9}$	416	0.12±0.0001	0.13±0.00010
31	Daidzin	$C_{21}H_{20}O_{9}$	416	0.12±0.0001	0.13±0.00010
32	Daidzein 4'-O- glucoside	$C_{21}H_{20}O_{9}$	416	0.12±0.0001	0.13±0.00010
41	Daidzein	$C_{15}H_{10}O_{4}$	254	1.62±0.0006	1.12±0.00055
42	3'-Methoxy-6"-O-xylosylpuerarin	$C_{27}H_{30}O_{14}$	578	4.14±0.0033	4.51±0.00333
44	3'Hydroxyl-4'-methyldaidzin	$C_{22}H_{22}O_{10}$	446	8.86±0.0328	10.11±0.03282
84	Sucrose	$C_{12}H_{22}O_{11}$	342	3.23±0.0185	1.74±0.01852

(Note: Select chemical components with peak area ≥ 0.1

3.4. FBP reverses H_2O_2-mediated decrease in T-AOC and increases in MDA in Zebrafish embryos

T-AOC is an indicator of total antioxidant capacity by measuring the total antioxidant level of various antioxidants and antioxidant enzymes. The results

showed that the T-AOC value of zebrafish embryos in the H_2O_2 model group(M) was 1.20 U/g, which was significantly lower than 1.92 U/g in the control group ($p < 0.05$). Compared with the M group, the AEP and FBP groups increased to 1.52 U/g and 1.61 U/g, respectively, indicating a significant increase in the total antioxidant capacity of FBP ($p < 0.05$) (**Fig. 3A**). Conversely, malondialdehyde is a byproduct of lipid peroxidation that results from the attack of polyunsaturated fatty acids in biofilms by reactive oxygen species. It serves as a reliable biomarker for detecting the presence of oxidative stress in organisms. As shown in Fig. 3B, the MDA concentrations of zebrafish embryos in the control, M, AEP, and FBP groups were 9.159,11.223,10.107 and 9.632 nmol/g, respectively. Compared with the control, the MDA concentration of zebrafish embryos in the M group was significantly increased by 22.5 % ($p < 0.05$). Compared with the M group, in the M and FBP groups, there was a decreased concentration of MDA in zebrafish embryos by 9.94 % and 14.18 % ($p < 0.05$) (**Fig. 3B**).

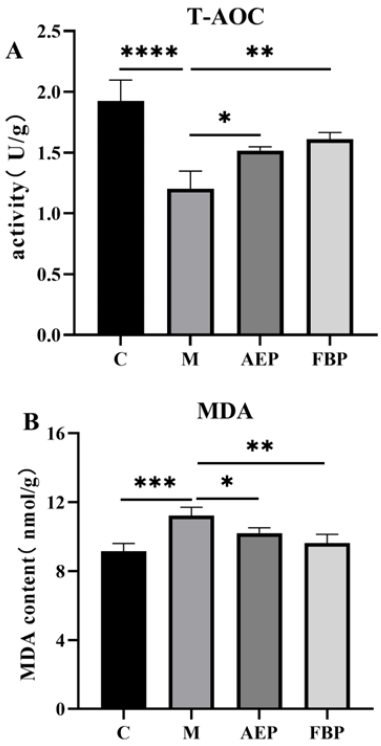

Fig. 3. Treatment of zebrafish embryos by FBP reverses H_2O_2-mediated decrease in T-AOC and increase in MDA. (A) T-AOC and (B) MAD. (*P < 0.05, **P < 0.01).

3.5. Effect of FBP on expression of Nrf2-ARE and plasma reticulum stress genes in Zebrafish under oxidative stress

The relative mRNA expression of Nrf2-ARE signaling pathway-related genes (Nrf2, SOD1, GPx, and GST) and endoplasmic reticulum stress-related genes (GADD45αa, C3) was measured to examine the role of the key genes and signaling pathways involved in the antioxidant action of FBP. H_2O_2 treatment significantly increased the mRNA expressions of SOD1, GPx, GST, Nrf2, GADD45αa, and C3 in H_2O_2-induced zebrafish by 3.21, 2.55, 2.16, 1.83, 1.64, and 4.48-fold, respectively ($p < 0.001$). The results presented in **Fig. 4** demonstrate that the mRNA levels in zebrafish subjected to FBP pretreatment (FBP group) were significantly reduced to levels that were comparable to those observed in the control (C) group ($p < 0.05$). These findings provide evidence that the antioxidant properties of FBP are associated with the suppression of the Nrf2/ARE signaling pathway.

4. Discussion

Puerariae radix extract is obtained from the root of Pueraria lobata, also known as kudzu, a plant used in traditional Chinese medicine for the treatment of a variety of ailments including, liver diseases (He et al., 2021), prevention of obesity (Jung et al., 2017), diminution of cerebral ischemia (Lim et al., 2013), high blood pressure (Wu et al., 2014), and alcoholism, to list a few(Liu et al., 2019, Liu et al., 2021). Its pharmacologic benefits have been attributed to the presence of active substances such as isoflavones, triterpenes, saponins, and polysaccharides (He et al., 2021). Isoflavones, the main functional components of *P. lobatae*, are higher in concentration than other active substances(Wang et al., 2020). Isoflavones can not only effectively scavenge free radicals, but also promote the proliferation of vascular endothelial cells, reduce the content of malondialdehyde in the liver (Gao et al., 2016), significantly increase the activity of antioxidant enzymes such as SOD, CAT, and GST in the blood of rats, prevent cell oxidative damage and apoptosis, and alleviate mitochondrial membrane potential disorder, so as to achieve good antioxidant effect (Gao et al., 2016, Abdel-Aleem et al., 2016, Thabet and Moustafa, 2017). This study LC-MS analysis of AEP and FBP showed

the presence of a variety of antioxidant active components such as Glycitin and 3'-Methoxypuerarin led by isoflavones (Table 2). In addition, the antioxidant components of the FBP are higher than the AEP. These data indicate that fermentation has a positive effect on enhancing the antioxidant components and exerting the antioxidant effects of *P. lobata*.

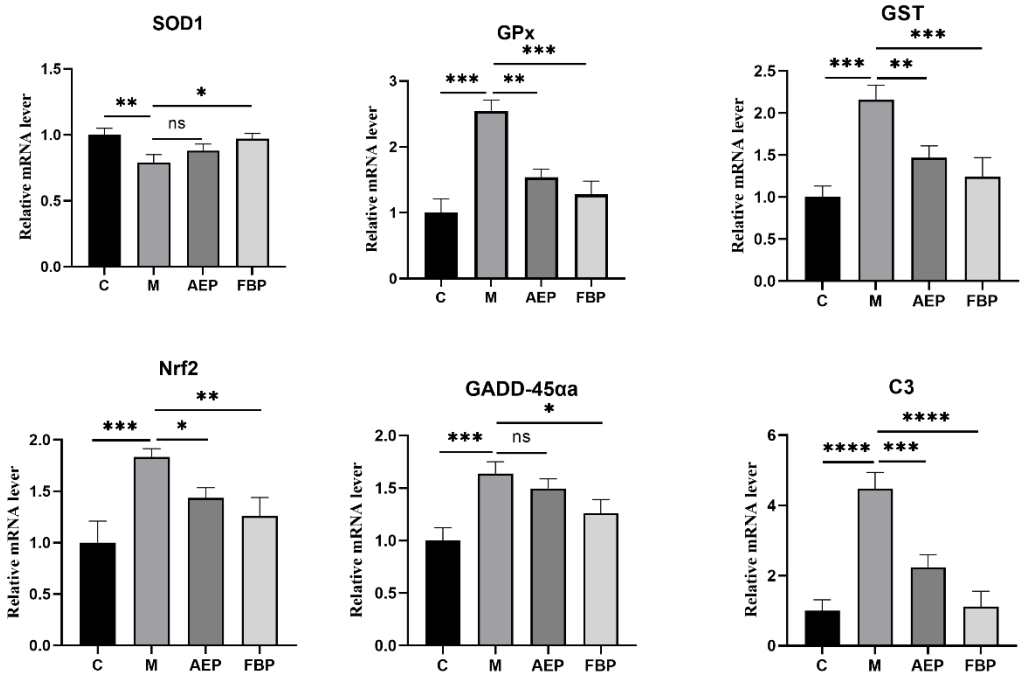

Fig. 4. Differential changes in expression levels of genes related to oxidative stress. (*p < 0.05，**p < 0.01，***p < 0.001).

Oxidative stress refers to a state of imbalance where oxidative capacity exceeds the antioxidant capacity (Liu et al., 2022, Jiang et al., 2023). When the level of ROS exceeds the physiological amount and the antioxidant system cannot be removed in time, excessive ROS causes excessive lipid free radicals such as malondialdehyde (MDA), which leads to a series of diseases or symptoms (Asgary et al., 2023). Therefore, MDA is also regarded as a marker of oxidative stress. The endogenous molecules of the antioxidant system include two parts: the enzyme system and non-enzyme system. The antioxidant enzyme system includes SOD, CAT, GSH-PX, GST, and others, which together constitute an important antioxidant system in the body that avoids the accumulation of ROS. Gene transcription analysis has shown that FBP can significantly alter the expression of ROS, T-AOC, SOD, CAT, and MDA caused by H_2O_2. In addition, the FBP group has a significant level of expression of SOD1, GST, and GCS genes, but the lowering effect of the AEP group is not as obvious as FBP (**Fig. 1**).

Nrf2/ARE signaling is one of the key pathways that the body uses to resist environmental stress, maintain the body's redox balance, and prevent oxidative stress injury (He et al., 2021). Nrf2 activates the expression of a series of antioxidant genes related to ARE by dissociation and nuclear transfer of Keap1 in the cytoplasm to protect the body from oxidative damage (Itoh et al., 1999). The downstream target proteins regulated by Nrf2 have been identified to fall into three main categories: phase II metabolic enzymes, antioxidant enzymes (SOD, CAT, and GPx), and molecular chaperones. Gadd45αa, as a cell cycle regulator and growth inhibitor can express genes after DNA damage (Messeha et al., 2020). While repairing damaged DNA, it also causes severe apoptosis and protects the body from damage (Liu et al., 2020a, Tong et al., 2020), Nrf2 and

GADD45αa are key biomarkers related to oxidative stress response and DNA damage. The relative expression of the genes linked to the Nrf2/ARE signaling pathway (*Nrf2*, *SOD1*, *GPx*, and *GST*) was verified in the zebrafish. The preventive treatment of AEP and FBP could reduce the expression levels of the above genes and the recovery effect of FBP was more significant. The findings clearly show that FBP's antioxidant effect is connected to lower expression of the Nrf2/ARE signaling pathway in zebrafish tissues.

5. Conclusion

The results of this study indicated that the antioxidant effect of *P. lobata* was related to its ingredients, which contain various flavonoid components and have beneficial health effects on zebrafish. Particularly, fermented *P. lobata* can promote an increase in the content of active components led by isoflavones. More importantly, after fermenting *P. lobata* with *P. kudriavzevii* the antioxidant capacity of zebrafish was improved. The effective control of the oxidative stress signaling pathway (Nrf2/ARE) and an increase in antioxidant components led by isoflavones were linked to this. Hence, this study highlights that the antioxidant effect of *P. lobata* can be improved via *P. kudriavzevii* fermentation, thus expanding the potential uses of *P. lobata* as a functional food.

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Conflict of Interest

The authors have no conflicts of interest to declare.

Ethical Statement

All experiments were conducted in accordance with the guidelines of the Experimental Animal Welfare and Ethics Committee of Gannan Normal University (protocol number: gnnu2022-0628) after obtaining relevant approval. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon request.

Supplementary data

The supplementary data of this article related to chemical constituents and their relative contents in AEP and FBP can be provided on request.

References

Abdel-Aleem GA, Khaleel EF, Mostafa DG, & Elberier LK, 2016. Neuroprotective effect of resveratrol against brain ischemia reperfusion injury in rats entails reduction of DJ-1 protein expression and activation of PI3K/Akt/GSK3b survival pathway. Arch Physiol Biochem, 122, 200-213.

Asgary S, Karimi R, Pour PM, Heydarpour F, Mostafaei S, Farzaei MH, Moradi S, & Aneva IY, 2023. Is consumption of pomegranate supplementation effective on oxidative stress biomarkers including MDA, ox-LDL, POX 1, GPX, TAC, and TBRAS? A systematic review and meta-analysis of randomized controlled trials. Curr Prob Cardiol, 48, 101198.

Chen R, Liu B, Wang XY, Chen K, Zhang KY, Zhang LF, Fei CZ, Wang CM, Liu YC, Xue FQ, Gu F, & Wang M, 2020. Effects of polysaccharide from *Pueraria lobata* on gut microbiota in mice. Int J Biol Macromol, 158, 740-749.

De Jong, J. L. O. & Zon, L. I. 2005. Use of the Zebrafish system to study primitive and definitive hematopoiesis. Annu Rev Genetics, 39, 481-501.

De Vuyst L, Harth H, Van Kerrebroeck S, & Leroy F, 2016. Yeast diversity of sourdoughs and associated metabolic properties and functionalities. Int J Food Microbiol, 239, 26-34.

Forman HJ, & Zhang H, 2021. Targeting oxidative stress in disease: promise and limitations of antioxidant therapy. Nat Rev Drug Discover, 20, 689-709.

Freeman BA, & Crapo JD, 1982. Biology of disease: free radicals and tissue injury. Lab Invest, 47, 412-26.

Gao Y, Wang X, & He C, 2016. An isoflavonoid-enriched extract from *Pueraria lobata* (kudzu) root

protects human umbilical vein endothelial cells against oxidative stress induced apoptosis. J Ethnopharmacol, 193, 524-530.

Geronikou A, Larsen N, Lillevang SK, & Jespersen L, 2023. Diversity and succession of contaminating yeasts in white-brined cheese during cold storage. Food Microbiol, 113, 104266.

He H, Peng S, Song X, Jia R, Zou Y, Li L, & Yin Z, 2021. Protective effect of isoflavones and triterpenoid saponins from *Pueraria lobata* on liver diseases: A review. Food Sci Nutri, 10, 272-285.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, & Yamamoto M, 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Develop, 13, 76-86.

Jiang X, Wang J, Liu J, Zhu H, Hu J, Sun X, & Zhou W, 2023. Resveratrol ameliorates penconazole-induced cardiotoxicity by inhibition of oxidative stress and apoptosis in Zebrafish larvae. Ecotoxicol Environ Saf, 256, 114865.

Jung H, Kang A, Kang S, Park YK, & Song M. 2017. The root extract of *Pueraria lobata* and its main compound, puerarin, prevent obesity by increasing the energy metabolism in skeletal muscle. Nutrients, 9, 33.

Kang MC, Kim SY, Kim EA, Lee JH, Kim YS, Yu SK, Chae JB, Choe IH, Cho JH, & Jeon YJ, 2015. Antioxidant activity of polysaccharide purified from *Acanthopanax koreanum* Nakai stems in vitro and in vivo Zebrafish model. Carb Polymer, 127, 38-46.

Kim M, Kim SH, & Yang W. 2014. Mechanisms of Action of phytochemicals from medicinal herbs in the treatment of alzheimer's disease. Plant Medi, 80, 1249-1258.

Lim D, Lee C, Kim IH, & Kim Y. 2013. Anti-Inflammatory effects of total isoflavones from *Pueraria lobata* on cerebral ischemia in rats. Mol, 18, 10404-10412.

Liu H, Xie J, Fan L, Xia Y, Peng X, Zhou J, & Ni X, 2022. Cryptotanshinone Protects against PCOS-induced damage of ovarian tissue via regulating oxidative stress, mitochondrial membrane potential, inflammation, and apoptosis via regulating ferroptosis. Oxidative Med Cell Longevi, 2022, 8011850.

Liu J, Shi YC, & Lee DYW, 2019. Applications of Pueraria lobata in treating diabetics and reducing alcohol drinking. Chinese Herb Med, 11, 141-149.

Liu JY, Fu WQ, Zheng XJ, Li W, Ren LW, Wang JH, Yang C, & Du GH, 2020a. Avasimibe exerts anticancer effects on human glioblastoma cells via inducing cell apoptosis and cell cycle arrest. Acta Pharmacol Sinica, 42, 97-107.

Liu X, Gao Y, Yang H, Li L, Jiang Y, Li Y, & Zheng J, 2020b. *Pichia kudriavzevii* retards fungal decay by influencing the fungal community succession during cherry tomato fruit storage. Food Microbiol, 88, 103404.

Liu YS, Yuan MH, Zhang CY, Liu HM, Liu JR, Wei AL, Ye Q, Zeng B, Li MF, Guo YP, & Guo L, 2021. *Puerariae Lobatae* radix flavonoids and puerarin alleviate alcoholic liver injury in Zebrafish by regulating alcohol and lipid metabolism. Biomed Pharmaco, 134, 111121.

Livak KJ, & Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods, 25, 402-408.

Maura YF, Balzarini T, Borges PC, Evrard P, De Vuyst L, & Daniel HM, 2016. The environmental and intrinsic yeast diversity of Cuban cocoa bean heap fermentations. Int J Food MicrobioL, 233, 34-43.

Messeha Ss, Zarmouh No, Asiri A, & Soliman Kfa 2020. Rosmarinic acid-induced apoptosis and cell cycle arrest in triple-negative breast cancer cells. Euro J Pharmacol, 885, 173419.

Nishimura Y, Inoue A, Sasagawa S, Koiwa J, Kawaguchi K, Kawase R, Maruyama T, Kim S, & Tanaka T, 2016. Using zebrafish in systems toxicology for developmental toxicity testing. Congen Anomal, 56, 18-27.

Park HJ, Ko HJ, Jeong H, Lee SH, Ko HJ, Bae JH, Sung BH, Han JI, & Sohn JH, 2018. Draft genome sequence of a multistress-tolerant yeast, *Pichia kudriavzevii* NG7. Genome Announce, 6, 10.1128/genomea. 01515-17.

Ruiz-Muñoz M, Cordero-Bueso G, Benítez-Trujillo F, Martínez S, Pérez F, & Cantoral JM, 2020. Rethinking about flor yeast diversity and its dynamic in the "criaderas and soleras" biological aging system. Food Microbiol, 92, 103553.

Scansani S, Van WN, Nader KB, Beisert B, Brezina S, Fritsch S, Semmler H, Pasch L, Pretorius IS, & Von Wallbrunn C, 2022. The film-forming *Pichia* spp. in a winemaker's toolbox: A simple isolation procedure and their performance in a mixed-culture fermentation of V*itis vinifera* L. cv. Gewürztraminer must. Int J Food Microbiol, 365, 109549.

Shashni B, Tamaoki J, Kobayashi M, & Nagasaki Y, 2023. Design of a new self-assembling antioxidant nanomedicine to ameliorate oxidative stress in

Zebrafish embryos. Acta Biomater, 159, 367-381.

Sies, H., Berndt, C. & Jones, D. P. 2017. Oxidative stress. Ann Rev Biochem, 86**,** 715-748.

Sook KY, Soo LI, & Sook KJ, 2014. Protective effects of *Puerariae radix* extract and its single compounds on methylglyoxal-induced apoptosis in human retinal pigment epithelial cells. J Ethnopharmacol, 152**,** 594-598.

Thabet NM, & Moustafa EM, 2017. Protective effect of rutin against brain injury induced by acrylamide or gamma radiation: role of PI3K/AKT/GSK-3β/NRF-2 signalling pathway. Arch Physiol Biochem, 124**,** 185-193.

Thannickal VJ, & Fanburg BL, 2000. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol, 279**,** L1005-28.

Tong RY, Wu X, Liu Y, Liu Y, Zhou JG, Jiang XY, Zhang L, He XY, & Ma LB, 2020. Curcumin-induced DNA demethylation in human gastric cancer cells is mediated by the DNA-damage response pathway. Oxidat Med Cell Longevit, 2020**,** 2543504.

Wang QS, Wang YL, Zhang WY, Li KD, Luo XF, & Cui YL, 2021. Puerarin from *Pueraria lobata* alleviates the symptoms of irritable bowel syndrome-diarrhea. Food Func, 12**,** 2211-2224.

Wang S, Zhang S, Wang S, Gao P, & Dai L, 2020. A comprehensive review on *Pueraria*: Insights on its chemistry and medicinal value. Biomed Pharmacotherap, 131**,** 110734.

Wong KH, Li GQ, Li KM, Razmovski-Naumovski V, & Chan K, 2011. Kudzu root: Traditional uses and potential medicinal benefits in diabetes and cardiovascular diseases. J Ethnopharmacol, 134**,** 584-607.

Woo K, Shih J, & Fraser SE, 1995. Fate maps of the Zebrafish embryo. Curr Opin Genet Develop, 5**,** 439-443.

Wu Q, Zhu W, Wang W, & Xu Y. 2015. Effect of yeast species on the terpenoids profile of Chinese light-style liquor. Food Chem, 168**,** 390-395.

Wu XD, Wang C, Zhang ZY, Fu Y, Liu FY, & Liu XH, 2014. Puerarin attenuates cerebral damage by improving cerebral microcirculation in spontaneously hypertensive rats. Evid Based Complement Alternat Med**,** 408501.

Xi C, Zhang M, Li B, Meng X, Xu S, Du H, Wang X, Xu J, Ke H, Cui Y, & Qu F, 2023. Metabolomics of the anti-inflammatory effect of *Pueraria lobata* and *Pueraria lobata* var. Thomsonii in rats. Journal Ethnopharmacol, 306**,** 116144.

Yang Y, Zheng K, Mei W, Wang Y, Yu C, Yu B, Deng S, & Hu J, 2018. Anti-inflammatory and proresolution activities of bergapten isolated from the roots of Ficus hirta in an in vivo Zebrafish model. Biochem Biophy Res Comm, 496**,** 763-769.

Zha Q, Chen S, Zhang J, Zhang XY, Ruan PJ, Amer H M, & Liu A, 2020. A reliable and rapid pharmacokinetic study of pueraria isoflavones using pueraria reference extractive substance in beagle plasma: Application to study of Yufeng Ningxin Tablets. Chinese Herb Med, 12**,** 88-94.

Zheng XW, Yan Z, Han BZ, Zwietering MH, Samson RA, Boekhout T, & Nout MR, 2012. Complex microbiota of a Chinese "Fen" liquor fermentation starter (Fen-Daqu), revealed by culture-dependent and culture-independent methods. Food Microbiol, 31**,** 293-300.

Zhou YX, Zhang H, & PENG C, 2014. Puerarin: a review of pharmacological effects. Phytotherap Res, 28**,** 961-975.