Extraction, isolation, structural characterization, and antioxidant activity of polysaccharides from elderberry fruit

Xinxin Wei1†, Junxiu Yao2†, Fangzhou Wang1,3, Dejun Wu2* and Rentang Zhang1*

1Key Laboratory of Food Processing Technology and Quality Control in Shandong Province, College of Food Science and Engineering, Shandong Agricultural University, Tai’an, China, 2Key Laboratory for Genetics and Breeding in Forest Trees of Shandong Province, Shandong Academy of Forestry Science, Jinan, China, 3Department of Food Science and Formulation, Gembloux Agro-Bio Tech, Université de Liège, Gembloux, Belgium

The isolation, purification, and antioxidant activity of polysaccharides extracted from elderberry fruits were studied. Two neutral polysaccharides (EFP-0 and EFP-1) and three acidic polysaccharides (EFP-2, EFP-3, and EFP-4) were isolated from elderberry. EFP-0, EFP-1, EFP-2, EFP-3, and EFP-4 all contain arabinose, galactose, glucose, and mannose, with molecular weights of $1.7981 \times 10^6$, $7.0523 \times 10^6$, $7.7638 \times 10^6$, $4.3855 \times 10^5$, and $7.3173 \times 10^5$ Da, respectively. Structural characterization showed that the backbone of EFP-2 consisted of →4)-Manp (1→4)-α-L-Araf (1→5) units, and T-α-L-Araf (1→ and T-β-D-Galp (1→ residues were detected by methylation analysis and NMR analysis. In addition, the MTT assay and zebrafish oxidative damage assay showed that EFP-2 had a protective effect on $\text{H}_2\text{O}_2$-damaged RAW264.7 cells in a dose-dependent manner, and zebrafish with the addition of EFP-2 would have low levels of ROS in vivo which showed significant antioxidant activity. Therefore, the results showed that the elderberry polysaccharides have antioxidant activity and can be used as potential antioxidants in functional foods.

KEYWORDS elderberry, polysaccharide, separation and purification, structural characterization, antioxidant

Introduction

The Sambucus williamsii Hance (elderberry), which belongs to the family Adoxaceae, is mainly distributed in Europe, Asia, and some parts of North Africa, and consists of seven different genera, divided into 5 to 30 species and 6 to 11 subspecies (1). Elderberry, black elderberry, European elderberry, and European black elderberry
are common names for elderberry (2). Studies have shown that
d elderberry was both an edible and medicinal plant, which was
the potential source of antioxidant ingredients (3) that can be
used as a healthy dietary supplement in the preparation of jams and yogurt, and also applied in the treatment of common
symptoms related to colds, fevers, coughs, and influenza (4).
In addition, elderberry contains sugars, flavonoids, phenolic
acids, lignans, triterpenoids, and other bioactive substances, so
everberies also have better physiological and pharmacological
effects in antioxidants (5), anti-influenza (6), antiviral (7), anti-
inflammatory (8), and anti-radiation (9).

Polysaccharides are a class of natural macromolecular
biopolymers composed of multiple monosaccharides through
glycosidic bonds (10). In recent years, plant, animal, and
microbial polysaccharides have received widespread attention
from the medical and food industries as bioactive ingredients and
food additives. It is widely believed that polysaccharides
extracted from natural products were less toxic and could be
used as raw materials or supplements for functional food
products (11), especially with antioxidants (12), anti-fatigue
(13), anti-tumor (14), modulation of human immune function
(15, 16), anti-inflammatory (17), hypolipidemic (18), and
inhibition of cell proliferation (19), it can prevent the harmful
effects of free radicals in the human body in terms of antioxidant
(20, 21). Studies have shown that oxidative stress leads to a
significant decrease in serum antioxidant activity and inhibition
of superoxide dismutase, glutathione (GSH), total antioxidant
activity, and glutathione peroxidase activities. Polysaccharides
play a key role as edible free radical scavengers in inhibiting
oxidative damage in organisms (22). Therefore, there was a need
to study the structural characteristics of polysaccharides and use
them as natural antioxidants to protect the body from excess
reactive oxygen species invasion.

Polysaccharides are one of the important active substances of
erberry, with a total sugar content of 7.86~11.50% and the
reducing sugar content of 2.8~8.55% in elderberry fruits (20).
And elderberry fruits have a large yield, good color stability, and
potential health-promoting effects, with wide applicability in
different food applications, especially in products with long shelf
life. Up to now, although polysaccharides extracted from various
natural products has attracted significant research attention
worldwide, little research has been reported on elderberry
polysaccharides (23–25). Currently, only 10 saccharides or
glycosides have been isolated from elderberry (26), and
Liu et al. (27) and Wu (28) performed only preliminary
extraction and purification of polysaccharides from Elderberry
leaves and stems, and Song and Fu (29) concluded that
Elderberry polysaccharides regulate insulin secretion from
pancreatic islet cells and play a hypoglycemic role. The
immunomodulatory effects of elderberry fruit polysaccharides
were investigated by Lu et al. (30), who confirmed that
everberry polysaccharides stimulate immune responses in
RAW 264.7 cells through the NF-kB pathway by the activity
of Escherichia coli lipopolysaccharides, and similarly Stich et al.
(31) who also demonstrated that polysaccharides in aqueous-
derived elderberry extracts induce effective immunomodulatory
effects. However, the isolation, purification, and structural
characterization of elderberry polysaccharides have not been
investigated. In this study, we isolated and purified elderberry
polysaccharides from elderberry fruit residues and investigated
their structural characteristics and antioxidant activity to
initially elucidate the relationship between their structure and
antioxidant activity.

Materials and methods

Materials and reagents

Elderberry fruits were harvested from the Qingzhou
Elderberry-nursery base in Shandong Province, China,
in August 2019, and the dark purple kernels growing
in clusters were obtained (Shandong, China). DEAE
cellulose 52 and Sephadex G-100 were provided by the
Shanghai Yuanye Biotechnology Co (Shanghai, China). The
monosaccharide standard methylation kit was provided
by the Borealis Biotechnology Co., Ltd (Jiangsu, China).
NaCl and trifluoroacetic acid (TFA) was provided by
ACROS (Belgium). ROS fluorescent staining reagent CM-
H2DCFDA was provided by the Beijing Biolab Biotechnology
Co. Glutathione (GSH) was provided by Wuhan Rongcan
Biotecnology Co., Ltd (Wuhan, China).

Preparation, isolation, and purification of elderberry polysaccharides

Preparation of crude polysaccharides from
erberry

The protocol of hot water extraction of polysaccharides
was according to the previous one with some modifications
(32). The extract was refluxed twice with hot water at
a material-to-liquid ratio of 1:20 (w/v) for 45 min each
time, and the clarified solution was combined and extracted
by filtration with a cloth funnel with diatomaceous earth
while hot, concentrated at 65°C, alcoholic precipitation
in 95% ethanol solution, followed by centrifugation at
10,000 × g for 10 min and freeze-dried to collect the
precipitate. Then, the obtained polysaccharides were defatted
and deproteinated by petroleum ether and Sevage reagent
(chloroform: n-butanol volume ratio = 4:1). After that,
the polysaccharide was alcoholically precipitated in 95%
ethanol, centrifuged after 12 h in the refrigerator at 4°C,
and the precipitate was used for freeze-drying to obtain
the defatted and deproteinized crude polysaccharide of
erberry, which was EFP.
Isolation and purification of elderberry polysaccharides

The polysaccharide separation and purification process were as shown in Figure 1. About 5.00 g of EFP was dissolved thoroughly in 100 mL of deionized water, followed by centrifugation at 10,000 × g for 10 min, and filtered through a 0.45-µm microporous filter membrane. The filtered crude polysaccharide solution was slowly added to a pre-equilibrated DEAE-cellulose 52 anion-exchange column (50 mm × 1,000 mm) and eluted with deionized water and 0.1, 0.2, 0.3, 0.4, and 0.5 mol/L NaCl solution, respectively, (33). Each tube was 50 mL with a flow rate of 5 mL/min, and 50 tubes of each were collected. The polysaccharide content of each tube was detected at 490 nm using the phenol-sulfuric acid method according to Ji et al. (33), and the elution curve was plotted with the number of tubes as the horizontal coordinate and the absorbance value as the vertical coordinate, and the polysaccharide eluates corresponding to the peaks (EFP-0, EFP-1, EFP-2, EFP-3, and EFP-4) were combined according to the peaks of the elution curve and spun off for concentration. 100 mL EFP-0 solution (0.5 mg/mL) was slowly added to Sephadex G-100 Sephadex column (16 mm × 2000 mm), eluted with deionized water, and 30 tubes of polysaccharide solution were collected at 10 mL per tube. The collected polysaccharides were lyophilized. EFP-1, EFP-2, EFP-3, and EFP-4 were purified by the above method.

Molecular weight determination

As previously described and improved upon Olawuyi et al. (34), the heavy mean molecular weight (Mw) distribution and polydispersity (Mw/Mn) of the five polysaccharide fractions were determined by high-performance gel permeation chromatography (HPGPC; 35). The samples and standards were weighed precisely, and the samples were prepared into a 5-mg/mL solution, centrifuged at 12,000 rpm for 10 min, then the supernatant was filtered through a 0.22-µm microporous membrane and the samples were transferred into a 1.8-mL injection vial. After that, 20 µL of sample solution was injected into a BRT105-104-102 series gel column (8 × 300 mm) with 0.05 mol/L NaCl solution as the mobile phase and the column temperature was 40°C at a flow rate of 0.6 mL/min. Using the detector RI-10A, the molecular weight size of each sample was calculated based on the standard curve.

Analysis of monosaccharide composition

The monosaccharide composition of purified polysaccharides was determined by ion chromatography (Thermo Fisher ICS5000, United States; 36). About 10 mg of lyophilized polysaccharide powder was placed in an ampoule, 10 mL of 3 mol/L TFA was added and hydrolyzed at 120°C for 3 h, and then the residual TFA was removed by rotary evaporator. A 10-mL acid hydrolyzed solution was accurately aspirated and blown dry in a tube, 10 mL of deionized water was added with vortex mixing, 100 µL of deionized water was aspirated, and 900 µL of deionized water was added and then centrifuged at 12,000 rpm for 5 min. The analysis was performed on a Dionex Carbowat TMPA20 (3 × 50 cm) column with H2O and 15 mol/L NaOH and 100 mol/L NaOAC as mobile phases at a flow rate of 0.3 mL/min, column temperature of 30°C, and injection volume of 5 µL. The retention times and peak areas of the 16 monosaccharide standards were compared, and the different sugars were identified and quantified separately.

Structure identification of elderberry polysaccharide

Infrared spectral analysis

The infrared spectral analysis was conducted following the methods described and improved by previous authors (20). The extracted and isolated purified elderberry polysaccharide was pressed into thin slices, and the slices were subjected to Thermo Nicolet IS10 Fourier transform infrared spectroscopy (FT-IR) with the setting of mid-infrared mode scan range of 4,000 to 400 cm−1, and the analysis was performed using OMSNIC 8.2 (Thermo Scientific™, United States) software.

X-ray diffraction spectrum

The maximum purity of polysaccharides in EFP-2 was obtained by isolation and purification, so the EFP-2 fraction was selected to investigate the structure and antioxidant activity of polysaccharides. As previously stated and modified (37), an X’Pert Pro X-ray diffractometer (PANalytical, Netherlands) was used to measure the crystallization properties of EFP-2 under 40 kV and 40 mA of radiation at Cu Kα (λ = 0.154 06 nm) on a copper target. The X-ray intensity was measured using a NaI crystal scintillation counter (scintillation counter) with a scanning range of 5° to 60°, a step size of 0.02°, and a scanning speed of 4°/min.

Scanning electron microscope

The study of the microstructure of elderberry polysaccharides was conducted by scanning electron microscopy (38). About 5 mg of the dried sample was taken, adhered to a conductive carbon film containing a double-sided adhesive, placed in the sample chamber of the ion sputterer, and sprayed with gold for about 40 s. After the sample was removed, it was placed in the scanning electron microscope observation chamber with an acceleration voltage of 2 kV for observation.

Methylation analysis

The methylation steps were mainly referred to Yang et al. (39) with some modifications. About 2 to 3 mg of EFP-2 sample was added to 1 mL of anhydrous DMSO, then the anhydrous base solution and iodomethane solution are quickly added and reacted for 60 min at 30°C in a magnetic stirring water bath,
and finally 2 mL of ultrapure water was added to terminate the methylation reaction. The methylated polysaccharide was taken and hydrolyzed by adding 1 mL of 2 M TFA for 90 min and evaporated dry by a rotary evaporator. About 2 mL of double-distilled water and 60 mg of sodium borohydride have been added to the residue to reduce for 8 h, then, glacial acetic acid was added to neutralize the residue, 1 mL acetic anhydride was added to acetylate the reaction at 100°C for 1 h, and cooled. After that, 3 mL of toluene was added to remove the excess acetic anhydride. The acetylated product was dissolved with 3 mL of CHCl₃ and the upper aqueous layer was removed. After, the CHCl₃ layer was dried with an appropriate amount of anhydrous sodium sulfate, and the volume was fixed at 10 mL and put into a liquid phase vial. Finally, an RXI-5 SIIL MS column of 30 m × 0.25 mm × 0.25 μm was used, the starting temperature was 120°C, ramped up to 250°C/min at 3°C/min, and held for 5 min. The injection port temperature was 250°C, the detector temperature was 250°C/min, and the carrier gas was helium at a flow rate of 1 mL/min.

Nuclear magnetic resonance spectroscopy

About 40 mg of EFP-2 was dissolved in D₂O and then loaded in an NMR tube. The NMR spectra were scanned on a high-resolution 700 MHz Bruker AVANCE III NMR spectrometer (Bruker, Germany). One-dimensional NMR spectra (¹H and ¹³C) and two-dimensional NMR spectra [correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond coherence (HMBC)] were adopted to analyze the structural features of polysaccharides (10).

Effect of polysaccharides on the antioxidant activity of H₂O₂-induced oxidative damage in RAW264.7 cells

Cell culture

RAW 264.7 cell line was obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium (with 10% fetal bovine serum and 100 U/mL each of penicillin and streptomycin) at 37°C, 5% CO₂, and saturated humidity in an incubator (41).

MTT test

The viability of RAW 264.7 cells was determined by a standard assay using the MTT method (42). Cells of logarithmic growth phase were collected, washed with PBS buffer, trypsin digested, and centrifuged after the termination of digestion, and the cells were made into cell suspension with culture medium, inoculated into 96-well plates at a density of 5 × 10⁵ cells/well, and cell dilution was added at 100 μL/well and incubated for 24 h at saturated humidity, 37°C, and 5% CO₂. The samples were diluted with culture medium separately, and 100 μL was added to each well; the solvent model group and control group (EFP-2 solution with different solubility) were also set up, and the final concentration of 1.2 mM H₂O₂ was added, respectively. After 4 h incubation, MTT solution was taken and added to the cells at 10 μL/well, and incubation was continued in the incubator for 4 h. After 4 h, the liquid in the wells was discarded, and 150 μL DMSO was added to each well to dissolve it. The absorbance at 490 nm was measured with an enzyme marker, and the survival rate was calculated according to the following Eq. (1).

\[
\text{Survival rate (100%)} = \frac{100\% \text{ Absorbance value of control group} - \text{Compound absorbance value}}{100\% \text{ Absorbance value of control group} - \text{Absorbance value of blank group}}
\]

Morphological observation

The differences in cell morphology and number between the different treatment groups were compared by fluorescence microscopy. RAW264.7 cells were inoculated in 6-well plates (5 × 10⁵ cells/mL) at 1 mL per well, and blank, VC (100 μg/mL), and control (12.5–200 μg/mL) were set up in the same way as the MTT assay, and after 24 h of incubation, respectively. Cells were observed and photographed under a 200× microscope using a fluorescent microscope to compare differences in cell morphology and number between different EFP-2 concentrations.

Flow cytometric analysis of apoptosis

The 12-well plates were seeded with good cells plastered overnight (5 × 10⁵ cells/well), and VC, EFP-2 in concentration gradient, and 1.2 mM H₂O₂ were added. Another sample was set as blank control and 1.2 mM H₂O₂ was used as the positive control, and the cells were incubated in the incubator for 4 h.

We transferred the collected cells to a 1.5 mL EP tube with PBS and divided it into two parts, one for the blank control group and the other part for the same volume as the sample tube, as the No stain group. Dilute 10× Binding Buffer with PBS, dilute BV421 Annexin V with Binding Buffer: BV421 Annexin V = 50:1 volume ratio, add 70 μL per tube to the sample tube, and place on ice for 30 min away from light (BV421 Annexin V and 7-AAD can also be stained at the same time, considering that BV421 Annexin V’s staining ability is lower than that of 7-AAD, BV421 Annexin V is stained first). Add PBS 800 μL/tube, centrifuge, discard the supernatant, and add 200 μL PBS to make cell suspension. Filter on the machine and flow cytometry for testing.
Protective effects of EFP-2 in a zebrafish model of oxidative damage

Zebrafish parents are kept in the zebrafish breeding system, quality control reference "B/T 39649-2020 experimental animals Experimental fish quality control." Ten pairs of parental fish, male and female, were temporarily reared in 3 L paired boxes, and fertilized eggs with basically synchronized development were obtained using the photoinduction method; 3 dpf embryos were selected as experimental organisms. The experiment was set up the control group (blank control group), the GSH group (positive drug control group, 100 µM) and the subject group (200 µg/L). The incubation was continued for 24 h after administration. The rest of the operation was performed according to the standard "Rapid assay for zebrafish model of antioxidant function of T/ZHCA health food (ROS method)." After the experiment, the live imaging of each group of pups was performed using a Nikon fluorescence microscope (Ci-S), and no less than 10 fish were imaged in each group; the image grayscale values were extracted using ImageJ software, and the ROS clearance rate was calculated.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of three measurements. Statistical analysis was performed by one-way analysis of variance (ANOVA) and t-test in SPSS software to assess the significance of differences, and p < 0.05 was considered significant.
Results and discussion

Elderberry polysaccharide isolation and purification

The crude polysaccharide (EFP) was extracted from elderberry fruits in $2.2 \pm 0.4\%$. Then five polysaccharides were isolated, two neutral polysaccharides (EFP-0 and EFP-1) and three acidic polysaccharides (EFP-2, EFP-3, and EFP-4), and were obtained by passing through a DEAE-52 cellulose ion-exchange column (Figure 2A), with recoveries of 6.74, 6.03, 5.83, 4.69, and 7.24%, respectively. Yuan et al. (43) reported that five polysaccharides were also isolated and purified from blackened jujube. These five polysaccharide fractions were then further purified on a Sephadex G-100 column, and the purified five polysaccharide fractions all showed single peaks (Figure 2B), and the purity of polysaccharides was $77.86 \pm 0.63$, $81.67 \pm 0.43$, $90.65 \pm 0.57$, $81.77 \pm 0.83$, and $82.25 \pm 0.56\%$, respectively, measured by phenol-sulfuric acid method.

![FIGURE 2](image-url)

Isolation and purification of five polysaccharide fractions from elderberry fruits. (A) Elution profile of elderberry polysaccharides on a DEAE cellulose-52 column. (B) Elution profiles of five fractions (EFP-0, EFP-1, EFP-2, EFP-3, and EFP-4) on a Sephadex G-100 column.
Molecular weight analysis

The molecular weight of EFP was determined by HPGPC, and the molecular weight distribution of polysaccharides is critical for biological activity (44). The results were shown in Table 1, and based on the standard curve equation: \( \lg M_w = -0.1889 t + 12.007 \) (\( R^2 = 0.9943 \)), it was calculated that among the five polysaccharides, EFP-2 had the highest heavy average molecular weight of 7.7638 \( \times 10^6 \) g/mol, followed by EFP-1 (7.0523 \( \times 10^6 \) g/mol), EFP-4 (7.3173 \( \times 10^5 \) g/mol), and EFP-0 (1.7981 \( \times 10^6 \) g/mol) and the lowest heavy average molecular weight was EFP-3 (4.3855 \( \times 10^5 \) g/mol) with retention times of 27.088, 32.518, 27.309, 33.695, and 30.451 min. Among them, the polydispersity index (\( M_w / M_n \)) of EFP-0, EFP-1, and EFP-2 are 1.931, 2.132, and 2.147, respectively, close to 2, indicating that the molecular weight distribution of EFP-0, EFP-1, and EFP-2 were relatively narrow and the polymer structure was more homogeneous, so we speculate that EFP-0, EFP-1, and EFP-2 had better hydrophilicity and solubility (45).

Analysis of monosaccharide composition

Monosaccharide composition analysis is a basic step in determining the structural characterization of polysaccharides, and Table 2 showed that the five polysaccharides are heteropolysaccharides, of which EFP-0 and EFP-1 do not contain galacturonic acid as neutral sugars, EFP-2, EFP-3, and EFP-4 all contain galacturonic acid as acidic sugars, and the proportion of galactoalic acid is 6.90%:22.70%:30.60%, respectively. EFP-0, EFP-1, EFP-2, EFP-3, and EFP-4 all contain Arabia (47.70%:55.00%:41.40%:19.20%:23.20%), Glucose (15.00%:16.30%:18.00%:15.40%:19.40%), Galactose (14.10%:22.30%:24.80%:10.10%:16.10%), Mannose (4.10%:2.40%:6.60%:7.50%:8.70%), and glucosamine hydrochloride (1.50%:4.00%:2.30%:1.10%:2.00%), except that EFP-0 and EFP-3 both contain fucose (17.60%:24.10%). Notably, although rhamnose is widely found in plant polysaccharides and pectins, none of the five polysaccharide fractions contained rhamnose. At the same time, the five components contain mannose and glucose, indicating that the polysaccharides isolated and purified by EFP have antioxidant activity and are all polymer heteropolysaccharides (46). Ferreira et al. (3) concluded that elderberry contains glucose, fructose, and a small amount of sucrose. This was similar to the monosaccharide composition of straw mushroom polysaccharides derived by Tian et al. (47), as they are all mainly composed of arabinose, mannose, glucose, and galactose. Data were suggesting that the combinations and ratios of monosaccharides were different, as they were not always the same even in the same genus (48).

Structural identification of elderberry polysaccharides

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy spectra revealed the main functional groups of polysaccharides. The FT-IR spectra as shown in Figure 3 indicated that the IR spectra of all five components contained similar absorption peaks in the

**Table 1** Molecular weight characteristic parameters.

| Sample | RT (min) | Mw (g/mol) | Mn (g/mol) | Mp (g/mol) | Mw/Mn |
|--------|----------|------------|------------|------------|-------|
| EFP-0  | 30.451   | 1.7981 \( \times 10^6 \) | 9.3108 \( \times 10^5 \) | 1.8289 \( \times 10^4 \) | 1.931 |
| EFP-1  | 27.309   | 7.0523 \( \times 10^6 \) | 3.3072 \( \times 10^6 \) | 1.2336 \( \times 10^4 \) | 2.132 |
| EFP-2  | 27.088   | 7.7638 \( \times 10^5 \) | 3.6156 \( \times 10^5 \) | 1.7536 \( \times 10^4 \) | 2.147 |
| EFP-3  | 33.695   | 4.3855 \( \times 10^5 \) | 2.5156 \( \times 10^5 \) | 2.2593 \( \times 10^4 \) | 1.743 |
| EFP-4  | 32.518   | 7.3173 \( \times 10^5 \) | 4.0443 \( \times 10^5 \) | 5.0097 \( \times 10^4 \) | 1.809 |

**Table 2** Composition and percentage of monosaccharides.

| Sample | Arabinose (%) | Galactose (%) | Glucose (%) | Mannose (%) | Fucose (%) | Galacturonic acid (%) | Glucosamine hydrochloride (%) |
|--------|---------------|---------------|-------------|-------------|------------|----------------------|-----------------------------|
| EFP-0  | 47.70         | 14.10         | 15.00       | 4.10        | 17.60      | 0.00                 | 1.50                        |
| EFP-1  | 55.00         | 22.30         | 16.30       | 2.40        | 0.00       | 0.00                 | 4.00                        |
| EFP-2  | 41.40         | 24.80         | 18.00       | 6.60        | 0.00       | 6.90                 | 2.30                        |
| EFP-3  | 19.20         | 10.10         | 15.40       | 7.50        | 24.10      | 22.70                | 1.10                        |
| EFP-4  | 23.20         | 16.10         | 19.40       | 8.70        | 0.00       | 30.60                | 2.00                        |
range of 4,000 to 500 cm$^{-1}$, only the absorption intensities differ. EFP-0, EFP-1, EFP-2, EFP-3, and EFP-4 can observe a broad and intense stretching peak in all FTIR spectrum around 3,200 to 3,400 cm$^{-1}$, which was mainly caused by polysaccharide molecules or intermolecular O-H stretching vibrations, indicating the presence of intermolecular hydrogen bonds (49, 50). The weak absorption bands around 2,900 to 2,800 cm$^{-1}$ (Figures 3A–E) were attributed to C-H, -CH$_2$, and
-CH$_3$- asymmetric stretching vibrations (51), and the presence of these two absorption peaks suggests that all five components purified from elderberry polysaccharides are carbohydrates, but EFP-0 is relatively strong (43). The absorption peak near 1,400 to 1,700 cm$^{-1}$ was due to the -COOH bending vibration or symmetric stretching vibration of C-O, indicating the presence of glucuronic acid in EFP and the presence of -COOH groups; in particular, EFP-2 has the largest carboxyl peak (52–54). EFP-3 has an absorption peak at 1,597.92 cm$^{-1}$, indicating the presence of N-H variable angle vibration in EFP-3 (49). The absorption peaks at 1,000 to 1,200 cm$^{-1}$ were related to the C-O-C stretch of the glycoside bonds, which have different spectral shapes for polysaccharides composed of different monosaccharides, where multiple absorption bands appeared in EFP-2, EFP-3, and EFP-4, but only 1–2 absorption bands in EFP-0 and EFP-1 (Figures 3A,B); and it is noteworthy that EFP-0, EFP-2, EFP-3, and EFP-4 (Figures 3A,C–E) have absorption peaks at 1,000 to 1,050 cm$^{-1}$, indicating the presence of the pyranose ring in these four polysaccharide fractions (55), and 1,024 cm$^{-1}$ is the peak of C-O-C stretching vibration (56). A peak around 900 cm$^{-1}$ indicates the presence of β-glycoside bonds in the polysaccharide chain, but the peaks of EFP-0 and EFP-1 are weaker, and there is an absorption peak around 850 cm$^{-1}$ indicating the presence of α-glycoside bonds in polysaccharides, but only EFP-0 has no absorption peak here (45, 57, 58) and (59). Thus, the FTIR spectra of these purified polysaccharide components all have typical absorption peaks of heteropolysaccharides.

**X-ray diffraction analysis**

EFP-2 was determined by X-ray diffraction to be an amorphous or crystalline structure. Generally, polysaccharides with sharp narrow diffraction peaks are crystalline structures, while polysaccharides with broad diffraction peaks are amorphous structures (60). As shown in Figure 4, the XRD profile of EFP-2 has a strong broad peak at 2θ = 20°, and a similar peak pattern was observed in the 2θ region by Zhao et al. (61) and Ji et al. (50). A new sharp crystal peak at approximately 2θ = 32, 46, and 56° each indicates that EFP-2 had an ordered crystal conception, and similar results were observed by Yuan et al. (62) and Bai et al. (63), which may be related to the content of glyoxylate, an acidic polysaccharide containing three peaks with sharp and narrow diffraction (64). This inference was consistent with the results for monosaccharide composition.

**Scanning electron microscope**

Scanning electron microscopy can be used as a qualitative tool to analyze the surface morphology of polysaccharides (64). As can be seen from Figure 5, the polysaccharide structure appears fragmented at 500 × magnification and had a rough surface, which may be caused by various hydroxyl and carboxyl groups (64). At 1,000 × magnification, the EFP-2 polysaccharide showed agglomerates or lamellar aggregates, but no regularity overall, which may be due to the magnetic field enhancement leading to different degrees of cellular tissue damage, resulting in wrinkling, cracking, and rupture (21). At 3,000 × magnification, the surface of EFP-2 polysaccharide was a smooth sheet-like structure with tighter binding and surface distribution, possibly due to stronger interactions between polysaccharide molecules (65, 66).

**Methylation analysis**

Methylation analysis is the most widespread and effective method for determining the type and linkage of polysaccharide
FIGURE 5
SEM photo of the surface structure of EFP-2.

Figure 6 showed that the total ion flow diagram of the methylation products and the analysis of the polysaccharide methylated glycol acetates results were listed in Table 3. It can be seen that EFP-2 included 12 glycosidic bond types, and 1,4-Glc (34.90%), 1,4-Man (32.70%), and 1,4,6-Glc (10.80%) were the three major glycosidic bond linkage mode, with small amounts of 1-Gal (5.10%), 1-Man (4.40%), 1-Ara (2.50%), 1-Glc (2.40%), 1,4,6-Gal (1.90%), 1,5-Ara (1.90%), 1,6-Gal (1.00%), and 1,3,6-Gal (0.50%) residues. It can be seen that the EFP-2 backbone may be joined by 1,4-α-Glc and 1,4-α-Man, and that 1,4-α-Glc produced a branched chain at the C-6 position.

As can be seen in the methylation results, the number of glycosidic bonds differs from the composition of the monosaccharides measured, mainly due to the fact that methylation analysis is more qualitative than quantitative, and the percentage content of GC-MS is different due to the lower sensitivity of ion chromatography. In addition, EFP-2 has good solubility, and the use of DMSO reagents can avoid the problem of insufficient methylation of polysaccharides, so the glycosidic bond type determined by the methylation experiment is reliable (68).

NMR spectroscopy analysis of EFP-2

According to the 1H NMR spectrum (Figure 7A), EFP-2 had five main anomeric proton signals at δ 5.22, 5.10, 5.09, 4.70, and 4.45, which were labeled A, B, C, D, and E, respectively. The chemical shifts from 3.21 to 4.44 ppm in the 1H NMR spectrum were assigned to protons from C-2 to C-6 in the residues. The corresponding anomeric carbon signals in the 13C NMR spectrum were labeled with reference to the HSQC data. All the 1H and 13C NMR (Figure 7B) of labeled residues were assigned with data from the 1H-1HCOSY (Figure 7C) and HSQC spectra (Figure 7D). In 13C NMR spectrum (Figure 7B), four isomers with heteropolyacid carbon resonance signals were at δC 90 to 110 ppm (107.30, 101.59, 107.48, 104.03, and 102.91 ppm). This indicated that EFP-2 contained both α and β configurations in its structure (69), which was consistent with the results of the FTIR spectrum. All the 1H and 13C signals were assigned as completely as possible according to 2D NMR analysis and literature values. The chemical shift in the anomeric proton of residue A was δ 5.22. The corresponding chemical shift in the anomeric carbon was δ 107.30. The other protons of residue A were assigned from the COSY spectrum. The other corresponding carbon and hydrogen signals were δ 79.65 (4.30), 76.38 (4.11), 84.03 (4.10), and 62.54 (3.82, 3.72), determined with HSQC (Figure 7D). Based on these NMR data, we inferred that T-α-L-Araf (1→ was a constituent unit of EFP-2 Combined with the analysis, we inferred that the →4) –Manp (1→ was assigned as residue B, →5) -α-L-Araf (1→ as residue C, T-β-D-Galp (1→ as residue D, and →4) -β-D-Glc (1→ as residue E (71–73). Taking the results of methylation analysis into consideration, it was supposed that the presence of two
different terminal signals was suggested as T-α-L-Araf (1→ and T-β-D-Galp (1→ in EFP-2, as well as comparing their NMR data with those in references (Table 4; 70). In the HMBC spectrum, some inter-residual cross-peaks were observed: B-H-1 to E-C-4 (Figure 7E). In the NOESY spectrum, some inter-residual cross-peaks were observed: B-1 to E-4/D-3 and C-5; E-1 to C-5/D-3 and E 4; D-1 to C-5/D-3 and E 4 (Figure 7F). The determination of the monosaccharide composition analysis confirmed that EFP-2 was mainly composed of galactose, arabinose, glucose, and mannose. Based on its monosaccharide composition and 1D and 2D NMR spectroscopy results, EFP-2 was proposed to comprise two units of (1→4) –Manp (1→4) -β-D-Glcp (1→ and (1→4) -β-D-Glcp (1→ 5) -α-L-Araf (1→.

**Protective effect of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress**

**Effect of EFP-2 on H<sub>2</sub>O<sub>2</sub>-induced cell competence in RAW264.7 cells**

Compared with the model group (Figure 8A), the survival rate of cells under 1.2 mM H<sub>2</sub>O<sub>2</sub> conditions both increased with the increase of polysaccharide concentration, which acted as a protective anti-free radicals in vitro, indicating that EFP-2 had a strong protective effect on RAW264.7 cells with H<sub>2</sub>O<sub>2</sub>-induced damage. It was shown that cell viability was enhanced with increasing concentrations, suggesting a dose-dependent relationship between polysaccharide concentration and cellular activity (12.5–200 µg/mL), which was consistent with the studies reported by Wang et al. (74) and Xie et al. (75). In Figure 8B, the cells were mostly round, with smooth surfaces and uniform size in clusters (76). Cell density and cell viability increased significantly after 24 h treatment with EFP-2 (12.5–200 µg/mL), which became more pronounced with increasing EFP-2 concentration, suggesting that FFP-2 could inhibit morphological changes in RAW264.7 cells and protect them from H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. This may be because the inhibitory effect of EFP-2 on cellular death induced by H<sub>2</sub>O<sub>2</sub> oxidative stress is determined by monosaccharides, which can provide the ability of cells or control oxidative stress (77). Studies have shown that many plant polysaccharides can scavenge excess free radicals and play an antioxidant role, and Li et al. (78) have concluded that red mushroom polysaccharides also have
a significant protective effect on the activity of \( \text{H}_2\text{O}_2 \)-induced RAW264.7 cells.

**Apoptosis analysis**

Studies have shown that \( \text{H}_2\text{O}_2 \)-induced oxidative stress can induce DNA damage, inflammation, tissue damage, and apoptosis, which is a key target of most chemotherapy drugs because it is the primary mechanism for clearing damaged cells (75). As shown in Figure 9, the percentage of apoptosis in the \( \text{H}_2\text{O}_2 \) group was 0.56%, the percentage of apoptosis in RAW264.7 cells treated with EFP-2 was significantly reduced to less than 0.50%, and the percentage of apoptosis in RAW264.7 cells gradually decreased to 0.48, 0.46, and 0.37% with the increase of EFP-2 concentration (12.5–50 \( \mu \)g/mL). Compared
TABLE 4 Assignments of $^1$H and $^{13}$C NMR spectra for EFP-2.

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| A | T-α-L-Araf (1→2) | C | 107.30 | 79.65 | 76.38 | 84.03 | 62.54 |
|   |   | H | 5.22  | 4.30  | 4.11  | 4.10  | 3.82/3.72 |
| B | →4)-Manp (1→3) | C | 101.59 | 68.69 | 71.10 | 76.49 | 75.77 | 61.16 |
|   |   | H | 5.10  | 4.24  | 3.76  | 3.98  | 3.46  | 3.86/3.76 |
| C | →5)-α-L-Araf (1→4) | C | 107.48 | 81.90 | 76.56 | 82.03 | 66.35 |
|   |   | H | 5.09  | 4.23  | 4.15  | 4.25  | 3.90  |
| D | T-β-D-Galp (1→3) | C | 104.03 | 72.01 | 73.59 | 69.15 | 75.12 | 61.16 |
|   |   | H | 4.70  | 3.58  | 3.84  | 4.02  | 3.72  | 3.86/3.76 |
| E | →4)-β-D-Glkp (1→5) | C | 102.91 | 76.27 | 74.31 | 82.10 | 72.43 | 61.16 |
|   |   | H | 4.45  | 3.42  | 3.58  | 3.85  | 3.72  | 3.86/3.76 |

with VC, RAW264.7 cells treated with EFP-2 (<25 μg/mL) had a weaker apoptotic activity, which was enhanced when the concentration of EFP-2 (50 μg/mL) was increased to a certain concentration. These results suggested that EFP-2 can participate in the process of programmed suicide to remove non-functional, unwanted, abnormal, and harmful cells, reducing the death of RAW264.7 cells. Thus, EFP-2 was shown to protect cells from H$_2$O$_2$-induced oxidative stress.

**Protective effect of EFP-2 polysaccharide on oxidative damage model in zebrafish**

Zebrafish are often used as biological models for in vivo studies of small molecules and drugs because of their fast growth rate, small size, transparency, and ease of handling (77, 78). Glutathione, as a key antioxidant element, with the formation of its disulfide dimer, can respond to chemical stress and clear ROS, thereby balancing the intracellular redox homeostasis, helping to prevent oxidative stress in cells, and maintain a normal immune system (74, 79). The intensity of green fluorescence is positively correlated with the level of ROS, and the higher the brightness, the higher the level of ROS. When the level of ROS increases in the body, it will consume the antioxidant substances in the body and disrupt the homeostasis of the antioxidant defense system in the body. If the synthesis rate of antioxidant substances in the body is slow or the clearance rate of ROS is not fast, it will cause the peroxidation level of lipids and proteins in the body to increase, leading to the occurrence of chronic diseases and causing aging and other physiological phenomena related to peroxidation in the body (22, 80). Compared with the blank control group (Control group), both the positive drug control group (GSH group) and the subject group (EFP-2 group) could significantly reduce the overall ROS level of zebrafish litter, and the ROS clearance rate was 50.51 and 44.62% (Table 5), respectively. After the addition of EFP-2, the fluorescence intensity of zebrafish was significantly reduced (Figure 10), and the ROS level in zebrafish was decreased, which improved its antioxidant level, directly reflecting that EFP-2 has an antioxidant ability. In addition to the direct antioxidant response, polysaccharides may also
FIGURE 9
Effect of EFP-2 on H$_2$O$_2$-induced apoptosis in RAW264.7 cells.

FIGURE 10
Partial fluorescence map in zebrafish.
TABLE 5 Fluorescence grayscale values for each group.

| Serial number | Blank group | GSH group | Subjects group |
|---------------|-------------|-----------|----------------|
| 1             | 434309      | 290555    | 205835         |
| 2             | 442634      | 195570    | 273728         |
| 3             | 325941      | 156175    | 155579         |
| 4             | 337943      | 215241    | 226494         |
| 5             | 344125      | 142899    | 243585         |
| 6             | 474124      | 242847    | 165656         |
| 7             | 433179      | 197831    | 209262         |
| 8             | 482588      | 199821    | 283857         |
| 9             | 460675      | 257933    | 251640         |
| 10            | 553025      | 149162    | 231248         |
| 11            | 302460      | 174076    | 239522         |
| 12            | 307248      | /         | /              |

Average value: 408188 ± 81619.53b 202010 ± 46949.15a 226037 ± 40112.29a

Values with different superscripts indicate significant differences (P < 0.05).

activate some signaling pathways that protect zebrafish from oxidative stress damage. For example, Yang et al. (40) added HJP-1a at doses of 5, 25, and 50 µg/mL, which can increase the fluorescent spots of zebrafish embryos by 60.8 to 83.3%, and then achieve the best effect on the protective effect of oxidative damage cells.

Conclusion

Five polysaccharide fractions, including two neutral polysaccharide fractions (EFP-0 and EFP-1) and three acidic polysaccharide fractions (EFP-2, EFP-3, and EFP-4), were isolated and purified from elderberry fruits using DEAE-52 anion-exchange chromatography and Sephadex G-100 dextran gel chromatography columns, and they differed in terms of monosaccharide composition and molecular weight. Both EFP-0 and EFP-1 were mainly composed of arabinose, galactose, and glucose, and fucose was also a major component of EFP-0. EFP-2, EFP-3, and EFP-4 were all acidic heteropolysaccharides, all containing arabinose (41.10, 19.20, 23.20%), galactose (24.80, 10.10, 16.10%), glucose (18.00, 15.40, 19.40%), and galacturonic acid (6.90, 22.70, 30.60%). The molecular weights were 1.7981 × 10^6, 7.0523 × 10^6, 7.7638 × 10^6, 4.3855 × 10^5, and 7.3173 × 10^5 g/mol, respectively. The backbone of EFP-2 consisted of two units, →4) –Manp (1→4) -β-D-GlcP (1→ and →4) -β-D-GlcP (1→5) -α-L-Araf (1→, and T-α-L-Araf (1→ and T-β-D-Galp (1→ residues were detected by methylation analysis and NMR analysis. In addition, the MTT method and zebrafish oxidative damage assay showed that EFP-2 had a protective effect on H2O2-damaged RAW264.7 cells in a dose-dependent manner, and zebrafish with the addition of EFP-2 would have low levels of ROS in vivo and also showed significant antioxidant activity. Therefore, the elderberry polysaccharide has antioxidant activity, which provides a theoretical basis for further studying the relationship between EFP-2 structure and antioxidant activity, and contributes to the development of EFP-2 as the most novel antioxidant. However, the disadvantage of this study is the lack of in-depth understanding of the relationship between polysaccharide structure and antioxidant activity.

Data availability statement

The original contributions presented in this study are included in the article supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

XW, RZ, and JY contributed to the conception, design, and writing of this study. DW and JY contributed funding and materials for this study. FW contributed to the data organization of the manuscript. All authors contributed to this article and approved the submitted version.

Acknowledgments

We gratefully acknowledged the key research and development plan of Shandong Province (Agricultural seed improvement project) "Breeding of new varieties of Sambucus with high yield and good quality" (2020LZGC0905).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.
synthesized by Lactobacillus casei ATCC 393. Carbohydr Polym. (2018) 195:576–85. doi: 10.1016/j.carbpol.2018.04.110
42. Wang MX, Kanako N, Zhang YQ, Xiao XL, Gao QF, Tetsuya K. A unique polysaccharide purified from Hericium erinaceus mycelium prevents oxidative stress induced by H2O2 in human gastric mucosa epithelial cell. PLoS One. (2017) 12:e0181546. doi: 10.1371/journal.pone.0181546
43. Yuan L, Qu Z, Yang Y, Liu C, Zhang R. Preparation, structural characterization and antioxidant activity of water-soluble polysaccharides and purified fractions from blackened jujube by an activity-oriented approach. Food Chem. (2022) 385:132637. doi: 10.1016/j.foodchem.2022.132637
44. Xiong QW, Luo GF, Zheng F, Wu K, Yang HN, Chen L, et al. Structural characterization and evaluation the elicitors activity of polysaccharides from Chrysochlamys indicum. Carbohydr Polym. (2021) 263:117994. doi: 10.1016/j.carbpol.2021.117994
45. Liu JY, Hou XX, Li ZY, Shan SH, Chang MC, Feng PG, et al. Isolation and structural characterization of a novel polysaccharide from Hericium erinaceus fructifying bodies and its effect on the S-phase in colon cancer cells. Int J Biol Macromol. (2020) 157:288–95. doi: 10.1016/j.ijbiomac.2020.04.162
46. Wang FF, Ye SH, Ding Y, Ma ZY, Zhao QK, Zang M, et al. Research on structure and antioxidant activity of polysaccharides from Ginkgo biloba leaves. J Mol Struct. (2022) 1522:121815. doi: 10.1016/j.molstruc.2021.121815
47. Tian WN, Xiao N, Yang YY, Xiao J, Zeng RP, Xie LH, et al. Structure, antioxidant and anticancer activity of a polysaccharide extracted by different methods. Int J Biol Macromol. (2020) 162:116–26. doi: 10.1016/j.jbimac.2020.06.150
48. Li MZ, Wen JJ, Huang XI, Nie QX, Wu XC, Ma WN, et al. Interaction between polysaccharides and toll-like receptor 4: primary structural role, immune balance perspective, and 3D interaction model hypothesis. Food Chem. (2022) 374:131856. doi: 10.1016/j.foodchem.2021.131856
49. Wu HQ, Ma ZL, Zhang DX, Wu P, Guo YH, Yang F, et al. Sequential extraction, characterization, and analysis of pumpkin polysaccharides for their hypoglycemic activities and effects on gut microbiota in mice. Front Nutri. (2021) 8:769181. doi: 10.3389/fnnut.2021.769181
50. Ji XL, Guo JH, Ding DQ, Gao J, Hao LR, Guo XD, et al. Structural characterization and antioxidant activity of a novel high-molecular-weight polysaccharide from Ziziphus jujuba cv. Muzao. J Food Meas Charact. (2022) 16:2022–30. doi: 10.1016/j.jfmc.2020.04.162
51. Guan Y, Sun HF, Chen HY, Li PJ, Shan Y, Li X. Phycobiocophysical characterization and the hypoglycemia effects of polysaccharide isolated from Passiflora edulis Sims peel. Food Funct. (2021) 12:4221–30. doi: 10.1039/d0fo02965c
52. Chen HY, Zeng JS, Wang B, Cheng Z, Xu J, Gao WH, et al. Structural characterization and antioxidant activities of Bletilla striata polysaccharide extracted by different methods. Carbohydr Polym. (2021) 266:118149. doi: 10.1016/j.carbpol.2021.118149
53. Gao J, Lin LZ, Sun BG, Zhao MM. A comparison study on polysaccharides extracted from Laminaria japonica using different methods: structural characterization and bile acid binding capacity. Food Funct. (2017) 8:3043–52. doi: 10.1039/c7fo00218a
54. Vasilevka T, Sigarev A, Kosyakov D, Ul’yanovskii N, Anikeenko E, Chuhchin V, et al. Preparation, structural characterization and bile acid-binding capacity of polysaccharides by pressurized hot water extraction on the yield and chemical characterization of polysaccharides from the bark of Avicennia marina. Carbohydr Polym. (2012) 87:1893–8. doi: 10.1016/j.carbpol.2011.08.059
55. Vasilieva T, Sigarev A, Kosyakov D, Ul’yanovskii N, Anikeenko E, Chuhchin V, et al. Chemical characterization of polysaccharides from the bark of Avicennia marina. Carbohydr Polym. (2012) 87:1893–8. doi: 10.1016/j.carbpol.2011.08.059
56. Xie LM, Shen MY, Wen PW, Hong YZ, Xu K, Tan JL, et al. Chemical structure and immunological activity of a highly branched 1-3-1-6 beta-D-glucan from Ginkgo biloba. Carbohydr Polym. (2014) 111:139–48. doi: 10.1016/j.carbpol.2014.04.077
57. Muschini T, Yoshida T. Structural analysis of galactomannans by NMR spectroscopy. Carbohydr Polym. (2012) 87:1893–8. doi: 10.1016/j.carbpol.2011.08.059
58. Zhu MQ, Huan HM, Weng R, Song Y, He BL, Tan JL, et al. Characterization, antioxidant activity and protective effect against cellular oxidative stress induced by H2O2 in RAW264.7 cells. J Food Meas Charact. (2020) 145:111754. doi: 10.1016/j.jfmc.2020.111754
59. Wang ZL, Xie JH, Kan LJ, Wang JQ, Shen MY, Li WJ, et al. Sulfated polysaccharides from Cyclocarya paliurus reduce H2O2-induced oxidative stress in RAW264.7 cells. Int J Biol Macromol. (2015) 80:418–7. doi: 10.1016/j.ijbiomac.2015.06.031
60. Xie LM, Shen MY, Wen PW, Hong YZ, Xu K, Xie JH, Preparation, characterization, antioxidant activity and protective effect against cellular oxidative stress of polysaccharides from Polypodium cymosum. J Funct Foods. (2022) 88:104866. doi: 10.1016/j.jff.2021.104866
61. Raguraman V, Abraham LS, Jyotina J, Palaniappan S, Gopal S, Thirunagasundambandam R, et al. Sulfated polysaccharide from Sargassum tenerrimum attenuates oxidative stress induced reactive oxygen species production in vitro and in zebrafish model. Carbohydr Polym. (2019) 203:441–9. doi: 10.1016/j.carbpol.2018.09.056
62. Li YL, Li XI, Chu Q, Jia RY, Chen W, Wang YX, et al. Effects of the steaming process on the structural properties and immunological activities of polysaccharides from Polypodium cymosum. Food Chem X. (2022) 12:e0181546. doi: 10.1016/j.carbpol.2022.12.e0181546
63. Hamed MA, Alkhalife TM, Alkhalife RE, Aremu AO, Oyedokun PA, Ghadamosi JA, et al. Glutamine restores testicular glutathione-dependent antioxidant defense and upregulates NO/CaGMP signaling in sleep deprivation-induced reproductive dysfunction in rats. Biomed Pharmacother. (2022) 148:112765. doi: 10.1016/j.biopha.2022.112765
64. Alkhalife RE, Ajayi A. The impact of reactive oxygen species in the development of cardiometabolic disorders: a review. Lipids Health Dis. (2021) 20:23. doi: 10.1186/s12944-021-01435-7