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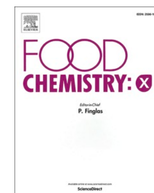

Flaxseed polyphenols: Effects of varieties on its composition and antioxidant capacity

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ABSTRACT

This study identified phenolic compounds in five flaxseed varieties and evaluated their antioxidant activities. Results showed significant variations in phenolic acids and flavonoids among the varieties. Longya 16 had the lowest flavonoid content, Longya 13 had the lowest phenolic acid content, while Longya 10 exhibited the highest content and diversity of polyphenols, including six flavonoids (vitexin, quercitrin, quercetin, apigenin, kaempfero1, (+)-dihydroquercetin) and five phenolic acids (gallic acid, vanillic acid, ferulic acid, sinapic acid, and 4-hydroxybenzoic acid). Antioxidant activity was assessed using DPPH and ABTS radical scavenging assays, and cell-based assays under tBHP-induced oxidative stress. Flaxseed polyphenol extracts significantly reduced ROS, MDA, and GSSG levels and increased SOD and CAT activities, preserving cell vitality and morphology. These findings confirmed the significant antioxidant activity of flaxseed polyphenols, providing a theoretical basis for their application in antioxidative functional areas.

1. Introduction

Flax (*Linum usitatissimum* L.) is an ancient herbaceous plant and is cultivated in over 50 countries and regions worldwide (Han, Yilmaz, & Gulcin, 2018). According to the statistical report of the Food and Agriculture Organization (FAO) of the United Nations, the total production of flaxseed is approximately 3.22 million tons, with the majority grown in the Northern Hemisphere. The largest portion is cultivated in Asia (49.5%), followed by Europe (25.4%), the Americas (21.7%), Africa (3.1%), and Oceania (0.3%) (Sharma & Saini, 2022). The main bioactive components and their contents in flaxseed may vary due to different genetic backgrounds and its environment (Kajla, Sharma, & Sood, 2015). The flaxseed has a crisp texture and rich nutty flavor, often presenting in reddish-brown color. Flaxseed was composed of approximately 40% fat, 35% dietary fiber, and 30% protein. It was also rich in various bioactive compounds, including omega-3 polyunsaturated fatty acids, lignans, cyclolinopeptides, and polysaccharides, among others (Doyen et al., 2014), which is a multi-functional nutritional health food raw material. Adequate intake can lower blood sugar, prevent diseases such as osteoporosis, and reduce the risk of cardiovascular and cerebrovascular diseases. Flaxseed polyphenols are a group of natural plant compounds extracted from flaxseed and are a class of secondary metabolites Flaxseed polyphenols include phenolic acids, flavonoids, lignans, and other compounds (Hajibabaie, Abedpoor, Safavi, & Taghian, 2022). Studies have found that the main compounds in flaxseed include ellagic acid, ferulic acid, quercetin, secoisolariciresinol (SECO), and secoisolariciresinol diglucoside (SDG) (Mechchate et al., 2021). The types and contents of polyphenols vary significantly among different varieties of flaxseeds. Mechchate et al. (2021) dentified 18 phenolic compounds from flaxseed, including oleuropein, hesperetin, ursolic acid, isorhamnetin-7-O-pentoside, luteolin-7-O-glucoside, trans-cinnamic acid, procyanidin, and naringin. Kyselka et al. (Kyselka et al., 2017) isolated ferulic acid, caffeic acid, and p-coumaric acid from flaxseed polyphenol extracts (Al-Jumaily & Al-Azawi, 2015) identified six phenolic substances from flaxseed polyphenol extracts, namely p-hydroxybenzoic acid, vanillin, p-coumaric acid, ascorbic acid, ferulic acid, and ellagic acid. In studies by Zorenc et al. (2017), it was found that compared to red currants, white currants contain no anthocyanins in their polyphenols, but have higher levels of hydroxycinnamic acids and flavonols. Jiang et al. (2021) revealed differences in polyphenol

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metabolites among different lychee varieties. The FZX variety showed significantly higher levels of catechins, anthocyanins, flavonols, quinic acid, hydroxycinnamoyl, proanthocyanidins, and total polyphenols compared to BTY, BYL, FC, and FZX ($p < 0.01$). Early-ripening FZX and BTY varieties exhibited higher polyphenols levels than late-ripening FC, YJQ, and BYL varieties. Bettaieb, Benabderrahim, Arcos, Araujo, and Elfalleh (2023) studied seven varieties of dates and found significant differences in total phenol, total flavonoid, and tannin contents. In the Hessa and Bouhattam varieties, the flavonoid content oscillated between 34.20 ± 0.34 and 94.46 ± 1.04 mg RE/g DM, and the total phenolic content ranged between 135.9 ± 12.1 and 284.86 ± 21.9 mg GAE/g DM. These differences may be attributed to geographical origin, genetic effects, and extraction conditions. The study indicates significant variations in polyphenols among different varieties of the same plant species.

Flaxseed polyphenols have attracted increasing attention due to their physiological functions such as antioxidant, anti-inflammatory, anti-atherosclerosis, and potential anti-cancer properties (Cakmak & Gulcin, 2019; İ. Gulcin, 2020; Gulcin, 2011). In recent years, consumers' awareness of the relationship between health and oxidative status has been increasing. Numerous studies have shown that oxidative damage led to structural damage to cells, lipids, proteins, and genetic material, resulting in chronic liver damage and fibrosis. It also caused lipid peroxidation to promote the occurrence of non-alcoholic fatty liver disease and liver cancer, as well as degenerative diseases such as Alzheimer's disease (Soltani, Moghadam, Nadalizadeh, Emami, & Javadi, 2021), Parkinson's disease, and cardiovascular diseases (Martinelli, Pucci, Battaglini, Marino, & Ciofani, 2020). Research has also found that oxidative stress was closely related to the level of cell apoptosis, that is, oxygen radicals such as superoxide anions and hydroxyl radicals were produced in large quantities in the body, inducing cell apoptosis (Kannan & Jain, 2000). Therefore, consumers' demand for antioxidants such as polyphenols continued to grow, particularly those derived from natural plants, due to their safety (Jiao, Quek, Gu, Guo, & Liu, 2020). Polyphenols such as flavonoids, flavonols, and coumarins have been shown to exert beneficial effects on various lifespan-related phenotypes, including ameliorating oxidative stress, inflammation, and cellular senescence (Chiorcea-Paquim, Enache, Gil, & Oliveira-Brett, 2020; Rolt & Cox, 2020). They assessed their antioxidant activity using chemical methods, and the results indicated that the antioxidant capacity of flaxseed polyphenol extracts was comparable to that of BHA and α-tocopherol. Cheng et al. (2019) incorporated flaxseed polyphenols into an emulsion system. By measuring the hydrogen peroxide and malondialdehyde content generated during emulsion oxidation, it was found that the oxidation product formation time in the nanoemulsion with added flaxseed polyphenols was delayed by 9 days compared to the blank control, indicating that flaxseed polyphenols can effectively inhibit lipid oxidation. In the study by Tadesse et al. (2023), comparing with the control group without supplementation, the addition of flaxseed polyphenol extract to the diets of laying hens resulted in significantly lower MDA content in the egg yolks. This indicated that flaxseed polyphenols extract prevented the production of oxidation stress related to the intake of diets rich in PUFA or reduced the formation of oxidative products in vivo. Pilar et al. (2017) fed secoisolariciresinol diglucoside (SDG) to metabolic syndrome (MS) rats and observed that SDG could enhance the antioxidant defense capacity of enzymes, preventing oxidative damage to lipids (Chera et al., 2022) observed the toxicity of flaxseed polyphenols on EAC (undifferentiated non-invasive cancer cells), which could not reverse cancer cells but significantly inhibited tumor growth. Flaxseed polyphenols reduced the production of ROS and MDA oxidative products in EAC mice. Flaxseed polyphenols achieved anti-inflammatory, antioxidant, and anti-tumor effects by reducing NF-κB signaling molecule levels and decreasing RNS and ROS levels in the body (Chera et al., 2022). There have been some studies on the antioxidant activities of flaxseed polyphenols, but compared with other plant polyphenols, the study of flaxseed polyphenols was still in the initial stage, and the identification and antioxidant studies of different varieties of flaxseed polyphenols were not systematic.

According to Wei (2016) research, the contents of α-linolenic acid, lignans, and total sterols in flaxseed from China were significantly higher than those in varieties from Egypt, Switzerland, and the United States. Moreover, the levels of these compounds were positively correlated with the antioxidant activity of flaxseed. Among the 32 Chinese flaxseed varieties studied, the Longya series flaxseeds exhibited significantly higher levels of p-coumaric acid, α-tocopherol, and total phenols compared to other varieties. The Longya series primarily grew in Gansu province, which endowed them with strong cold tolerance and resistance to adverse conditions, leading to high yield stability and higher oil content. These characteristics indicated a higher commercial value for the Longya series. Therefore, this study selected the Longya series varieties as the experimental subjects. The main objective of this study is to identify the types and contents of phenolic compounds in seeds of five different varieties of flaxseeds and to determine their antioxidant activity using an in vitro mammalian cell-based assay system. Specifically, the antioxidant capacity of flaxseed polyphenol extracts was assessed by evaluating their effects on the viability of RAW 264.7 cells, intracellular ROS levels, generation of intracellular oxidative products, and intracellular antioxidant enzyme activity. This paper aims to elucidate the differences in antioxidant capacity among polyphenols from different varieties of flaxseed and their protective effects against cellular oxidative stress.

2. Materials and methods

2.1. Chemicals and materials

The flaxseeds were provided by Henan Forest Holiday Food Technology Development Co., LTD. The five varieties of flaxseed used in this experiment were Longya 10, Longya 13, Longya 14, Longya 15, and Longya 16. Kits for measuring oxidized glutathione (GSSG) content, reduced glutathione (GSH) content, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, BCA protein concentration, Folin phenol reagent, ethanol, methyl alcohol, and Na_2CO_3 were obtained from Beijing Solaibao Biology Co., LTD. The hydrogen peroxide assay kit, total antioxidant capacity assay kit, reactive oxygen species assay kit, DMSO, MTT were provided by Shanghai Biyuntian Biotechnology Co., LTD. The RAW264.7 cells used in this experiment were gifted by the Key Laboratory of Animal Immunology, Henan Academy of Agricultural Sciences. DMEM complete medium was obtained from Zhejiang Noble Biological Products Co., LTD.

2.2. Preparation of flaxseed polyphenol extract

Phenolic compounds were extracted based on the method previously described by Wang et al. (2024). The subtle milled sample (1.0 g) was extracted with 70% ethyl alcoholl (10 mL) and shaken with a laboratory rotary shaker under nitrogen and dark conditions at 200 g and 25 °C for 1 h. The mixture was subsequently centrifuged at 10,000 ×g for 10 min at 4 °C. Following centrifugation, the ethyl alcoholl supernatant was filtered, and extraction process was repeated three times. The combined supernatant were then evaporated under vacuum to a final volume of 5 mL at 40 °C using a rotary vacuum evaporator. The flaxseed polyphenol extract has been freeze-dried at −20 °C for further use.

2.3. Analysis of polyphenol extracts from flaxseed

2.3.1. Determination of phenolic acid content

The HPLC method used was slightly modified from Ma, Wang, Gu, Sun, and Yang (2022). Filtered through a 0.22 μm organic filter membrane, 10 μL of the aforementioned extracts were analyzed using high performance liquid chromatography (Agilent Technologies Inc., St. Clara, CA, USA) and a diode array detector (DAD). Analytical column

3.0 *150 mm, 2.7 μm diameter, C18 column (Phenomenex, Torrance, California, USA). The conditions of the high performance liquid phase are as follows: the flow rate is 0.3 mL/min, the mobile phase A solution is 0.1% acetic acid aqueous solution, and the B solent is 0.1% acetic acid methanol solution. At a constant temperature of 35 °C and a flow rate of 0.3 mL/min, the gradient elution procedure is as follows: 0–11 min, 9%–14% B; 11–14 min, 14%–15% B; 14–17 min, 15% B; 17–24 min, 15%–16.5% B; 24–28 min, 16.5%–19% B; 28–30 min 19%–25% B; 30–36 min, 25%–26% B; 36–38 min, 26%–28% B; 38–41 min, 28%–35% B; 41–46 min, 35%–40% B; 46–48 min, 40%–48% B; 48–50 min, 9% B. The wavelength was measured at 280 nm.

2.3.2. Determination of flavonoid content

Filtered through a 0.22 μm organic filter membrane, 10 μL of the aforementioned extracts were analyzed using high performance liquid chromatography (Agilent Technologies Inc., St. Clara, CA, USA) and a diode array detector (DAD). Analytical column 3.0 *150 mm, 2.7 μm diameter, C18 column (Phenomenex, Torrance, California, USA). The conditions of the high performance liquid phase are as follows: the flow rate is 0.3 mL/min, the mobile phase A solution was 0.1% acetic acid aqueous solution, and the solvent B was methanol solution. At a constant temperature of 35 °C and a flow rate of 0.3 mL/min, the gradient elution procedure was as follows: 0–4 min, 10%–35% B; 4–27 min, 35%–65% B; 27–30 min, 65%–10% B. The wavelength was measured at 280 nm.

2.4. Determination of antioxidant activity assay

2.4.1. DPPH radical scavenging activity

The scavenging activity of DPPH free radicals was determined according to the method described by Gulcin and Alwasel (2023). A volume of 200 μL of flaxseed polyphenol extract (appropriately diluted) was added to 3.8 mL of DPPH solution, vortexed, and left to react in the dark at room temperature for 30 min. The absorbance (A) was measured at 515 nm. A 50% ethanol solution was used as a blank control (A-control). A standard curve was prepared using Trolox, and the DPPH scavenging capacity was expressed as μmol TE/g DW.

2.4.2. ABTS radical cation scavenging activity

A volume of 100 μL of flaxseed polyphenol extract (appropriately diluted) was added to 3.0 mL of ABTS solution, vortexed, and left to react in the dark at room temperature for 30 min. The absorbance (A) was measured at 734 nm. A 50% ethanol solution was used as a blank control (A-control). A standard curve was prepared using Trolox, and the ABTS scavenging capacity was expressed as μmol TE/g DW.

2.5. Cell culture

RAW 264.7 cells were purchased from the Key Laboratory of Animal Immunology, Henan Academy of Agricultural Sciences (Zhengzhou, Henan, China). Cells were cultured in high glucose DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.6. Cell viability assay

RAW264.7 cells were seeded at a density of 5×10^4 cells/mL, with 200 μL per well, in a 96-well plate and cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. After removing the old culture medium, the control group received no polyphenol extract, while the experimental groups received 100 μL of tBHP solution (final concentration of 200 μmol/L), 20 μL of different varieties of flaxseed polyphenol extract (final concentrations of 0.625, 1.25, 2.5, 5, and 10 mg/mL), and 80 μL of fresh medium. The cells were then cultured for an additional 24 h. After discarding the culture medium, 20 μL of MTT solution (concentration of 5 mg/mL) was added to each well, followed by incubation at 37 °C with 5% CO_2 for 2 h. The MTT solution was then removed, and 200 μL of DMSO was carefully added to each well. The plate was placed on a thermostatic shaker at 100 rpm for 15 min at 37 °C, and 150 μL of the resulting solution from each well was transferred to a new 96-well plate. The absorbance at 550 nm was measured using an enzyme-linked immunosorbent assay reader. The cell viability was calculated using the following Eq. (1).:

$$\text{Cell viability}(\%) = \text{As}/\text{Ac} \times 10 \tag{1}$$

“As” denotes the absorbance value of the cells after treatment;

“Ac” denotes the absorbance value of the wells containing cells from the untreated control group under normal culture conditions;

The cell viability of the untreated control group is set as 100%;

The treatment groups consist of cells treated with tBHP solution or different concentrations of polyphenol extracts.

2.7. Intracellular ROS level

RAW 264.7 cells were seeded in 6-well plates (1×10^6 cells/well) and treated with different concentrations of flaxseed polyphenol extracts from various cultivars and tBHP or individual components as described above for 24 h. After discarding the old culture medium, flaxseed polyphenol extracts (final concentrations of 2.5, 1.25, 0.625 mg/mL) and tBHP solution (final concentration of 200 μmol/L) were added, and the cells were incubated for 6 h. Subsequently, the old culture medium was removed, and the cells were treated with a final concentration of 10 μmol/L DCFH-DA probe solution prepared in serum-free culture medium. After washing the cells with PBS to remove excess DCFH-DA, fluorescence microscopy was immediately used for qualitative analysis. Fluorescence intensity was quantitatively analyzed using a fluorescence spectrophotometer with excitation wavelength set at 488 nm and emission wavelength set at 525 nm.

2.8. Intracellular MDA/GSH/GSSG/T-AOC content

RAW 264.7 cells were seeded in 6-well plates (1×10^6 cells/well) and treated with different concentrations of flaxseed polyphenol extracts from various cultivars and tBHP or individual components as described above for 24 h. After discarding the old culture medium, flaxseed polyphenol extracts (final concentrations of 2.5, 1.25, 0.625 mg/mL) and tBHP solution (final concentration of 200 μmol/L) were added, and the cells were cultured for 24 h. The cells were then collected into 1.5 mL centrifuge tubes, and the levels of T-AOC, GSH, GSSG, and MDA were assessed using commercial assay kits according to the manufacturer's instructions. The protein concentration of the harvested cells was determined using a BCA protein assay kit.

2.9. Intracellular SOD/CAT activity

RAW 264.7 cells were seeded in 6-well plates (1×10^6 cells/well) and treated with different varieties and concentrations of flaxseed polyphenol extracts and tBHP, as described above, or individual components for 24 h. After removing the old culture medium, flaxseed polyphenol extracts (final concentrations of 2.5, 1.25, 0.625 mg/mL) and tBHP solution (final concentration of 200 μmol/L) were added, and the cells were cultured for 24 h. Subsequently, the cells were collected in 1.5 mL centrifuge tubes, and the activity of CAT and SOD was assessed using commercial assay kits according to the manufacturer's instructions. The protein concentration of the cells obtained was determined using a BCA protein assay kit.

2.10. Statistical analysis

Each experiment was measured three times in parallel. Analysis of variance (ANOVA) was performed using SPSS 20.0. Data are expressed as mean ± standard deviation. Significant differences ($p < 0.05$) between the treatments were determined according to Duncan multiple

range test. Charts were completed by Origin 2021.

3. Results

3.1. Analysis results of polyphenol extracts from flaxseed

This study determined the phenolic compounds and total phenolic content in seeds of five flaxseed varieties, as shown in Fig. 1(a) and Fig. 1 (b), respectively. Among the five varieties, Longya 10 exhibited the highest diversity of phenolic compounds. Longya 10 was found to contain 5 phenolic acids (ferulic acid, vanillic acid, sinapic acid, gallic acid, 4-hydroxybenzoic acid) and 6 flavonoids (vitexin, quercetrin, quercetin, apigenin, kauniol, and (+) dihydroquercetin), Longya 13 contained 3 phenolic acids (gallic acid, vanillic acid, 4-hydroxybenzoic acid) and 5 flavonoids ((+) dihydroquercetin, kaempferol, quercetrin, quercetin, apigenin), Longya 14 contained 3 phenolic acids (gallic acid, vanillic acid, 4-hydroxybenzoic acid) and 5 flavonoids ((+) dihydroquercetin, kaempferol, quercetrin, apigenin, naringenin), Longya 15 contained 4 phenolic acids (gallic acid, vanillic acid, 4-hydroxybenzoic acid, syringic acid) and 4 flavonoids ((+) dihydroquercetin, kaempferol, quercetrin, vitexin), and Longya 16 contained 4 phenolic acids (gallic acid, vanillic acid, 4-hydroxybenzoic acid, ferulic acid) and 1 flavonoid ((+) dihydroquercetin). The highest content of phenolic compounds in Longya 10, Longya 13, Longya 14, Longya 15, and Longya 16 were found to be syringic acid (26.71 ± 0.75 μg/g DW), vanillic acid (27.89 ± 1.22 μg/g DW), quercitrin (22.52 ± 0.43 μg/g DW), quercitrin (16.47 ± 0.33 μg/g DW), and ferulic acid (20.47 ± 0.91 μg/g DW), respectively.

3.2. Antioxidant activity of flaxseed polyphenol extract

DPPH and ABTS react with antioxidant compounds and can easily donate hydrogen atoms (Tohma & Gulcin, 2010). As shown in Fig. 2(a) and 2(b), flaxseed polyphenols exhibit effective scavenging activity against free radicals, with DPPH and ABTS radical scavenging capacities of 12.37, 10.71, 13.80, 10.20, 11.52, and 12.01, 10.86, 12.99, 10.50, 11.41 μmol TE/g DW for Longya 10–16, respectively. There were no significant differences in the radical scavenging capacities of flaxseed polyphenol extracts from different varieties.

3.3. Effects of flax polyphenol extract on RAW264.7 cell viability under tBHP

Based on the Fig. 3, it was be observed that 200 μmol/L tBHP reduces cell viability to 52.52%. Within the concentration range of 0.625 to 2.5 mg/mL, all five varieties of flaxseed polyphenol extracts enhanced cell viability. Under the concentration gradients of 0.625, 1.25, and 2.5 mg/mL, the cell viability for Longya 10 is 103.28%, 93.38%, and 75.49%, respectively, for Longya 13 it was 105.05%, 93.38%, and 75.49%, respectively, for Longya 14 it was 87.11%, 64.22%, and 58.79%, respectively, for Longya 15 it was 79.50%, 58.96%, and 51.56%, respectively, and for Longya 16 it was 89.53%, 74.68%, and 68.04%, respectively. Therefore, it can be concluded that the protective effects of Longya 10 (103.28%) and Longya 13 (105.05%) were stronger than those of the other three varieties.

3.4. Effects of flax polyphenol extract on ROS levels in RAW264.7 cells treated with tBHP

The nonpolar DCFH-DA penetrated the cell membrane and then was hydrolyzed by cellular esterases to form polar DCFH. The intracellular DCFH was rapidly oxidized by free radicals, resulting in the formation of fluorescent DCF. As shown in Figs. 4 and 5, RAW 264.7 cells produced ROS upon tBHP stimulation. Following oxidative damage induced by tBHP, the intracellular ROS levels increased by 17.79-fold compared to the control group. Pre-treatment with flaxseed polyphenols of different concentrations and varieties significantly reduced ROS levels ($p < 0.01$), demonstrating that flaxseed polyphenol extracts from different varieties effectively and dose-dependently reduced tBHP-induced ROS accumulation. This indicates that flaxseed polyphenols could scavenge reactive oxygen species and upregulate protective antioxidant defense networks to inhibit ROS generation, thereby achieving antioxidation.(Liu, Luo, & Wei, 2019).

3.5. Effects of flax polyphenol extract on MDA/GSH/GSSG/T-AOC content in RAW264.7 cells treated with tBHP

MDA, GSSG, GSH, and T-AOC were important indicators for assessing oxidative damage. The experimental results showed that after treatment with 200 μmol/mL tBHP, the levels of MDA and GSSG in the cell culture supernatant significantly increased by 13.60% and 398.33%, respectively, while the levels of GSH and T-AOC significantly decreased by 92.30% and 33.33%, respectively, indicating that tBHP induced oxidative stress in RAW 264.7 cells.The results, as shown in the Fig. 6(a), indicate that the MDA content in the tBHP-treated group significantly increased ($p < 0.01$), reaching 4.99 times that of the control group. Samples treated with Longya 10, 13, 14, 15, and 16 flaxseed polyphenol

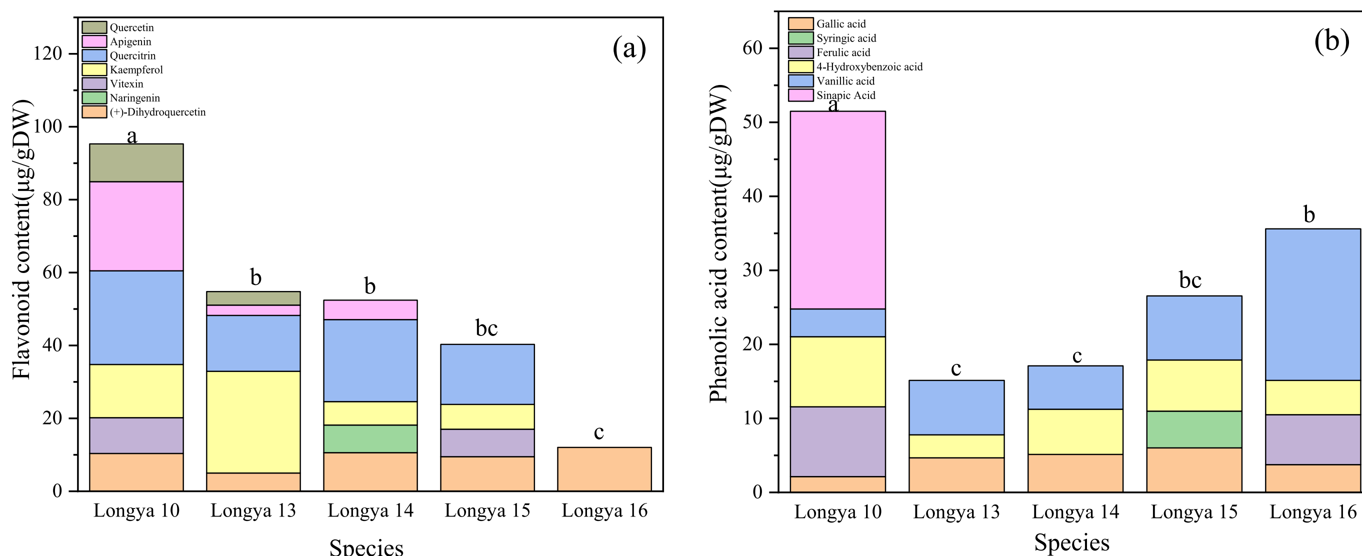

Fig. 1. Analysis of flavonoids (a) and phenolic acid compounds (b) in polyphenol extracts of flaxseed of different varieties.

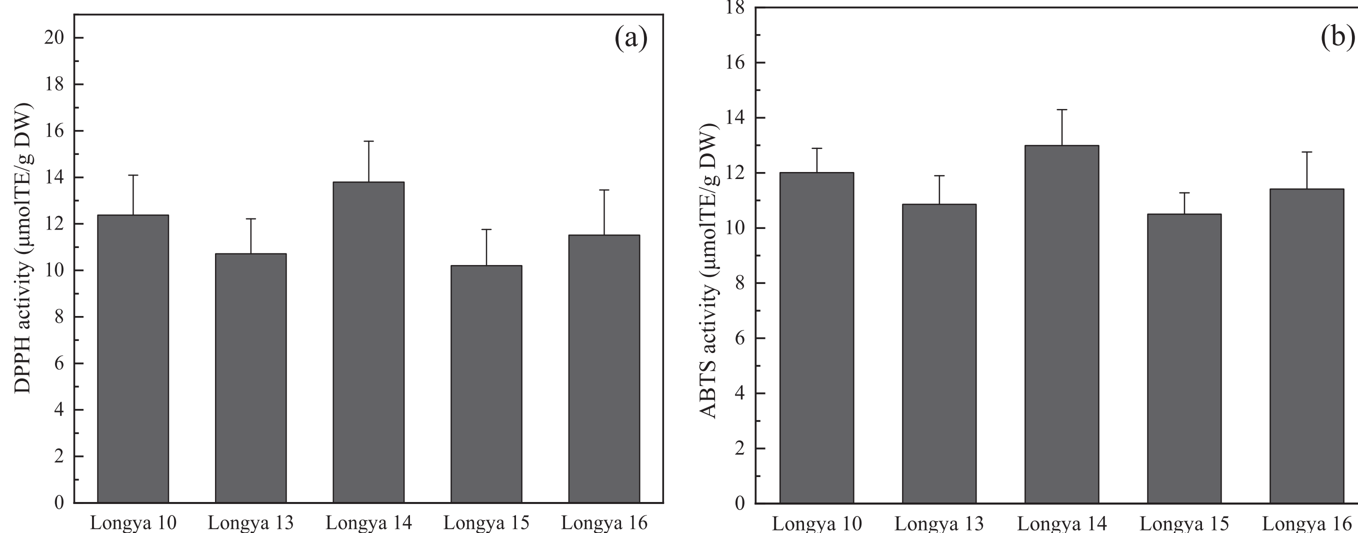

Fig. 2. DPPH (a) and ABTS (b) free radical scavenging rates of polyphenol extracts from flaxseed of different varieties.

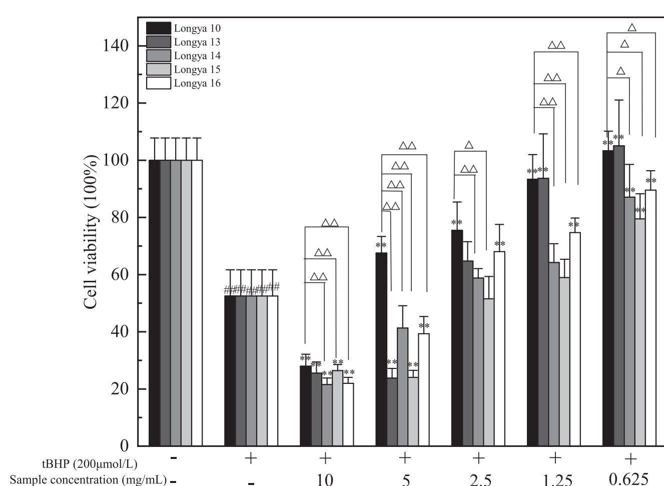

Fig. 3. Effects of polyphenol extracts from different varieties of flax on cell survival under tBHP treatment.
In the tBHP group compared with the control group, # represents a significant difference, and * represents a significant difference in the sample group compared with the tBHP group, while △ represents a comparison among the five varieties of flaxseed polyphenol extracts at the same concentration. # (*, △) indicates a significant difference, i.e., $p < 0.05$, and ## (**, △△) indicates an extremely significant difference, i.e., $p < 0.01$; $n = 6$ (same below).

extracts within the concentration range of 0.625 to 2.5 mg/mL all significantly reduced MDA content (p < 0.01), with a decrease observed as concentration increased, reaching the lowest at 2.5 mg/mL, at 0.17, 0.42, 0.40, 0.17, and 0.31 times that of the tBHP-treated group, respectively. Moreover, samples treated with different varieties of flaxseed polyphenol extracts significantly increased GSH content within the experimental concentration range, reaching the maximum at 2.5 mg/mL, at 21.82, 19.59, 21.00, 19.00, and 18.15 times that of the tBHP-treated group, respectively Fig. 6(b). As shown in Fig. 6(c) and 6(d), after *co*-incubation with flaxseed polyphenols and tBHP, the GSSG content in the cell supernatant decreased, while T-AOC significantly increased.In summary, tBHP induced a significant increase in MDA production in RAW264.7 cells, causing cell lipid oxidation and depleting GSH, thus reducing ROS clearance and resulting in the accumulation of ROS, leading to oxidative damage to cells. The addition of flaxseed polyphenol extracts significantly reduced MDA content, effectively inhibited lipid oxidation, increased GSH content, and provided protection against tBHP-induced oxidative damage to RAW264.7 cells.

3.6. Effects of flax polyphenol extract on SOD and CAT activity in RAW264.7 cells treated with tBHP

The results, as shown in Fig. 7(a) and Fig. 7(b), demonstrate the variation in antioxidant enzyme activity. In RAW264.7 cells treated with tBHP, the activities of SOD and CAT enzymes were significantly lower than those in the control group 0.53-fold and 0.56-fold of the control group, respectively. However, in the samples treated with Longya 10, 13, 14, 15, and 16 flaxseed polyphenol extracts within the concentration range of 0.625 to 2.5 mg/mL, the activities of both enzymes increased, showing significant differences compared to the tBHP group ($p < 0.01$). The peak activities of SOD and CAT enzymes were observed at 2.5 mg/mL. For the groups treated with Longya 10, 13, 14, 15, and 16 flaxseed polyphenol extracts, the maximum SOD enzyme activities were 1.96-fold, 1.80-fold, 2.82-fold, 2.18-fold, and 2.29-fold of the tBHP-treated group, respectively; while the maximum CAT enzyme activities were 1.53-fold, 1.87-fold, 1.79-fold, 1.89-fold, and 1.68-fold of the tBHP-treated group, respectively. These results indicatee that tBHP can disrupted the oxidative-antioxidative balance by reducing the activities of SOD and CAT enzymes in cells, thereby reducing the antioxidant capacity in the body and causing damage to cells. However, the addition of flaxseed polyphenol extracts significantly increased the activities of these two antioxidant enzymes within the experimental concentration range of 0.625 to 2.5 mg/mL, thereby protecting RAW264.7 cells from oxidative damage.

4. Discussion

The total phenol content of flaxseed of different varieties was different (Fig. S1). Separate standard curves for flavonoids and phenolic acids and mixed standard curves were presented in previous studies (Wang et al., 2024). Based on the HPLC results obtained in this experiment, it was observed that flaxseed polyphenols constitute a complex mixture abundant in phenolic compounds, including ferulic acid, gallic acid, and sinapic acid (Fig. 1). Among the varieties tested, Longya 10, 13, 14, 15, and 16 exhibited varying levels of phenolic acids, with sinapic acid being the most abundant in Longya 10, followed by ferulic acid in Longya 13, 14, 15, and 16. Similarly, the predominant flavonoids differed among the varieties, with apigenin, kaempferol, quercitrin and (+) dihydroquercetin being the most abundant. Notably, Longya 10

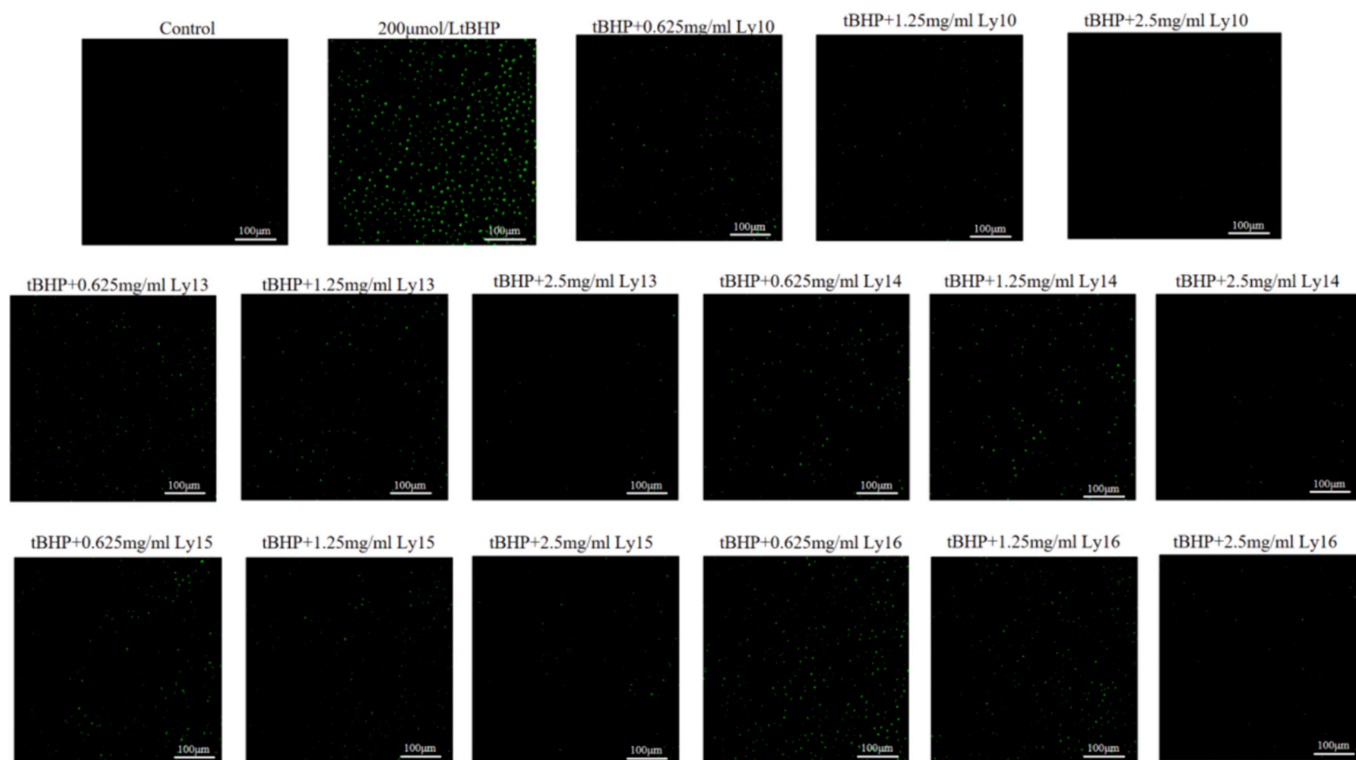

Fig. 4. Effects of polyphenol extracts from different varieties of flax on cell ROS production under tBHP treatment.

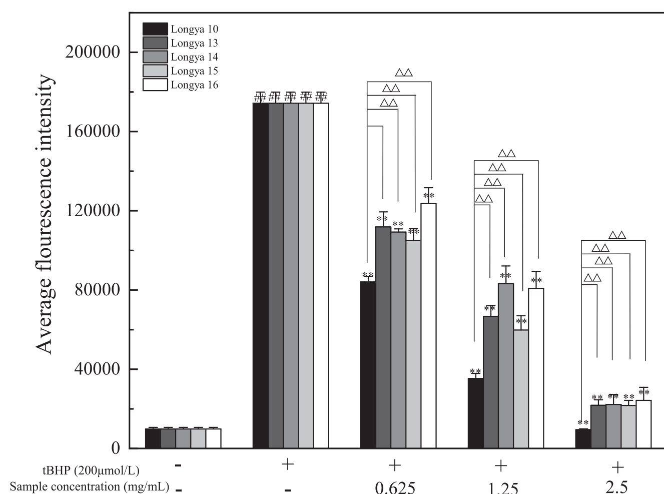

Fig. 5. Effects of polyphenol extracts from different flax varieties on ROS content in cells treated with tBHP.

demonstrated higher concentrations of ferulic acid, sinapic acid, kaempferol, quercetin, and apigenin compared to the other varieties. In the study by Mechchate et al. (2021), the polyphenols with the highest content in flaxseed, identified as Oleuropein and Ursolic acid, were found in the flaxseed sample labeled BPRN57. Contrary to these findings, Beejmohun et al. (2007) reported that in the Barbara variety of flaxseed, the content of ferulic acid glucoside was 3.7 ± 0.2 mg/g, while that of coumarin glucoside was 4.1 ± 0.2 mg/g. The results of these two studies differed from the present experiment, which found that in the flaxseed of the Longya 10 variety, the content of coumarin was 9.44 ± 0.2 μg/gDW. This indicated significant differences in both the types and amounts of polyphenols present in flaxseed among different varieties. Additionally, Longya 10 exhibited 11 significantly higher phenolic acids and flavonoids compared to the other four varieties, with a content of hesperidin at 25.72 ± 0.32 μg/gDW, which was 1.68 times, 1.14 times, 1.56 times, and 25.72 times higher than that in Longya 13, 14, 15, and 16, respectively.

Plant polyphenols, recognized as secondary metabolites inherently synthesized by plants (Sharma, Shahzad, Rehman, Bhardwaj, & Zheng, 2019), have been extensively studied for their potential in treating various chronic diseases associated with oxidative stress, including cancer, inflammation, diabetes, and hyperlipidemia, rendering them as promising natural antioxidants (Yan et al., 2019). Among these, flaxseed polyphenols emerged as significant bioactive compounds present in flaxseeds, with numerous investigations elucidating their diverse pharmacological activities, prominently highlighting their antioxidative properties (Al-Jumaily & Al-Azawi, 2015). It was widely acknowledged that the presence of hydroxyl groups in polyphenols contributed to their antioxidative prowess. Notably, ferulic acid has been shown to mitigate endoplasmic reticulum stress and nitric oxide (NO) production in polarized Caco-2 and T84 cells, thereby reducing membrane permeability in these cell types (Hwang et al., 2022). Polyphenols have been shown to increase the concentration of GSH and the activity of SOD in the liver, reduce blood lipids and hepatic cholesterol levels, and overall enhance the potential of the antioxidant system (Yeh, Lee, Hsieh, & Hwang, 2009). Ellagic acid was demonstrated to prevent oxidants from entering the lipid bilayer, thereby preventing oxidants from entering the interior of cells and undergoing oxidation reactions (Suwalsky et al., 2016). This result was consistent with the findings of our experiment (Fig. 2), indicating that flaxseed polyphenols had high free radical scavenging capacity. RAW 264.7 cells, known for their high versatility in sensing and responding to their surrounding or systemic environment by altering their morphology and functionality, were widely employed as suitable screening cell models for studying the antioxidant activity of natural products (Chen et al., 2023). In this study, the antioxidant activity of flaxseed polyphenols was investigated using RAW 264.7 cells. It was found that after treatment with 200 μmol/L tBHP, the cell viability of RAW 264.7 cells was only 52.5% (Fig. 3). However, *co*-incubation

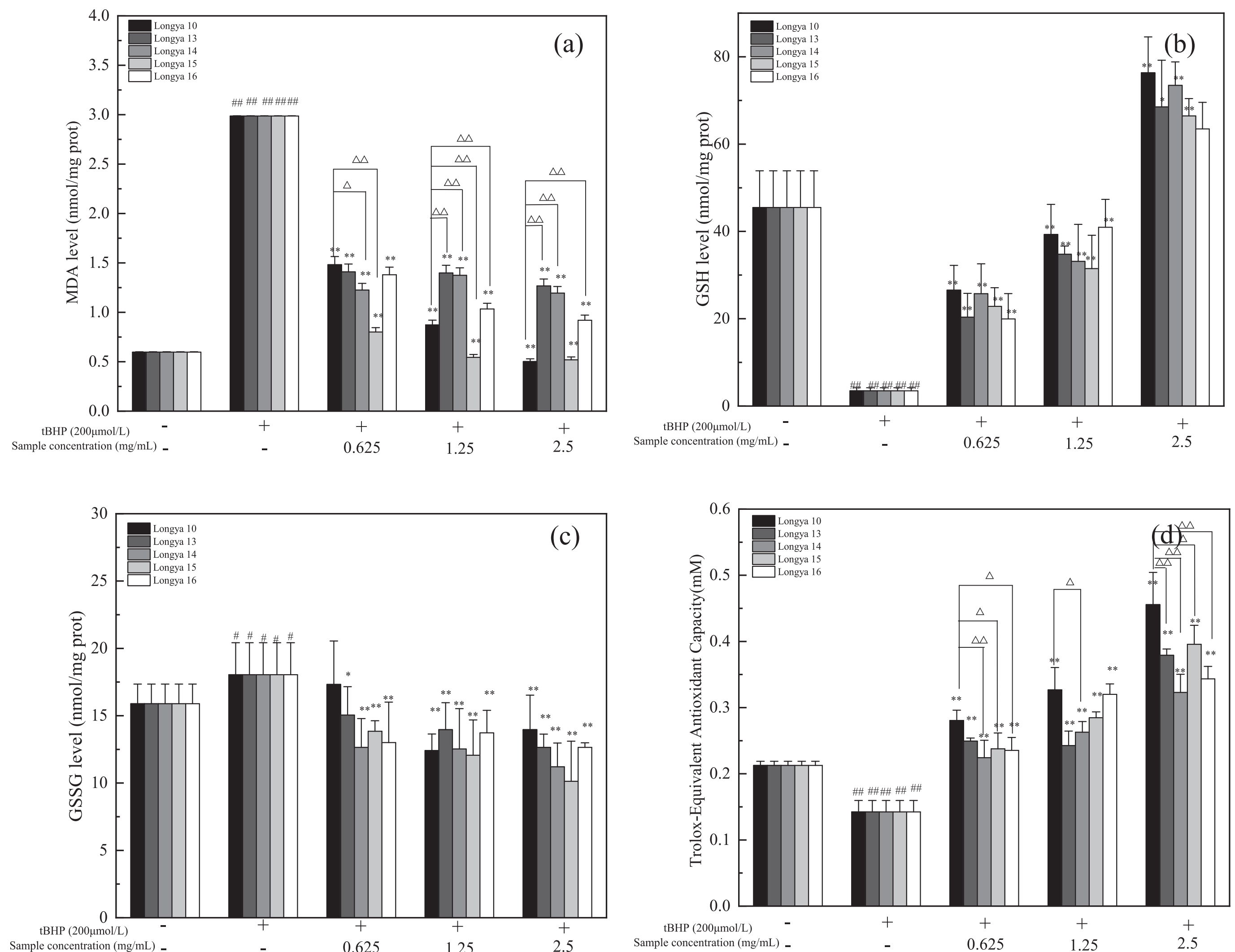

Fig. 6. Effects of polyphenol extracts of different flax varieties on intracellular MDA(a), GSH(b), GSSG(c) and T-AOC(d) under tBHP treatment.
In the tBHP group compared with the control group, # represents a significant difference, and * represents a significant difference in the sample group compared with the tBHP group, while △ represents a comparison among the five varieties of flaxseed polyphenol extracts at the same concentration. # (*, △) indicates a significant difference, i.e., p < 0.05, and ## (**, △△) indicates an extremely significant difference, i.e., p < 0.01; n = 6 (same below).

with polyphenol extracts from Longya 10–16 significantly enhanced the activity of RAW 264.7 cells, maintained cell morphology, and significantly increased the number of viable cells ($p < 0.05$). The cell viability reached 103.28%, 105.05%, 87.11%, 79.50%, and 89.53%, respectively, indicating a protective effect of flaxseed polyphenol extracts against tBHP-induced cellular oxidative damage. Pre-treatment with flaxseed polyphenols of different concentrations and varieties significantly reduced ROS levels ($p < 0.01$) (Fig. 4, Fig. 5). This finding was consistent with the results reported by Fuentes et al. (2019), who found that metabolites of quercetin in onion water extract could protect Caco2 cells exposed to ROS from oxidative damage. Similarly, in line with the findings of Yan and Zheng (2017), oxidative damage was mainly attributed to excessive ROS production. Therefore, reducing the level of free radicals in the body could mitigate the risk of related complications. Mulberry flower extract, rich in polyphenolic compounds, was shown to alleviate oxidative stress damage in HepG2 cells by reducing ROS accumulation and mitigating the excessive production of damaged free radicals.

In this experiment, a tBHP-induced oxidative stress model in RAW 264.7 cells was utilized to investigate the protective effects of polyphenol extracts from five varieties of flaxseed against cellular oxidative stress (Fig. 6, Fig. 7). In organisms, MDA served as the primary product of long-chain polyunsaturated fatty acid (PUFA) oxidation, which could induce cross-linking polymerization of macromolecules such as proteins and nucleic acids, consequently exhibiting cytotoxicity. The level of MDA reflected the extent of lipid peroxidation and indirectly indicated the degree of cellular oxidative stress damage. SOD played a pivotal role in maintaining the balance between oxidation and antioxidation in the body. By scavenging intracellular superoxide anion radicals, SOD protected cells from damage. In essence, the activity of SOD could be indicative of the body's antioxidant capacity (Xu et al., 2017). Therefore, to evaluate the extent of lipid oxidation damage in organisms or cells, the level of lipid peroxidation product MDA needs to be assessed to determine the severity of free radical attack on cells. Additionally, the activity of antioxidant enzymes such as SOD and CAT had to be measured to assess the ability of drugs to scavenge oxygen free radicals. In this experiment, the levels of MDA, SOD, GSH, GSSG, T-AOC, ROS, and CAT in the cell culture supernatant were measured. The results revealed that the levels of MDA and ROS were significantly elevated in the tBHP control group, while the levels of SOD, CAT, and T-AOC were markedly reduced ($p < 0.01$). The treatment groups administered with various varieties of flaxseed polyphenols effectively decreased the levels of MDA and ROS, and increased the levels of SOD, GSH, and CAT ($p < 0.05$), indicating that flaxseed polyphenols have the potential to

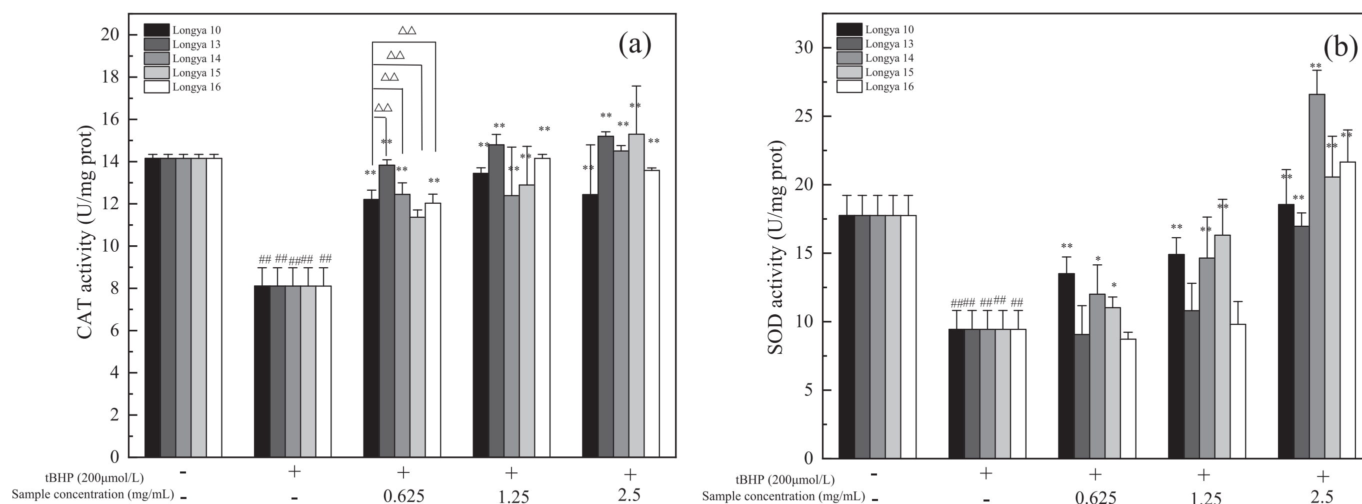

Fig. 7. Effects of polyphenol extracts from different flax varieties on the activity of CAT(a) and SOD(b) enzymes in cells treated with tBHP.
In the tBHP group compared with the control group, # represents a significant difference, and * represents a significant difference in the sample group compared with the tBHP group, while △ represents a comparison among the five varieties of flaxseed polyphenol extracts at the same concentration. # (*, △) indicates a significant difference, i.e., $p < 0.05$, and ## (**, △△) indicates an extremely significant difference, i.e., $p < 0.01$; n = 6 (same below).

enhance the antioxidant capacity of RAW 264.7 cells, mitigate the extent of oxidative stress damage, and demonstrate a dose-dependent effect. These findings were in line with those of Lin et al. (2021), who reported that seaweed polyphenols exhibited a protective effect against oxidative damage induced by H_2O_2 in HepG2 cells.

In summary, the flaxseed polyphenol extract effectively protected RAW 264.7 cells from tBHP-induced oxidative stress damage. Pre-incubation with flaxseed polyphenols significantly increased cell viability and improved cell morphology in RAW 264.7 cells. Furthermore, flaxseed polyphenols enhanced the activity of SOD, GSH, and CAT in RAW 264.7 cells, reduced the oxidative product MDA, thereby inhibiting tBHP-induced oxidative stress damage, enhancing the antioxidant levels of the cells, and providing robust protection against oxidative stress damage. These findings suggest a promising avenue for the development of natural active substances derived from flaxseed polyphenols.

5. Conclusion

The analysis focused on the phenolic acids and flavonoids content in flaxseeds, revealing significant variations in polyphenol types and quantities among different flaxseed varieties. Notably, sinapic acid, kaempferol, quercitrin, quercitrin, and ferulic acid were found to be the most abundant phenolic compounds in Longya 10, 13, 14, 15, and 16, respectively. In the tBHP-induced oxidative damage model, flaxseed polyphenols effectively restored the decreased cell viability of RAW 264.7 cells caused by tBHP. Moreover, they mitigated the decrease in intracellular GSH levels and the increase in ROS and MDA levels induced by tBHP, thereby alleviating damage to RAW 264.7 cells. Additionally, flaxseed polyphenols upregulated the activities of SOD, GSH, and CAT, contributing to their protective effects against oxidative damage. These findings suggest that flaxseed polyphenols can counteract oxidative damage induced by external stimuli by modulating the cellular redox system and scavenging intracellular reactive oxygen species. However, it's worth noting that the number of flaxseed samples and the choice of varieties used in this study may be relatively limited. Future research endeavors could encompass a broader spectrum of sample sources to explore the diversity of flaxseed polyphenols. Overall, this study laid the foundation for further understanding of the composition, quantity and antioxidant properties of phenolic compounds in flaxseeds.

CRediT authorship contribution statement

Xianqing Huang: Writing – review & editing, Investigation, Funding acquisition. **Nan Wang:** Writing – original draft, Methodology, Formal analysis. **Yan Ma:** Writing – review & editing, Investigation, Funding acquisition. **Xiaoyong Liu:** Resources, Conceptualization. **Hongtao Guo:** Supervision, Resources. **Lianjun Song:** Supervision, Methodology. **Qiuyan Zhao:** Supervision, Methodology. **Dan Hai:** Data curation. **Yongxia Cheng:** Data curation. **Ge Bai:** Methodology. **Qi Guo:** Methodology.

Declaration of competing interest

The authors declare that they have no known competing financialinterests or personal relationships that could have appeared to influencethe 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.fochx.2024.101597.

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