Research article

Dicliptera chinensis-derived polysaccharide enhanced the growth activity of submandibular gland cells *in vitro* after radiotherapy

Lixiang Zhao [a,b,1], Yanchun Zhu [a,b,c,1], Lihua Zhang [d], Yude Huang [a,b], Yiyang Fan [a,b,e], Linjin Gao [a,b], Yanfei Zhao [a,b], Xian Wang [a,b], Dongqing Mo [a,b], Haoyu Lu [a,b], Daiyou Wang [a,b,*]

[a] *College & Hospital of Stomatology, Guangxi Medical University, NO. 10 Shuangyong Road, Nanning, Guangxi, 530021, China*
[b] *Guangxi Key Laboratory of Oral and Maxillofacial Rehabilitation and Reconstruction, Guangxi Clinical Research Center for Craniofacial Deformity, Guangxi Key Laboratory of Oral and Maxillofacial Surgery Deformity, Nanning, 530021, China*
[c] *Xiaolan People's Hospital, ZhongShan, 528415, China*
[d] *Liuzhou People's Hospital, Liuzhou, 545000, China*
[e] *Yichang City Hospital of Traditional Chinese Medicine, Yichang, 443000, China*

ARTICLE INFO

ABSTRACT

Objective: Radiotherapy for head and neck can damage the salivary gland cells, which can easily result in xerostomia. No effective treatment for radiation-induced salivary gland dysfunction currently exists. Thus, we aimed to study the protective effect of *Dicliptera chinensis* polysaccharides (DCP) on the prevention of submandibular gland (SMG) cell damage caused by radiotherapy in Sprague-Dawley rats.
Design: Mechanical enzyme digestion was used to extract primary rat SMG cells. A radiation injury model was established by treating these cells with a dose of 8 Gy, followed by intervention using different DCP concentrations. The cell counting kit 8 assay was used to determine the inhibition rate of SMG cells in each group. The rates of apoptosis and cell cycle progression were detected using flow cytometry. Expression of the Mre11/Rad50/Nbs1 complex (MRN) was detected using western blotting.
Results: DCP increased the proliferation of SMG cells after irradiation, and cell growth activity positively correlated with polysaccharide concentration. Flow cytometry analysis of SMG cell apoptosis revealed that DCP markedly reduced the total apoptosis rate after irradiation, especially the early apoptosis rate. Cell cycle results suggested that DCP reduced the number of cells in the S and G2 phases after irradiation and alleviated the S and G2 blocks. Western blot results indicated that the expression of Mre11, Rad50, and Nbs1 decreased in the radiation-injured group, whereas their expression increased after DCP treatment.
Conclusions: DCP can protect the rat SMG cells after radiation and be used as a protective agent against salivary gland cell damage caused by radiotherapy.

1. Introduction

Radiation therapy is one of the most commonly used standard treatments for malignant head and neck cancer [1]. It is commonly utilised in conjunction with both surgical and chemotherapy treatments. Approximately 60–80 % of patients with head and neck tumours require radiotherapy [2]. Radiotherapy has fewer side effects on the system outside the target but has substantial side effects on the neighbouring healthy tissues [3]. Almost all degrees of salivary gland function loss occur after radiotherapy in patients with head and neck cancer [4]. The related symptoms mainly include dry mouth, sputum, and radioactive osteomyelitis [5]. The quality of life of patients is profoundly influenced by these symptoms and the disease [6]. Therefore, promptly prevention and treatment of these complications is crucial.

No particularly effective treatment for radiation-induced salivary gland dysfunction currently exists; however, following clinical prevention and control measures have been applied: treatment by amifostine (an active oxygen scavenger) [7]; and, use of intensity-modulated radiation therapy (IMRT) [8]. Nonetheless, these methods have drawbacks [9]. Amifostine can scavenge oxidis free radicals and accelerate tissue repair [10], but can result in serious adverse side effects, including nausea, vomiting, anaphylaxis, and hypotension, leading to the need to discontinue treatment in up to 40 % of cases [11,12]. IMRT is a targeted method devised to avoid direct radiation to the parotid gland, while being beneficial in maintaining saliva flow rate. Patients may still experience symptoms of dry mouth, which may be due to altered saliva composition [13], and IMRT may not be feasible depending on the location of the tumour [14]. Therefore, finding new ways to solve radiation-induced salivary gland damage is imperative.

Double-strand break (DSB) is a form of DNA damage that can be produced during DNA replication, or when the cells are treated with physicochemical factors, such as ionising radiation and cisplatin. Mre11/Rad50/Nbs1 complex (MRN) is among the initial molecules to induce and bind to the cleavage site upon occurrence of DSB; it plays a pivotal role in DNA recombination [15].

With the advancement of biochemical technology, it has become evident that polysaccharides possess complex and multifaceted biological activities. These activities include lowering blood sugar levels, and blood pressure, enhancing immunity, and exhibiting anti-ageing, anti-tumour, and anti-radiation activities. Sun et al. [16] reported that polysaccharides from *Angelica sinensis* can protect lymphocytes from radiation and can be used for acute radiation protection. Chen et al. [17] demonstrated that black bean-derived polysaccharides have anti-radiation effects. Furthermore, animal experiments have shown that polysaccharides can stimulate haematopoietic stem cells and promote leukocyte-macrophage colony formation and produce blood-forming cells in the spinal cord.

Dicliptera chinensis is widely distributed in South China, Vietnam, India, Bangladesh and other countries, as food and traditional Chinese medicine [18]. *D.chinensis* exhibits various effects including heat clearance, detoxification, diuretic effects, and anti-radiation effects [19]. *D. chinensis* polysaccharides (DCP) is the component extracted from *D. chinensis*, which exhibits the abovementioned effects [20].

In this study, we aimed to investigate whether DCP could protect against submandibular gland (SMG) cell injury after irradiation and to elucidate the associated signalling pathways and mechanisms of action.

2. Materials and methods

2.1. Preparation

2.1.1. Preparation of DCP
D. chinensis was purchased from the Medicinal Botanical Garden of Guangxi University of Traditional Chinese Medicine. DCP was extracted and isolated using a water extract–ethanol precipitate and Sephadex column chromatography [21]. The phenol-concentrated sulfuric acid method was utilised to determine the concentration of DCP [22].

2.1.2. Preparation of SMG cells
The experimental protocol was approved by the Laboratory Animal Welfare and Ethics Committee, Guangxi Medical University, in accordance with the Guide for the Care and Use of Laboratory Animals. Three-day-old female Sprague-Dawley (SD) rats were purchased from the Experimental Animal Centre of Guangxi Medical University (production licence number: SCXK Gui 2014-0002, experimental animal use licence number: SYXK Gui 2014-0003). The SMGs of rats were isolated and primary cells were extracted by enzyme digestion using 0.125 % pancreatic enzymes. The cells were shaken into a uniform mixture and cultured in Dulbecco's modified Eagle medium containing high glucose (4.5 g/L), 10 % foetal bovine serum with 5 μg/mL insulin, 5 μg/mL transferrin, 0.01 μg/mL EGF, 0.1 μg/mL hydrocortisone, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5 % CO_2. Primary cultured SMG cells were combined with the differential digestion and adherence methods to obtain first-generation SMG cells [23]. To identify the SMG cells, immunocytochemistry was performed using α-Amylase (1:200, BIOSS, Beijing, China) and Pan cytokeratin (1:200, BIOSS). For this study, we utilised the second-generation SMG cells for detection.

2.1.3. Radiation
After referring to the radiation intensity of other previously reported experiments [24,25], SMG cell damage was induced by local radiation at a single dose of 8 Gy using an electronic linear accelerator with X-ray (Mevatron MD, Siemens Medical Laboratories Inc., Germany).

0

3. Results

3.1. SMG cells identification

After purification and passaging, SMG cells in the first and second generation were in good shape and exhibited strong proliferation and growth ability. However, upon reaching the third generation, the cells began to age and experienced a decline in their growth rate. Pan-CK (Fig. 1A) and α-amylase (Fig. 1B) immunohistochemical staining results were positive, and the cells were triangular or polygonal in shape, well stretched, and had a clear nuclear membrane and envelope.

3.2. Effect of radiation on the growth rate and viability of SMG cells

Preliminary experiments revealed that the cell viability of each irradiated group was the lowest at 96 h (Fig. 2A); therefore, this time was chosen to monitor the effect of DCP on the growth viability of SMG cells after radiation-induced damage. We observed that the cell growth inhibition rate was significantly lower after radiotherapy compared with that in cells in the Ctrl group. Furthermore, the cell viability following radiotherapy exhibited a positive correlation with the concentration of DCP (Table 1; Fig. 2B).

3.3. Apoptosis rate in SMG cells

Flow cytometry analysis revealed that the late apoptotic rate did not significantly increase in cells after radiotherapy compared with that in untreated cells; however, the early apoptotic rate significantly increased. Counterintuitively, the early apoptotic rate was significantly lower in DCP-treated radiotherapy cells than those in untreated cells (Fig. 3A). As shown in Fig. 3B, the overall apoptosis rate of DCP- treated radiotherapy cells was significantly lower than those cells in IR group.

3.4. DCP affects the cell cycle of SMG cells following irradiation

Flow cytometry analysis indicated that the G2 and S phases were prolonged, and the G1 phase was shortened in SMG cells in the IR group compared with that in the Ctrl group. The G2 phase was significantly shortened and the G1 phase was prolonged in DCP-treated cells compared with those treated with IR alone (P < 0.05, Fig. 4A&B).

3.5. DCP alters the expression of proteins in the MRN complex

The expression of MRN significantly decreased in SMG cells in the IR group, and the decrease in the Nbs1 level was more prominent compared with that in the Ctrl group. The expression of MRN increased to varying degrees in the SMG cells in the DCP- treated groups compared with that in the IR group (Fig. 5A-D, Supplementary File-1).

4. Discussion

Compared to the IR group, the absorbance values of all DCP groups exhibited a significant increase, indicating that DCP exerted a radioprotective effect on SMG cells. CCK-8 experiments consistently demonstrated that DCP effectively shielded SMG cells from ionising radiation-induced damage, resulting in reduced cell growth inhibition rate and enhanced cell survival rate. Furthermore, DCP restored cellular proliferation capacity.

Cell division genes and cell cycle checkpoints (G1/S, S/G2, and G2/M checkpoints) regulate and monitor cell cycle progression
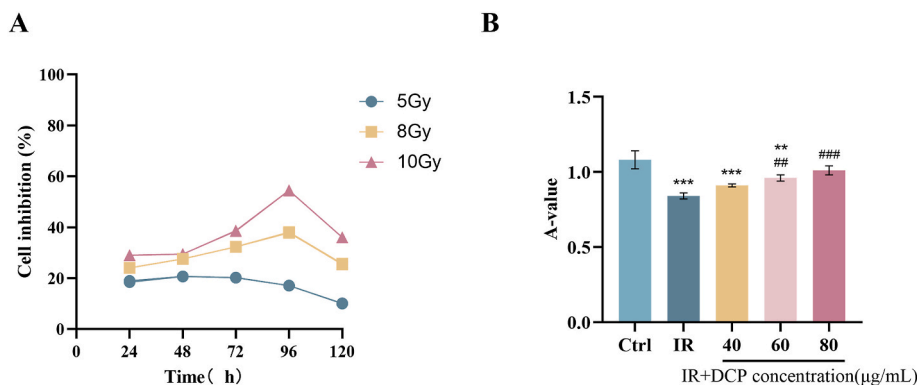
Fig. 2. A) Effect of different radiation doses on SMG cell inhibition rate is depicted here, B) SMG cell proliferation 96 h after irradiation, the result shown is the standard deviation average of three independent experiments. The significance was analysed using one-way analysis of variance. (**P < 0.01, ***P < 0.001 VS. Control; ##P < 0.01, ###P < 0.001 VS. IR). SMG, submandibular gland.

Table 1

Growth inhibition rates of SMG cells in each group.

Group	Radiation dose (Gy)	Drug dose (µg/mL)	A-value	Growth inhibition rate (%)
Ctrl	0	0	1.08 ± 0.06	0
IR	8	0	0.84 ± 0.02#	24.58
DCPs-L	8	40	0.91 ± 0.01#★	17.34
DCPs-M	8	60	0.96 ± 0.02#★	11.32
DCPs-H	8	80	1.01 ± 0.03#★	6.86

#P < 0.05, compared with the Ctrl group; ★P < 0.05, compared with the IR group.

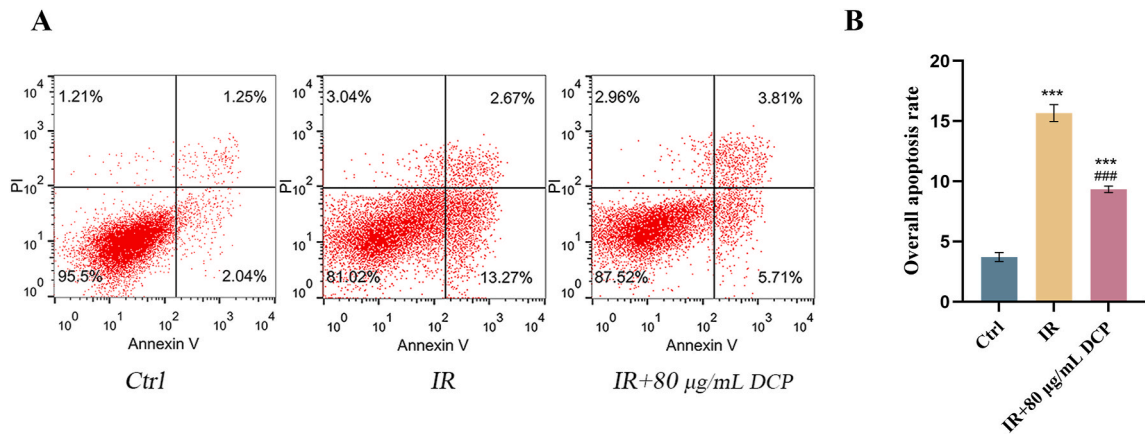(x ± s; n = 5). Ctrl, control; DCP, *Dicliptera chinensis* polysaccharides; H, high; IR, irradiation; L, low; M, medium; SMG, submandibular gland.


Fig. 3. A) Effect of *Dicliptera chinensis* polysaccharides on apoptosis of SMG cells after Annexin V-PI double staining. B) Overall apoptosis rate. The result shown is the standard deviation average of three independent experiments. The significance was analysed using one-way analysis of variance. (***P < 0.001 VS. Control; ###P < 0.001 VS. IR). SMG, submandibular gland.
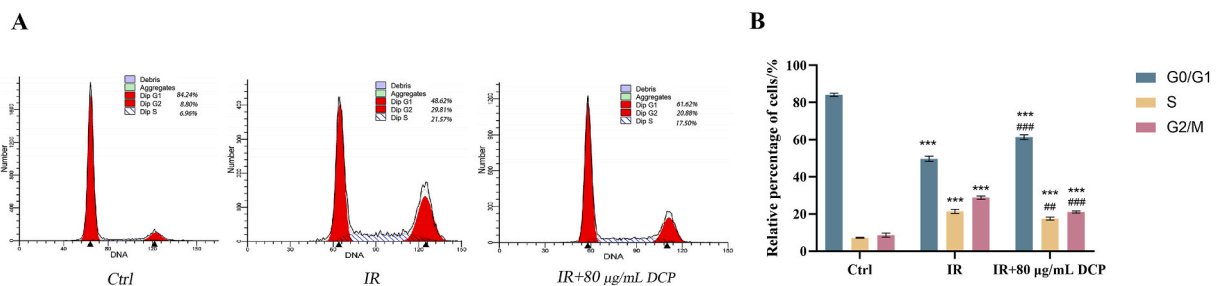
Fig. 4. A) Cell cycle changes of three groups of SMG cells. B) Relative percentage of cells. The result shown is the standard deviation average of three independent experiments. The significance was analysed using one-way analysis of variance. (***P < 0.001 VS. Control; ##P < 0.01, ###P < 0.001 VS. IR). SMG, submandibular gland.

[26]. The damage of cells in each cycle without complete repair at the G2 phase activates the G2/M checkpoint to block the initiation of mitosis [27]. A long-term evolutionary mechanism has advanced to maintain the integrity of the genome following radiation-induced DNA damage and other types of damage [28]. DNA damage activates certain factors, thereby arresting the cell cycle to facilitate DNA repair [29]. However, unrepaired damaged DNA activates apoptosis-related genes, thereby leading to apoptosis. Radiation doses higher than 2 Gy may lead to G2 phase arrest in mouse skin foetal fibroblasts [30]. Our findings suggested that the G2 and S phases of the cell proliferation cycle were prolonged, and the apoptosis rate significantly increased in the experimental-, irradiated, and DCP groups compared with that in the control group. The total apoptosis rate in the DCP group was significantly lower than that of the irradiated group. These results suggest that DCP reduces radiation-induced DNA damage in SMG cells, and the cells may enter the next cell cycle through the S and G2/M checkpoints. Collectively, we demonstrated that DCP protects SMG cells against radiation by reducing the number of apoptotic cells and attenuating the arrest of SMG cells in the S and G2 phases after irradiation.

In this study, the expression of each subunit of the MRN complex positively correlated with the radiation dose within a certain dose range. The ability to repair cell damage is reduced when this range is exceeded; this causes metabolic disorders or metabolic arrest, and its expression may decrease [15]. Our results revealed that the expression of MRN was significantly lower in the IR group than that in
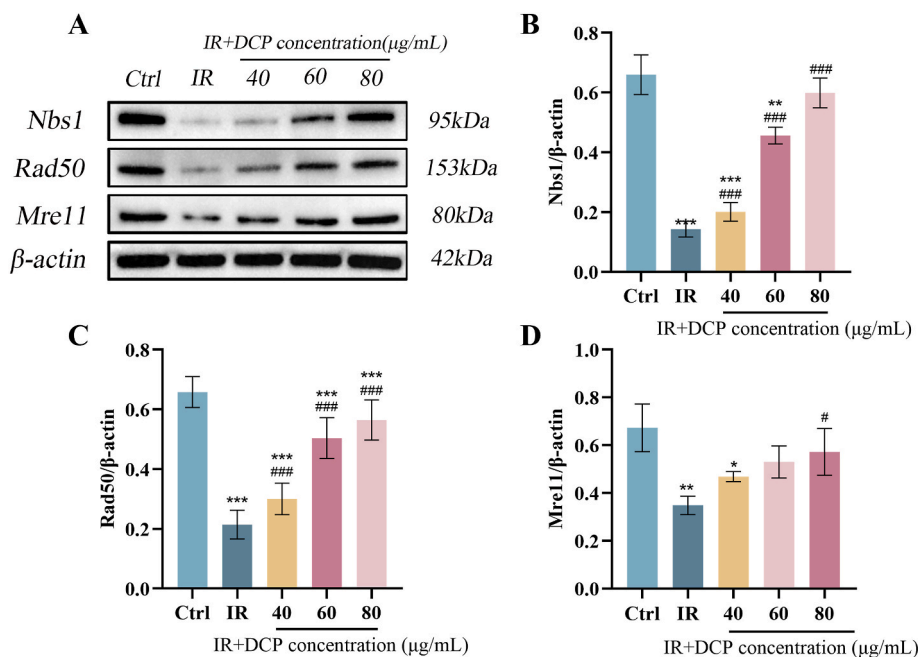
Fig. 5. A) Results of western blotting analysis of Mre11, Rad50, and Nbs1 proteins in SMG cells of all groups. B-D) Statistical analysis of Fig. 5A. The result shown is the standard deviation average of three independent experiments. The significance was analysed using one-way analysis of variance. (*P < 0.05, **P < 0.01, ***P < 0.001 VS. Control; #P < 0.05, ##P < 0.001 VS. IR). SMG, submandibular gland.

the Control group (P < 0.05). We hypothesised that the expression of the MRN complex subunits decreased when SMG cells were exposed to radiation doses >8 Gy because it caused irreversible damage to some cells. Therefore, the expression of MRN in the IR group decreased to different degrees compared with that in the control group. The expression of the three subunits of the MRN complex increased at higher DCP concentrations and promoted the repair of DSB by regulating the MRN complex compared with that in the IR group.

We conclude that the use of DCP to pre-treat SMG cells improves apoptosis caused by the loss of the MRN complex following ionising radiation. Furthermore, activation of the MRN complex results in the synthesis of Nbs1, MRE11, and RD50 proteins to bind to and repair the damaged DNA. The S and G2/M phase checkpoints are activated to restore the cell cycle after the synthesis of the MRN complex.

This study has some limitations. This study only verified that DCP promote the repair of SMG cell damage by radiation using in vitro experiments. Future experiments should explore the repair ability of DCP on salivary gland damaged by radiation through in vivo experiments.

5. Conclusion

In summary, DCP may reduce radiation-induced SMG cell injury by alleviating S and G2 phase arrest to reduce apoptosis and regulate MRN complex expression to promote the repair of cell proliferation. We demonstrated that DCP has a pivotal role in promoting cell damage repair caused by ionising radiation. A follow-up study is required to explore the optimal dose, onset time, and mechanism of DCP drugs and determine the potential value of DCP as an anti-radiation drug.

Funding

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Lixiang Zhao: Writing – original draft, Data curation. **Yanchun Zhu:** Software, Conceptualization. **Lihua Zhang:** Visualization, Investigation. **Yude Huang:** Supervision. **Yiyang Fan:** Validation, Software. **Linjin Gao:** Methodology. **Yanfei Zhao:** Validation,

Software. **Xian Wang:** Supervision. **Dongqing Mo:** Writing – review & editing. **Haoyu Lu:** Methodology. **Daiyou Wang:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31005.

References

[1] J.J. Caudell, et al., The future of personalised radiotherapy for head and neck cancer, Lancet Oncol. 18 (5) (2017) e266–e273.
[2] F. Siddiqui, B. Movsas, Management of radiation toxicity in head and neck cancers, Semin. Radiat. Oncol. 27 (4) (2017) 340–349.
[3] L. Barazzuol, R.P. Coppes, P. van Luijk, Prevention and treatment of radiotherapy-induced side effects, Mol. Oncol. 14 (7) (2020) 1538–1554.
[4] I. Brook, Early side effects of radiation treatment for head and neck cancer, Cancer Radiother. : Journal de La Societe Francaise de Radiotherapie Oncologique 25 (5) (2021) 507–513.
[5] A. Vissink, et al., Clinical management of salivary gland hypofunction and xerostomia in head-and-neck cancer patients: successes and barriers, Int. J. Radiat. Oncol. Biol. Phys. 78 (4) (2010) 983–991.
[6] P. Strojan, et al., Treatment of late sequelae after radiotherapy for head and neck cancer, Cancer Treat Rev. 59 (2017) 79–92.
[7] J.S. Choi, et al., Radioprotective effect of epigallocatechin-3-gallate on salivary gland dysfunction after radioiodine ablation in a murine model, Clinical and Experimental Otorhinolaryngology 9 (3) (2016) 244–251.
[8] A. Vallard, et al., Intensity-modulated radiotherapy or volumetric-modulated arc therapy in patients with head and neck cancer: focus on salivary glands dosimetry, Head and Neck-Journal for the Sciences and Specialties of the Head and Neck 38 (7) (2016) 1028–1034.
[9] A. Eisbruch, Reducing xerostomia by IMRT: what may, and may not, be achieved, J. Clin. Oncol. : Official Journal of the American Society of Clinical Oncology 25 (31) (2007) 4863–4864.
[10] M.I. Koukourakis, V. Danielidis, Preventing radiation induced xerostomia, Cancer Treat Rev. 31 (7) (2005) 546–554.
[11] D. Rades, et al., Serious adverse effects of amifostine during radiotherapy in head and neck cancer patients, Radiother. Oncol. : Journal of the European Society For Therapeutic Radiology and Oncology 70 (3) (2004) 261–264.
[12] E. Obrador, et al., Radioprotection and radiomitigation: from the bench to clinical practice, Biomedicines 8 (11) (2020).
[13] T. Dijkema, et al., MUC5B levels in submandibular gland saliva of patients treated with radiotherapy for head-and-neck cancer: a pilot study, Radiat. Oncol. 7 (2012) 91.
[14] X. Wang, A. Eisbruch, IMRT for head and neck cancer: reducing xerostomia and dysphagia, J. Radiat. Res. 57 (Suppl 1) (2016) i69–i75. Suppl 1.
[15] Anuranjani, M. Bala, Concerted action of Nrf2-ARE pathway, MRN complex, HMGB1 and inflammatory cytokines - implication in modification of radiation damage, Redox Biol. 2 (2014) 832–846.
[16] Y. Sun, et al., Water-soluble polysaccharides from Angelica sinensis (Oliv.) diels: preparation, characterization and bioactivity, Int. J. Biol. Macromol. 36 (5) (2005) 283–289.
[17] H.-F. Liao, Y.-J. Chen, Y.-C. Yang, A novel polysaccharide of black soybean promotes myelopoiesis and reconstitutes bone marrow after 5-flurouracil- and irradiation-induced myelosuppression, Life Sci. 77 (4) (2005) 400–413.
[18] F.R.P.S.E. Committee, Flora Republicae Popularis Sinicae, Science Press, Beijing, 2002.
[19] J.C.o.N. Medicine, Dictionary of Chinese Materia Medica, Shanghai People's Republic of China, 1977.
[20] Y. Xu, et al., Isolation, characterization and bioactivities of the polysaccharides from Dicliptera chinensis (L.) Juss, Int. J. Biol. Macromol. 101 (2017) 603–611.
[21] Q. Xu, et al., Study on the protective effect and mechanism of Dicliptera chinensis (L.) Juss (Acanthaceae) polysaccharide on immune liver injury induced by LPS, Biomed. Pharmacother. 134 (2021).
[22] X.-Q. Zhou, et al., Efficacy of gecko polysaccharide on suppressed immune response induced by cyclophosphamide in mice, Journal of Traditional Chinese Medicine = Chung I Tsa Chih Ying Wen Pan 41 (4) (2021) 539–545.
[23] L.Q. Chen Long, Binling Yi, Zhe Sun, Daiyou Wang, Effects of silencing single-stranded DNA-binding protein 1 gene on proliferation and DNA repair of submandibular gland cells after irradiation, Chin J Radiol Med Prot 37 (9) (2017) 645–650, in Chinese.
[24] V. Mohan, et al., Dose response modelling of secretory cell loss in salivary glands using PSMA PET, Radiother. Oncol. 177 (2022) 164–171.
[25] S.Q. Li, et al., Inorganic nitrate alleviates irradiation-induced salivary gland damage by inhibiting pyroptosis, Free Radic. Biol. Med. 175 (2021) 130–140.
[26] A.M. Abreu Velez, M.S. Howard, Tumor-suppressor genes, cell cycle regulatory checkpoints, and the skin, N. Am. J. Med. Sci. 7 (5) (2015) 176–188.
[27] J. Neumann, et al., Mangrove dolabrane-type of diterpenes tagalsins suppresses tumor growth via ROS-mediated apoptosis and ATM/ATR-Chk1/Chk2-regulated cell cycle arrest, Int. J. Cancer 137 (11) (2015) 2739–2748.
[28] E.A. Prokhorova, et al., The DNA-damage response and nuclear events as regulators of nonapoptotic forms of cell death, Oncogene 39 (1) (2020).
[29] A. Campos, A. Clemente-Blanco, Cell cycle and DNA repair regulation in the damage response: protein phosphatases take over the reins, Int. J. Mol. Sci. 21 (2) (2020).
[30] M. Beck, et al., X-irradiation induces cell death in fetal fibroblasts, Int. J. Mol. Med. 30 (1) (2012) 114–118.