

The Seed Extract of *Orychophragmus Violaceus* Protects Against Radiation-induced Intestinal Injury

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Abstract *Orychophragmus violaceus* (OV), also known as the February orchid, has been used in traditional Chinese medicine for thousands of years. It possesses various physiological activities. In this study, the major components of OV seed alkaloid extract (OVS-2) were identified through liquid chromatography-mass spectrometry (LC-MS). The protective effect of OVS-2 against radiation-induced oxidative stress in rat intestinal crypt epithelial IEC-6 cells was determined. C57 mice underwent abdominal irradiation with ⁶⁰Co gamma rays to induce radiation-induced intestinal injury (RIII), followed by OVS-2 treatment. The efficacy of OVS-2 against RIII was evaluated based on changes in survival rate, body weight, histopathological staining, and intestinal biomarkers. Serum inflammatory cytokine levels and mRNA expression levels in the tissues were also measured. Potential mechanisms were explored using transcriptomic sequencing. LC-MS analysis revealed that the top 20 monomeric components of OVS-2 were primarily alkaloids. We demonstrated that the expression levels of malondialdehyde (MDA) and lipid peroxidation markers in OVS-2-treated IEC6 cells were significantly reduced under radiation-induced oxidative damage, while maintaining mitochondrial integrity. OVS-2 treatment improved the survival rate of irradiated mice, as evidenced by increased villus length and crypt depth in the histopathological sections. Increased expressions of intestinal biomarkers Ki67, Muc2, and lysozyme were observed after OVS-2 treatment, along with decreased expressions of inflammatory cytokines IL-6, IL-1 β , and TNF- α in serum and intestinal tissues. Transcriptomic sequencing of intestinal tissues revealed the enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were mainly related to immune regulation. Results suggest that the active components of OV mainly consist of alkaloids that exhibit antioxidant, anti-inflammatory, and intestinal mechanical barrier-protective effects.

Keywords: February orchids, RIII, alkaloid monomer, oxidative stress, transcriptomic sequencing

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1. Introduction

RIII is one of the most severe complications of abdominal and pelvic tumor radiotherapy and is almost unavoidable during the treatment of abdominal and pelvic malignancies [1]. After radiotherapy, patients often experience symptoms such as vomiting, weight loss, loss of appetite, diarrhea, and infection [2,3,4]. In severe cases, patients may die of septic shock [5]. The underlying mechanisms of radiation enteritis are complex and include epithelial cell death, crypt stem cell injury, mucosal barrier dysfunction, and inflammation, none of which can be cured by bone marrow transplantation. Although pathogenesis of RIII is still not fully understood, various studies reported that epithelial injury, impaired vascular systems as well as the disordered gut immune and

microbiota are involved in RIII. At present, no unified and effective methods are available for clinical prevention and treatment of RIII. Therefore, there is an urgent need to develop new RIII-protective drugs with good efficacy, stability, and low toxicity. Treatment mechanisms for RIII mainly include radiation protection, antioxidation, and anti-inflammation. In recent years, many Chinese herbal medicines or natural extracts have been widely used in radiotherapy and protection due to their effectiveness and low toxicity. Traditional Chinese medicine (TCM) is an effective method for treating radiation-induced intestinal injury [6]. *Orychophragmus violaceus* (L.) O.E.Schulz, commonly known as the February orchid or Zu-Ge-Cai in Chinese, is an annual or perennial herbaceous plant belonging to the *Brassicaceae* family. Previous domestic and foreign studies have demonstrated that OV, a natural antioxidant, possesses significant physiological activities [7]. Compounds found in OV seeds mainly include

alkaloids, flavonoids, and triterpenoid saponins, which exhibit antioxidation, antibacterial, anti-tumor, and hepatoprotection activities [8,9,10,11].

However, how OV affects RIII and its potential mechanism of action remain unclear. This study aimed to elucidate the radioprotective pharmacological activity of an OV seed alkaloid extract (OVS-2). First, the impact of OVS-2 on the survival rate of lethally irradiated mice was examined. Subsequently, the protective effect of OVS-2 on RIII was evaluated, and its potential mechanism in radiation protection was explored.

2. Materials and Methods

2.1. Extraction and Chemical Composition Analysis of OVS-2

The herbal material was obtained from Shuyang County, Anhui Province, China, and was identified as the seeds of OV by Associate Researcher Li Bin of the Institute of Radiation Medicine, Academy of Military Medical Sciences, China. Specimens were preserved in our laboratory.

Dried OV seeds (40 kg) were extracted three times with 70% ethanol at a ratio of 6:1 (w/v) for 2 h each time. The combined extracts were then concentrated to obtain the total extract. The total extract (OVS-1) was adsorbed onto D001 anion exchange resin and washed with water at a flow rate of 1 bed volume per hour (BV/h) to remove impurities. Elution was performed with 95% ethanol containing 0.5% ammonia at a flow rate of 1 BV/h to obtain the total alkaloids of OVS-2.

The separation process was carried out utilizing a Dionex™ UltiMate™ 3000 ultra-high performance liquid chromatography (UPLC) system, with the specific conditions as follows. A Waters UPLC HSS T3 column (1.8 μ m, 2.1 mm $\times$ 100 mm) was employed for the chromatographic separation. The mobile phase consisted of Phase A, composed of water with 0.1% formic acid, and Phase B, comprising acetonitrile. Elution gradient conditions were referenced from Table 1. The flow rate was maintained at 0.3 mL/min, with an injection volume of 10.0 μ L. Additionally, to ensure optimal separation, the column temperature was set at 40 °C throughout the analysis.

Table 1. Elution gradient

Time(min)	Mobile phase	
	A (v%)	B (v%)
0	2	98
1.0	2	98
41.0	100	0
50.0	100	100
50.1	2	98
52.0	2	98

2.2. Cell Culture and Grouping

The IEC-6 cell line was obtained from Procell Life Sciences, Ltd. Cells were cultured in high-glucose dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified environment at 37°C with 5% CO₂.

IEC-6 cells were irradiated with an X-RAD 320iX biological irradiator (Precision X-ray, North Branford, CT, USA) at a dose rate of 1.175 Gy/min to establish an *in vitro* model of intestinal epithelial cell injury. The radiation dose was set to 8 Gy. Irradiated cells were further incubated in a humidified environment at 37°C.

Untreated IEC-6 cells served as the control group (CON group). Cells subjected to radiation alone were designated as the model group (IR group). Cells pretreated with OVS-2 (100 μ g/mL) for 12 h before irradiation were designated as the treatment group (OVS-2 group).

2.3. Cell Viability Assay

IEC-6 cells were seeded in 96-well plates at a density of 5000 cells/well and cultured overnight. After appropriate treatments, 10 μ L of Cell Counting Kit-8 (CCK8) reagent was added to each well containing 100 μ L of culture medium. After incubation for 3 h, the optical density (OD) was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

2.4. Measurement of MDA, SOD, and Lipid ROS levels

Malondialdehyde (MDA) and superoxide dismutase (SOD) levels were determined using kits provided by Shanghai Beyotime Biotechnology Co. Ltd. Reactive oxygen species (ROS) levels were assessed by incubating treated cells with 5 μ M BODIPY 581/591 C11 fluorescent probe. After 30 min of incubation in a humidified incubator (37°C, 5% CO₂), the cells were harvested, and the fluorescence intensity was measured using a flow cytometer (BD FACSCelesta, BD Biosciences, California, USA).

2.5. Serum Inflammatory Factor Detection

Tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-1 β levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (provided by Multisciences Biotech Co., Ltd., China) following the manufacturer's instructions.

2.6. Transmission Electron Microscopy (TEM)

Cells were harvested 24 h after appropriate treatments, fixed with 2.5% glutaraldehyde at room temperature in the dark for 30 min, dehydrated, embedded in epoxy resin, and cut into ultrathin sections. Sections were observed using a TEM (Hitachi, Tokyo, Japan).

2.7. Mitochondrial Morphology Assessment

IEC-6 cells were seeded in 24-well plates, allowed to adhere overnight, and treated with different drugs for 24 h. Mitochondria were stained with MitoTracker® Green CM-H2XROS according to the manufacturer's instructions, and mitochondrial morphology was observed using a confocal microscope (Olympus Corp.).

2.8. Animal Model and Treatment

C57 mice were obtained from SPF (Beijing)

Biotechnology Co., Ltd. and acclimated for 7 days under standardized conditions of temperature, humidity, and a 12/12-h light/dark cycle. C57 mice were feed by irradiated with ⁶⁰Co-irradiated Maintaining feed. feed (SPF-F02-002) was purchased from SPF (Beijing) Biotechnology Co., Ltd. Formula: First-grade flour 29.00%, High-gluten flour 4.10%, Puffed corn 40.84%, Fish meal (imported, steamed) 16.06%, Soybean oil 2.00%, Soybean oil 6.00%. Mice were anesthetized intraperitoneally with sodium pentobarbital for irradiation. Abdominal irradiation was performed using a ⁶⁰Co irradiator (Beijing Institute of Radiation Medicine, Beijing, China) at a dose rate of 74.22 cGy/min. The Animal Laboratory of Experimental Animal Center, Military Medical Research Institute approved all animal experiments (Ethic number IACUC-DWZX-2023-P502, Approval date: 29 January 2022).

Next, 50 mice were randomly divided into a control group (CON group), a model group (MOD group), and low, medium, and high OVS-2 groups (L-OVS-2, M-OVS-2, and H-OVS-2 groups, respectively). The CON group received physiological saline via oral administration, whereas the MOD and OVS-2 groups received different drugs simultaneously with irradiation and treatment. The radiation dose was 17 Gy, and the OVS-2 groups received OVS-2 dissolved in physiological saline and administered orally 24 h before irradiation and 0.5 h, 24 h, and 48 h after irradiation at doses of 50 mg/kg, 100 mg/kg, or 200 mg/kg. The CON group was administered physiological saline solution. Mouse survival was monitored for 30 days.

The radiation dose was 14 Gy, and 35 mice were randomly divided into 5 groups, as described previously, for dissection and collection of intestinal tissue on the 3.5th day.

2.9. Histopathological Staining

Mice were euthanized by cervical dislocation on the 3.5th day post-irradiation, and intestinal tissues from the stomach end to the beginning of the cecum were collected, washed with physiological saline, fixed in 4% paraformaldehyde solution, stained with hematoxylin and eosin (HE), and sectioned. The intestinal mucosal structure was observed under an optical microscope, and the villus height and crypt depth were measured using ImageJ software.

2.10. Immunohistochemistry

For immunohistochemistry, tissue sections were blocked with 3% bovine serum albumin at room temperature for 30 min and incubated overnight with primary antibodies at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 50 min. Immunostaining was performed using a diaminobenzidine staining kit (Servicebio), and counterstaining was performed with hematoxylin.

2.11. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from the cells using the TRIzol reagent according to the manufacturer's instructions. Reverse transcription-quantitative real-time

polymerase chain reaction (RT-qPCR) was performed using the SYBR Green method with β-actin as the internal reference for amplification, and mRNA expression levels were analyzed using the 2^{-ΔΔCT} method. RNA levels were normalized to β-actin as the internal control. All experiments were repeated four times. RT-qPCR primer sequences are showned in Table 2.

Table 2. RT-qPCR primer sequences

Gene name	Primer sequence (5'-3')
CD5-FORWARD	GTAAGTCTCTGCAGTGTGGCT
CD5-REVERSE	TCCCCACATAGCTCTTCCCA
Isg15-FORWARD	TGGTACAGAACTGCACGAG
Isg15-REVERSE	CAGCCAGAACTGGTCTTCGT
Slc7a5-FORWARD	AAGGGCAGGGATTTCATGGTG
Slc7a5-REVERSE	GTAGGGGTGTCTTTCAGGGC
Cxcl10-FORWARD	GGTCTGAGTCCTCGCTCAAG
Cxcl10-REVERSE	GTCGCACCTCCACATAGCTT
Nampt-FORWARD	GTTGTGTGAATTCACCGGC
Nampt-REVERSE	GGGGATGGCCAAATACTGCT
Bst2-FORWARD	CACAGGCAAACCTCTGCAAC
Bst2-REVERSE	TCCTGGTTCAGCTTCGTGAC
Ccl3-FORWARD	CCATATGGAGCTGACACCCC
Ccl3-REVERSE	GAGCAAAGGCTGCTGGTTTC
CD74-FORWARD	GGCTCCACCTAAAGAGCCAC
CD74-REVERSE	CGTGTCTGGGACGATGAAA
Tnfsf13b-FORWARD	CCACCGTGCCTCTGTTTTTG
Tnfsf13b-REVERSE	TTCGTATAGTCGGCGTGTCTG
Ccl5-FORWARD	TGCTCCAATCTTGACAGTCGT
Ccl5-REVERSE	GCAAGCAATGACAGGAAGC
ACTB-FORWARD	GCAGGAGTACGATGAGTCCG
ACTB-REVERSE	ACGAGCTCAGTAACAGTCC
TNF-α-FORWARD	GACGTGGAAGTGGCAGAAGAG
TNF-α-REVERSE	TTGGTGGTTGTGAGTGTGAG
IL-1β-FORWARD	GCAACTGTTCTGAACTCAACT
IL-1β-REVERSE	ATCTTTTGGGGTCCGTCAACT
IL6-FORWARD	CCAAGAGGTGAGTGCTTCCC
IL6-REVERSE	CTGTTGTTTCAGACTCTCTCCCT

2.12. Transcriptome Sequencing

Three mice intestinal tissue samples were randomly selected from the MOD group and the OVS-2 group for transcriptome sequencing. The intestinal tissue samples were divided into two portions. One portion was sent to Beijing Novogene Bioinformatics Technology Co., Ltd. for transcriptome sequencing, while the other portion was stored at -80°C for subsequent validation of differentially expressed key genes. Primer sequences are listed in Table 1. Raw reads obtained from sequencing contained low-quality sequences, which were analyzed and filtered using the Fastp software to obtain clean reads, ensuring data quality and reliability. After removing sequences containing adapter sequences, low-quality sequences, sequences with all A bases, and sequences with unknown base ratios of >10%, the effective data were all >99%, indicating good sequencing data quality. RSEM was used to calculate the expression levels of all genes in each sample, and differential gene expression analysis was performed using the DESeq2 method, with screening criteria set as FDR < 0.05 and |log2(FC)| > 1. The biological functions and signaling pathways of the

differentially expressed genes were enriched and analyzed using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.8. Statistical Analysis

Statistical analyses were performed using ImageJ and GraphPad Prism software, version 9.0.1. Data are reported as means $\pm$ standard deviations (SDs). One-way analysis of variance (ANOVA) and Tukey's post-hoc test was used to analyze intergroup differences for comparisons involving more than two groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Extraction and Principal Component Analysis of OVS-2

Table 3. Chemical composition identified by OV extract

Number	Compound name	Molecular formula	Molecular weight
1	orychophragmuspine A	C ₂₆ H ₃₃ N ₃ O ₅	512.2404
2	orychophragmuspine D	C ₂₈ H ₃₅ N ₃ O ₆	554.2508
3	hypargyresulide B	C ₂₄ H ₄₆ O ₄	397.3322
4	orychophragmuspine G	C ₂₇ H ₃₅ N ₃ O ₅	526.2561
5	orychophragmuspine F	C ₂₇ H ₃₅ N ₃ O ₅	480.2506
6	(+)-isolariciresinol-9'-O- β -D-glucopyranoside	C ₂₆ H ₃₄ O ₁₁	521.2033
7	orychophragmuspine H	C ₂₅ H ₃₃ N ₃ O ₄	438.2401
8	orychophragine A	C ₁₄ H ₁₇ N ₃ O ₄	290.1150
9	β -D- glucopyranosyl-12-hydroxyjasmonate	C ₁₈ H ₂₈ O ₉	387.1663
10	demethylorychophragine A	C ₁₃ H ₁₅ N ₃ O ₄	276.0990
11	orychophragine B	C ₁₆ H ₁₉ N ₅ O ₅	360.1317
12	orychophragmuspine C	C ₂₆ H ₃₃ N ₃ O ₅	512.2405
13	(-)-5'-methoxyisolariciresinol-9'-O- β -D-glucopyranoside	C ₂₇ H ₃₆ O ₁₂	551.2141
14	orychophragine D	C ₁₆ H ₁₈ N ₆ O ₄	357.1320
15	11-deoxykompasinal A	C ₂₅ H ₂₄ O ₇	435.1449
16	Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	312.0954
17	orychophramarin C	C ₁₆ H ₁₂ O ₆	299.0564
18	orychovioside A	C ₂₂ H ₃₈ O ₁₀	507.2449
19	orychophragmuspine E	C ₂₈ H ₃₅ N ₃ O ₆	508.2457
20	orychophragmuspine I	C ₂₆ H ₃₅ N ₃ O ₅	468.2501

The OV seeds were refluxed with 70% ethanol to obtain the total OVS-1 extract, which was subjected to adsorption on D001 cation exchange resin. Impurities were removed via elution with water at a flow rate of 1 BV/h. Subsequently, OV seed extract OVS-2 was obtained by elution with 95% ethanol containing 0.5% ammonia at a flow rate of 1 BV/h. Liquid chromatography-mass spectrometry (LC-MS) offers advantages such as rapid analysis, high sensitivity, and

excellent specificity. It combines the efficiency, speed, and wide applicability of liquid chromatography with the high sensitivity of mass spectrometry, making it increasingly popular in the field of TCM. We applied LC-MS to analyze and identify the chemical components in the OVS-2 extract of OV seeds and identified 79 compounds (Table 3). Among them, the top 20 compounds based on peak area were as follows: orychophragmuspine A, orychophragmuspine D, hypargyresulide B, orychophragmuspine G, orychophragmuspine F, (+)-isolariciresinol-9'-O- β -D-glucopyranoside, orychophragmuspine H, orychophragine A, β -D-glucopyranosyl-12-hydroxyjasmonate, demethylorychophragine A, orychophragine B, orychophragmuspine C, (-)-5'-methoxyisolariciresinol-9'-O- β -D-glucopyranoside, orychophragine D, 11-deoxykompasinal A, Adenosine, orychophramarin C, orychovioside A, orychophragmuspine E, and orychophragmuspine I. Notably, the main components are monomers of alkaloids.

3.2. Antioxidant Effects of OVS-2 on IEC-6 Cells

First, we measured the cytotoxicity of OVS-2, and the results are demonstrated in Figure 1. As shown in Figure 1A, no significant difference was observed between the OVS-2 groups ranging from 0 to 300 μ g/mL, compared with the CON group. OVS-2 at concentrations of ≤ 400 mg/L showed no cytotoxicity in IEC-6 cells ($*P < 0.05$, compared with the CON group). To determine whether OVS-2 can rescue ionizing radiation (IR)-induced intestinal epithelial cell death, we used rat IEC-6 cells to establish an *in vitro* radiation injury model by subjecting them to 8 Gy irradiation. Cells were pretreated with the optimal concentration of 100 μ g/mL OVS-2 and irradiated with 8 Gy IR. Furthermore, TEM revealed a reduction or disappearance of mitochondrial ridges, outer membrane rupture, and mitochondrial vacuolization in IEC-6 cells after 24 h of irradiation. These are typical morphological features of radiation-induced cell damage (Figure 1D), and OVS-2 was able to reduce the occurrence of these changes. Moreover, the observation of Mito-Tracker Green-labeled mitochondria showed a decrease in the intracellular mitochondrial number induced by ionizing radiation, which OVS-2 was able to improve (Figure 1C). In conclusion, OVS-2 exhibits a radioprotective effect on IEC-6 cells, characterized by a strong antioxidative ability, and can maintain cellular mitochondrial morphology. MDA, SOD, and ROS levels were also measured. After pretreatment with OVS-2, the MDA content in irradiated cells significantly decreased (Figure 1E), whereas the SOD content significantly increased (Figure 1F). Additionally, a significant increase was observed in ROS levels in the IR group (Figure 1G), which OVS-2 was able to reduce in IEC-6 cells. OVS-2 has low toxicity and strong antioxidant capacity, thereby protecting mitochondrial oxidative damage.

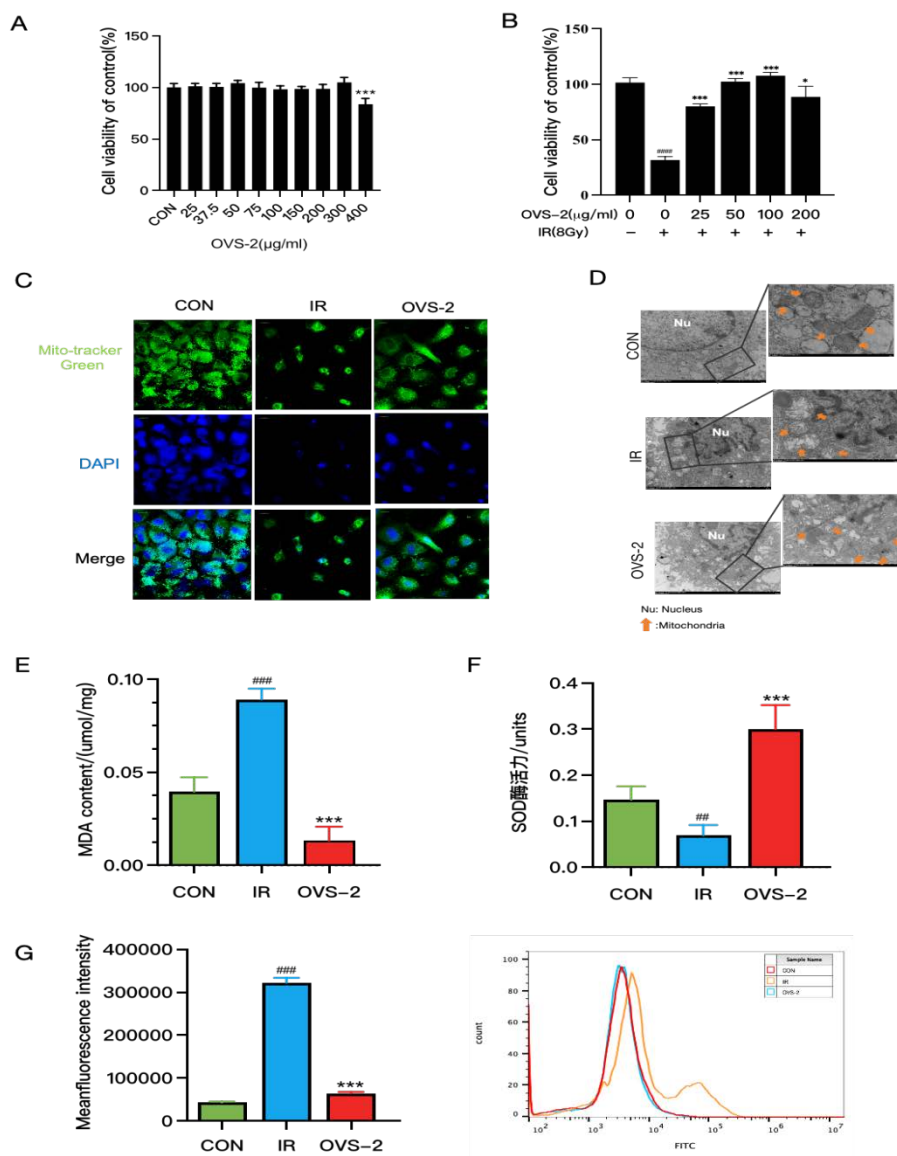


Figure 1. Antioxidant Effects of OVS-2 on IEC-6 Cells (A) Cytotoxicity of OVS-2 against IEC-6 cells. (B) Cell viability of IEC-6 cells treated with different OV seed extracts under 8 Gy. (C) Mito-Tracker Green fluorescent probe labeling of mitochondria (bar=10 μm). (D) Morphological images of IEC-6 cells observed by TEM (horizontal bar=500 nm). (E–F) Detection of MDA and SOD levels in MOD and SOD content using MDA and SOD assay kits (n=3); (G) C11 BODIPY fluorescent probe used to detect intracellular ROS levels (n=3). (* $P<0.05$, ** $P<0.01$ vs. CON group; # $P<0.05$, ## $P<0.01$ vs. IR group)

3.3. OVS-2 Attenuates Radiation-Induced Intestinal Injury in Mice

To investigate the effect of OVS-2 on RIII in mice, we subjected mice to high-dose abdominal irradiation (ABI) at a dose of 17 Gy, which induces acute intestinal crypt injury and fatal crypt damage[12,13]. We assessed the survival rate of the mice treated with OVS-2 at a lethal dose of 17 Gy. C57 mice received 17 Gy ABI on day 0 and were orally administered different doses of OVS-2 (50, 100, or 200 mg/kg) or saline 24 h before irradiation and 0.5 h, 24 h, and 48 h after irradiation. Mouse survival was monitored for 30 days (n=10). The HE staining results are as shown Figure in 2A. The results showed that oral administration of OVS-2 effectively delayed the time of death in mice receiving a lethal dose. All mice in the MOD group died within 4 days, whereas mice in the L-OVS-2, M-OVS-2, and H-OVS-2 treatment groups

showed delays in time to death by 1, 3, and 4 days, respectively, compared with the MOD group (Figure 2B). Additionally, we evaluated the beneficial effects of OVS-2 on radiation-induced intestinal tissue damage at a nonlethal dose of 14 Gy. C57 mice received 14 Gy ABI on day 0 and were orally administered different doses of OVS-2 (50, 100, or 200 mg/kg) or saline 24 h before irradiation and 0.5 h, 24 h, and 48 h after irradiation. Intestinal tissues were collected on day 4 post-ABI. Notably, oral administration of OVS-2 improved the morphology of intestinal tissue, as demonstrated by HE staining of intestinal sections (Figure 2C). In mice treated with L-OVS-2, M-OVS-2, and H-OVS-2, the crypt length and number observed by HE staining were significantly higher than those in the MOD group ($P<0.05$) (Figure 2D–E). The H-OVS-2 group was more effective than the other two experimental groups. These data suggest that OVS-2 may alleviate post-radiation injury to the intestinal villus and crypt structure in mice.

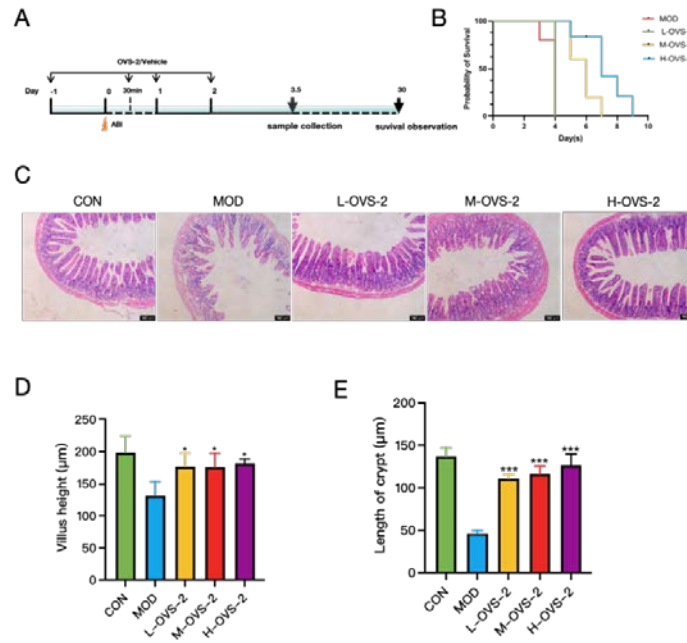


Figure 2. OVS-2 Attenuates RIII in C57 Mice (A) Experimental scheme. Mice were intraperitoneally injected with vehicle (saline) or OVS-2 (50, 100, or 200 mg/kg) for 3 consecutive days before and after 17 Gy-ABI (n=10). Intestinal samples were collected 3.5 days after 14 Gy-ABI (n=7). (B) Survival of irradiated mice was observed until 30 days post-irradiation (n=10). (C) Representative images of HE staining of intestinal sections from solvent- or OVS-2-treated mice 3.5 days after 14 Gy-ABI. All villi on the circumference of each sample were measured (scale bar=100 μm). (D) Statistical graph of villus length of each group 7 days after ABI (n=7). (*P<0.05, **P<0.01 vs. MOD group)

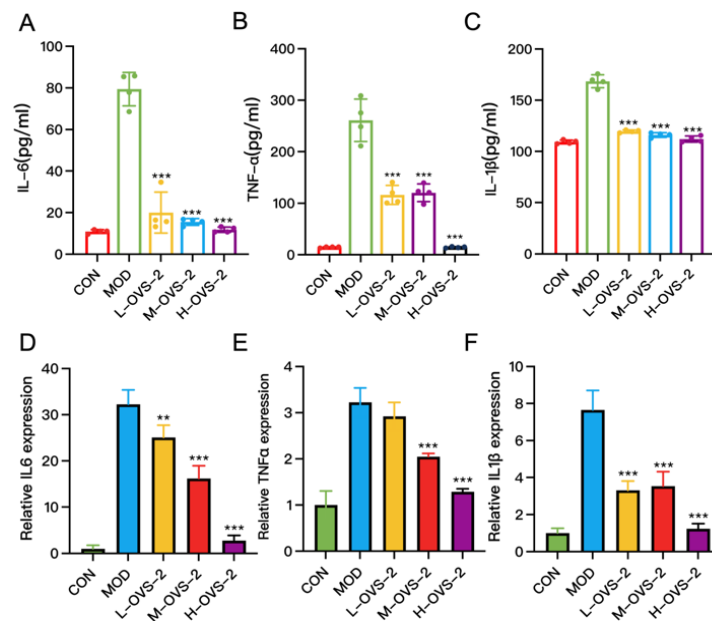


Figure 3. Anti-inflammatory Effects of OVS-2 in ABI Mice (B) Serum levels of inflammatory cytokines including IL-6, IL-1β, and TNF-α were determined by ELISA 3.5 days after ABI (n=4). Values are presented as means ± SDs (*P<0.05, **P<0.01 vs. MOD group). (C) mRNA expression levels of IL-6, IL-1β, and TNF-α in intestinal tissues were measured by RT-qPCR (n=4). (*P<0.05, **P<0.01, ***P<0.001 vs. MOD group)

3.4. Anti-Inflammatory Effects of OVS-2 on RIII Mice

Serum levels of inflammatory factors IL-6, IL-1β, and TNF-α were measured. As shown in Figure 21, the levels of IL-6, IL-1β, and TNF-α in the MOD group were significantly higher than those in the CON group. The levels of IL-6, IL-1β, and TNF-α in the L-OVS-2, M-OVS-2, and H-OVS-2 groups were significantly lower than those

in the MOD group, especially in the H-OVS-2 group (Figure 3A-C). mRNA levels in mouse intestinal tissues were also measured, and the results showed that the mRNA expression levels in the intestinal tissues of each group were consistent with the levels of inflammatory factors expressed in the serum, indicating that OVS-2 has strong anti-inflammatory activity, especially in the high-concentration OVS-2 group (Figure 3D-F). These results suggest that OVS-2 can reduce RIII by inhibiting the expression of cellular inflammatory cytokines.

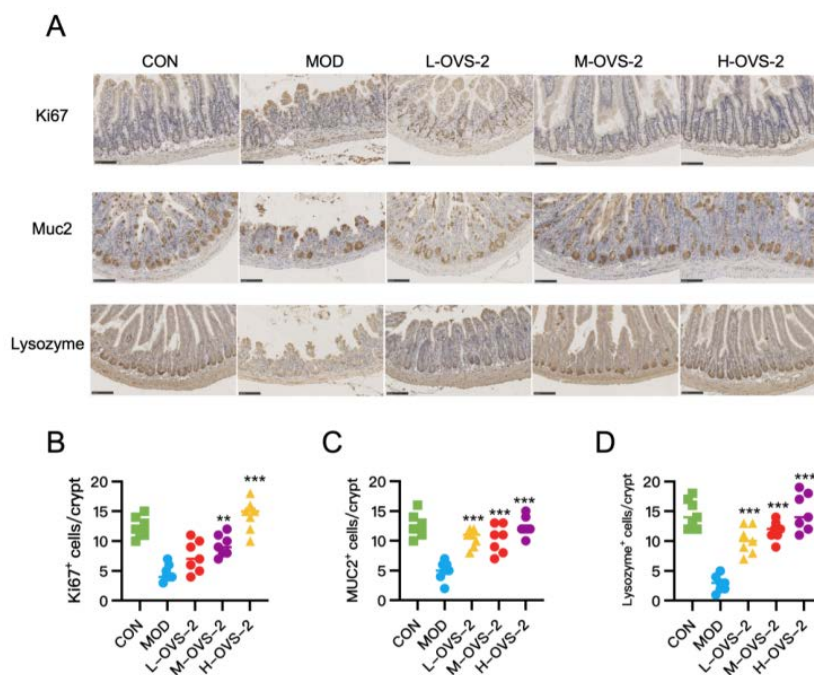


Figure 4. OVS-2 Improves Radiation-Induced Intestinal Barrier Damage (A) Immunohistochemical staining showing expression of Ki67⁺, Muc2⁺, and Lysozyme⁺ in cross-sections of small intestines. Histogram showing the number of Paneth cells expressing Lysozyme, Ki67⁺ cells in crypts, and surviving crypts, as well as the number of Muc2⁺ cells in crypts per week (n=7). (*P<0.05, **P<0.01, ***P<0.001 vs. MOD group)

3.5. OVS-2 Improves Radiation-Induced Intestinal Barrier Damage

Immunohistochemical staining was performed for small intestinal markers. The expressions of Ki67, Muc2, and lysozyme significantly decreased in the intestinal sections after irradiation (Figure 4A). Immunohistochemical staining showed a significant increase in the number of Ki67-positive cells in crypts treated with OVS-2 and the positivity of Paneth cell lysozyme and goblet cell Muc2 in the intestinal sections (Figure 4B–D). In conclusion, OVS-2 improve the function of small intestinal epithelial cells and promote mucosal barrier repair by improving the proliferative capacity of small intestinal epithelial cells.

3.6. Transcriptome Sequencing of Small Intestine Tissues Treated With OVS-2 is Mainly Enriched in Immune Pathways

Using data provided by the sequencing company, data on differentially expressed genes (DEGs) were obtained for each group, and conditional functions (if otherwise) were used to add significance to the expression of DEGs in each group. The criteria for judging significance were $\log_2(\text{foldchange}) > 1$ and $P < 0.05$ for significant upregulation, and $\log_2(\text{foldchange}) < -1$ and $P < 0.05$ for significant downregulation of gene expression. Differential gene selection and statistical analyses were performed for each group, and differential gene visualization was performed. Histograms were used to intuitively show the total number of differentially expressed genes between the groups, as presented in Figure 5A. The results showed that, compared with those of the MOD group, 802 upregulated DEGs and 979 downregulated DEGs were present in OVS-2 (Figure 5A). Differential gene clustering was used to determine the

variation of differential genes among the groups. Cluster analysis of genes based on the similarity of gene expression in each sample was used to visualize the expression of genes in different samples. Variation was small among the OVS-2 and MOD groups, but large differences in gene expression were determined between groups (Figure 5D). GO analysis summarizes and analyzes the relationships among genes, related gene functions, and biological processes from three aspects: cellular components, biological processes, and molecular functions. KEGG is a comprehensive database of genome, chemical, and system function information that classifies and counts the most likely signaling pathways and functions involved in DEGs. We used GO function enrichment analysis on the KEGG pathway enrichment analysis platform of the Cloud Atlas website to analyze the functional enrichment of DEGs. The results showed that all differentially expressed genes were enriched in immune responses, immune system processes, responses to external biological stimuli, immune receptor activity, and cytokine receptor activity. KEGG analysis revealed that the DEGs were mainly enriched in cytokine-cytokine receptor interactions, the intestinal immune network for IgA production, the NOD-like receptor signaling pathway, inflammatory bowel disease, and immune system processes. Furthermore, to verify the reliability of the transcriptome sequencing results, we conducted RT-qPCR validation of immune- and chemokine-related genes (Ccl3, Ccl5, CD5, CD74, Tnfsf13b, Bst2, Cxcl10, Isg15, Nampt, and Slc7a5) and compared them with the transcriptome sequencing results. RT-qPCR results (Figure 5E) showed that the upregulation or downregulation trends of the selected 10 genes were consistent with the sequencing results, indicating that the sequencing results were reliable (Figure 5E). These results suggest that OVS-2 can regulate the immune response and the chemokines signaling pathway to reduce RIII.

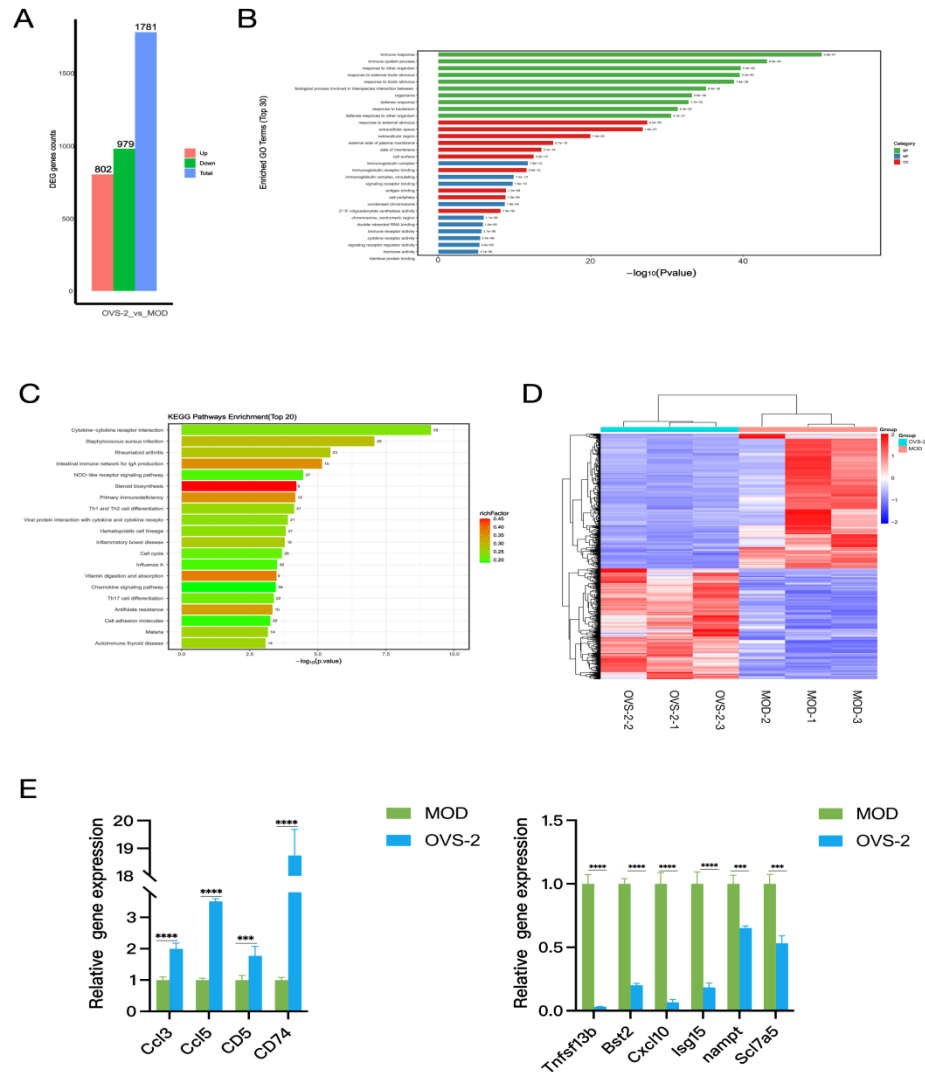


Figure 5. Transcriptome Sequencing of Small Intestine Tissues Treated With OVS-2 is Mainly Enriched in Immune Pathways (A) Histogram of differentially expressed genes, DEGs with $|\log_2FC| > 1$ and $Padj < 0.05$ were considered significantly differentially expressed genes. (B) Significant enrichment analysis of GO functional annotation. The abscissa represents the $-\log_{10}$ transformed P-value after processing, and the entries are sorted in ascending order of P-values. The three major categories of GO classification are represented by bars of different colors (green represents biological processes, blue represents molecular functions, and red represents cellular components), and the ordinate is the description information of specific functions. (C) Histogram of KEGG enrichment of differentially expressed genes. (D) Clustering diagram of differential genes. Each column represents a sample, and each row represents a gene. Red represents relatively highly expressed genes, and blue represents relatively lowly expressed genes. The dendrogram on the top represents the clustering of samples, and closer branches of two samples indicate closer expression patterns of all differential genes in the two samples. The dendrogram on the left represents the clustering of genes, and closer branches of two genes indicate closer expressions. (E) RT-qPCR validation of DEGs ($n=3$) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. MOD group)

4. Discussion

In this study, a new function for the active ingredient, OVS-2, in OV seeds was discovered. The present study unveils the protective effect of OVS-2, an active compound derived from OV seeds, against RIII. In an *in vitro* model, OVS-2 alleviated the oxidative stress induced by IR. Using an *in vivo* RIII mouse model, we evaluated the therapeutic effects of OVS-2 on RIII. The OV extract significantly improved histological damage, protected against intestinal mechanical barrier damage, and reduced the levels of inflammatory cytokines, and these effects may have been achieved through the modulation of immune pathways.

During radiotherapy, radiation can directly affect DNA,

membrane proteins, and other structures of intestinal epithelial cells, leading to lysis, apoptosis, and disruption of the mechanical barrier integrity of the intestine [14]. Furthermore, due to the high sensitivity and poor tolerance of intestinal stem cells to radiation, the process of proliferation and differentiation of intestinal stem cells into intestinal epithelial cells is hindered, resulting in a sharp decrease in the number of intestinal epithelial cells and further exacerbating damage to the intestinal mechanical barrier [15,16]. Radiation can also interact with water in tissue cells to generate ROS and other substances, inducing oxidative stress reactions that causes DNA strand breaks, inhibits cell proliferation, disrupts the integrity of intestinal epithelial cells, and promotes the expression of pro-apoptotic gene P53, activating pro-apoptotic pathways and inducing apoptosis [17,18]. By

modulating signaling pathways such as NF- κ B, mitogen-activated protein kinases (MAPK), and extracellular regulated protein kinases (ERK), radiation increases the expression levels of cytokines such as IL-6, IL-1 β , and TNF- α , activating a cascade effect of mucosal inflammation [19,20,21]. Our study confirms many of these points and explores the relationship between radiation and oxidative stress. After confirming the protective effect of OVS-2 against radiation, our research suggests that the active ingredients of OVS-2 can suppress radiation-induced oxidative stress by modulating levels of MDA, SOD, and ROS. *In vivo* experiments have shown that OVS-2, a novel alkaloid extract from OV seeds, can significantly improve histological damage, reduce levels of inflammatory cytokines IL-6, IL-1 β , and TNF- α , and regulate intestinal barrier function by increasing the expression levels of small intestinal biochemical markers Ki67, Muc2, and Lysozyme, possibly through modulation of immune pathways.

Analysis of its main components revealed that OVS-2 is rich in various alkaloid monomers. Among them, orychofragmuspine A-H has inhibitory effect on apoptosis and antioxidant effects. Furthermore, orychofragmuspine A, F, G, and H are included in the main components of OVS-2. Zhang et al. found that orychofragine D exhibited significant radioprotective activity on the survival rate of irradiated human umbilical vein endothelial cells (HUVEC). *In vivo* experiments showed that orychofragine D not only significantly improved the survival rate of irradiated mice within 30 days but also significantly promoted blood system recovery in irradiated mice [22]. Another study showed that orychofragmuspine I exhibited a protective effect against H₂O₂-induced oxidative damage in HepG2 cells [23]. The impact on RIII of OVS-2 may be attributed to these monomeric components.

This study provides a basis for investigating the efficacy and active components of OV seeds, laying the groundwork for future research directions and exploring the efficacy components of other parts of OV. Based on this work, it is necessary to continue: (1) systematically evaluating the biological effects of OVS-2 and further study its mechanisms of action; (2) further characterize the monomeric components of OVS-2 with radioprotective activity, laying the foundation for subsequent experiments.

5. Conclusion

Our study suggests that the active components of OV mainly consist of alkaloids, which exhibit antioxidative, anti-inflammatory, and intestinal mechanical barrier-protective effects. These findings highlight the potential of OV alkaloid extract as an oral therapeutic agent for RIII. Further research is warranted to fully elucidate its mechanisms of action and to advance its development as a promising drug for the treatment of RIII.

Statement of Competing Interests

The authors declare that they have no competing financial interests or personal relationships that may have

influenced the work reported in this study.

Abbreviations

ABI, abdominal irradiation; ANOVA, analysis of variance; BV/h, bed volume per hour; DEG, differentially expressed gene; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; CCK8, cell counting kit-8; FBS, fetal bovine serum; GO, Gene Ontology; HE, hematoxylin and eosin; IL, interleukin; IR, ionizing radiation; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS, liquid chromatography-mass spectrometry; MDA, malondialdehyde; OD, optical density; OV, *Orychophragmus violaceus*; OVS-2, *Orychophragmus violaceus* seed alkaloid extract; RIII, radiation-induced intestinal injury; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ROS, reactive oxygen species; SD, standard deviation; SOD, superoxide dismutase; TCM, traditional Chinese medicine; TEM, transmission electron microscopy; TNF, tumor necrosis factor; UPLC, ultra-high performance liquid chromatography.

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