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Research article

Mechanism of Bazi Bushen capsule in delaying the senescence of mesenchymal stem cells based on network pharmacology and experimental validation

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ABSTRACT

Ageing is becoming an increasingly serious problem; therefore, there is an urgent need to find safe and effective anti-ageing drugs.

Aims: To investigate the effects of Bazi Bushen capsule (BZBS) on the senescence of mesenchymal stem cells (MSCs) and explore its mechanism of action.

Methods: Network pharmacology was used to predict the targets of BZBS in delaying senescence in MSCs. For *in vitro* studies, MSCs were treated with D-gal, BZBS, and NMN, and cell viability, cell senescence, stemness-related genes, and cell cycle were studied using cell counting kit-8 (CCK-8) assay, SA-β-galactosidase (SA-β-gal) staining, Quantitative Real-Time PCR (qPCR) and flow cytometry (FCM), respectively. Alkaline phosphatase (ALP), alizarin red, and oil red staining were used to determine the osteogenic and lipid differentiation abilities of MSCs. Finally, the expression of senescence-related genes and cyclin-related factors was detected by qPCR and western blotting.

Results: Network pharmacological analysis suggested that BZBS delayed cell senescence by interfering in the cell cycle. Our *in vitro* studies suggested that BZBS could significantly increase cell viability ($P < 0.01$), decrease the quantity of β-galactosidase^{+} cells ($P < 0.01$), downregulate p16 and p21 ($P < 0.05$, $P < 0.01$), improve adipogenic and osteogenic differentiation, and upregulate *Nanog*, *OCT4* and *SOX2* genes ($P < 0.05$, $P < 0.01$) in senescent MSCs. Moreover, BZBS significantly reduced the proportion of senescent MSCs in the G_0/G_1 phase ($P < 0.01$) and enhanced the expression of CDK4, Cyclin D1, and E2F1 ($P < 0.05$, $P < 0.01$, respectively). Upon treatment with HY-50767A, a CDK4 inhibitor, the upregulation of E2F1 was no longer observed in the BZBS group.

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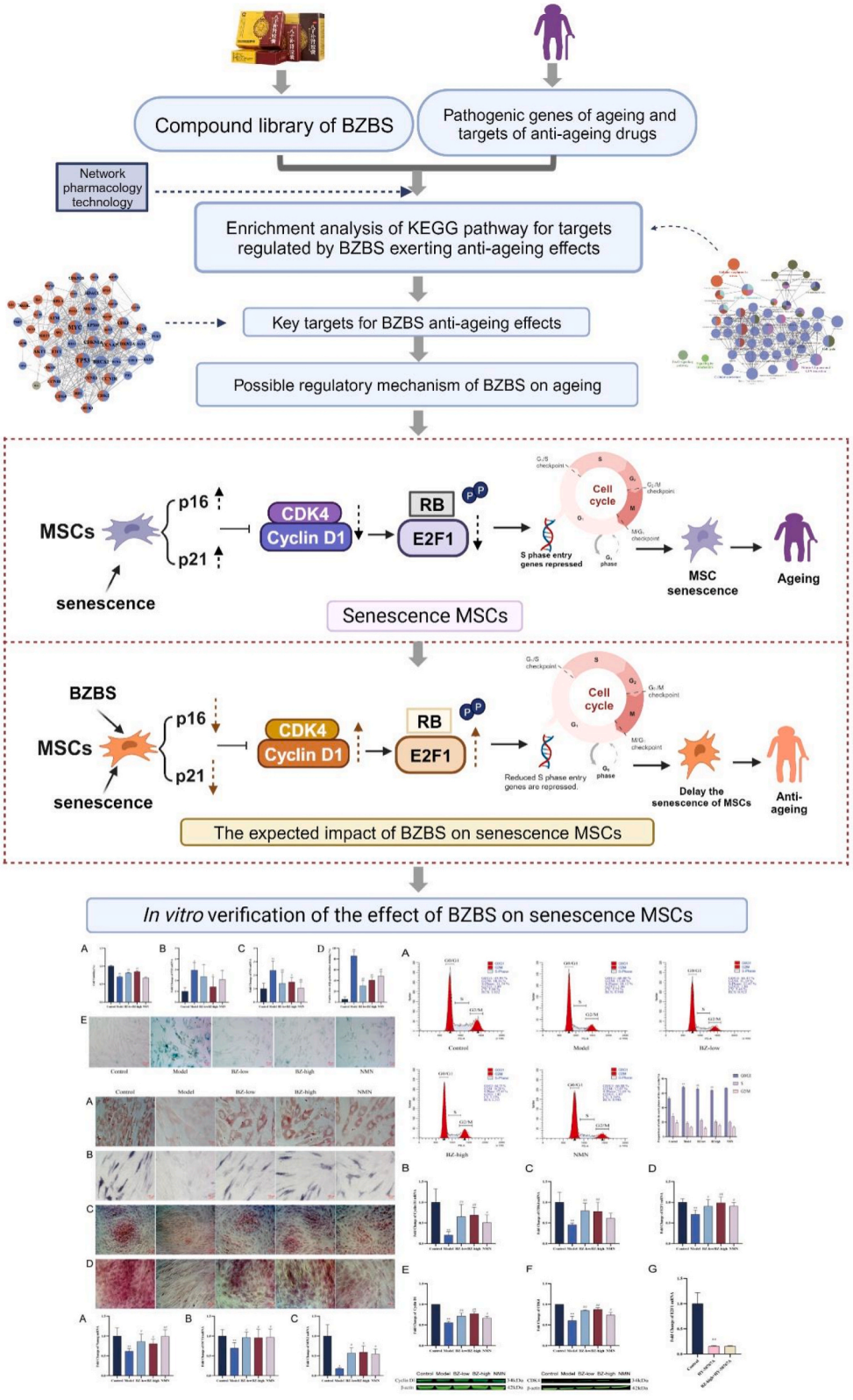

Fig. 1. Graphical abstract of anti-ageing validation of BZBS based on network pharmacology and *in vitro* experiments (Created with BioRender.com).

Conclusions: BZBS can protect MSCs against D-gal-induced senescence, which may be associated with cell cycle regulation via the Cyclin D1/CDK4/E2F1 signalling pathway.

1. Introduction

In recent years, with the increase in human life expectancy, the world's population has gradually aged, resulting in serious social, health, and economic problems [1]. Ageing is a gradual process that is part of the life cycle of every organism [2]. This is a natural, evolutionarily programmed phenomenon characterised by degenerative events such as tissue degeneration, telomere shortening, dementia, cognitive deficits, functional impairment, and chronic diseases [3]. Experts have proposed 12 markers of ageing, including cellular senescence, stem cell exhaustion, and genomic instability [4]. Senescence and exhaustion of stem cells are the core mechanisms of ageing [5]. An increasing number of studies have shown that delaying the senescence of stem cells can help to effectively resist overall ageing and prolong life span [6]. Therefore, delaying stem cell senescence may improve phenotypes associated with ageing. MSCs are primary pluripotent cells isolated from various tissues [7]. It plays crucial roles in haematopoiesis, immune regulation, and tissue repair [8]. The decreased ability of adult stem cell populations to proliferate and regenerate is one of the main causes of the human ageing process [5]. Current anti-ageing drugs exhibit potent anti-ageing effects; however, some drawbacks remain regarding their safety and long-term use [9–11]. Therefore, it is necessary to find anti-ageing drugs that are not only safe and effective for longer usage periods.

The basic theory of traditional Chinese medicine (TCM) believes that the deficiency of '*kidney essence*' is the fundamental cause of ageing [12]. BZBS, as a representative Chinese patent medicine, consists of 16 herbs [13,14]; it contains *Cuscuta chinensis* Lam. (Tu-Si-Zi), *Lycium harharum* L. (Gou-Qi-Zi), *Schisandra chinensis* (Turcz.) Baill (Wu-Wei-Zi), *Cnidium monnieri* (L.). Cusson (She--Chuang-Zi), *Rosa Laevigata* Michx. (Jin-Ying-Zi), *Rubus chingii* Hu (Fu-Pen-Zi), *Allium tuberosum* Rottler ex Spreng. (Jiu-Cai-Zi), *Toosendan fructus* (Chuan-Lian-Zi), *Epimedium brevicornu* Maxim. (Yin-Yang-Huo), *Morindae officinalis* radix (Ba-Ji-Tian), *Cistanche deserticola* Ma (Rou-Cong-Rong), Rehmannia root (Di-Huang), *Cyathula officinalis* K. C. Kuan (Chuan-Niu-Xi), *Panax ginseng* C. A. Mey. (Ren-Shen), *Cervus nippon* Temminck (Lu-Rong), and *Hippocampal Kelloggi* (Hai-Ma). Prescription tonic of '*kidney essence'* balances '*Yin and Yang*' and enhances archaeus, making the body healthy and full of spirit. Previous studies have shown that BZBS has anti-ageing effects [14–19], such as inhibiting premature senescence in mice, slowing methylation, and maintaining telomere length. Furthermore, it is believed that the '*kidney essence*' is closely related to stem cells. However, the mechanism by which BZBS effectively alleviates senescence in MSCs remains unclear.

Recently, network pharmacology has been widely accepted as an efficient research strategy to explore TCM from the perspective of biological network balance [20,21]. In recent years, great progress has been made in the application of network pharmacology methods to study the scientific connotation of TCM, such as the identification of new targets, biological processes, and signalling

Table 1
Abbreviation list.

Full name	Abbreviation
Mesenchymal stem cells	MSCs
Bazi Bushen capsule	BZBS
Nicotinamide mononucleotide	NMN
D-galactose	D-gal
SA-β-galactosidase	SA-β-gal
Alkaline phosphatase	ALP
Traditional Chinese medicines	TCM
Disease Gene Network	DisGeNET
Comparative Toxicogenomics Database	CTD
Therapeutic Target Database	TTD
Universal Protein	UniProt
Protein-protein Interaction Network	PPI network
Kyoto Encyclopedia of Genes and Genomes	KEGG
Flow Cytometry	FCM
Quantitative Real-Time PCR	qPCR
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH
Polyvinylidene fluoride	PVDF
Radio-Immunoprecipitation Assay	RIPA
Phosphate buffer saline	PBS
Analysis of variance	ANOVA
Cell Counting Kit-8	CCK-8
Least significant difference	LSD
Senescence-Associated Secretory Phenotype	SASP
Cyclin-dependent kinase inhibitor 1A	p21
Cyclin-dependent kinase inhibitor 2A	p16
Cyclin-dependent kinase 4	CDK4
E2F transcription factor 1	E2F1

pathways, the discovery of potential active compounds, and elucidation of the mechanism of action [22–27]. Furthermore, previous studies using network pharmacology predict that BZBS can alleviate the cognitive impairment caused by ageing [14,15]. Therefore, it is possible to discover the core mechanisms of approved Chinese medicines through network pharmacology. In this study, we hypothesised that BZBS exerts anti-ageing effects on MSCs. This study is the first to investigate the effects and mechanism of action of BZBS on MSCs senescence using network pharmacology and *in vitro* cytology (Fig. 1). The abbreviations in this article can be found in Table 1.

2. Materials and methods

2.1. Network pharmacology

Using 'cellular ageing' or 'cell senescence' as search terms, ageing-related genes were retrieved from six sources: DisGeNET [28], Open Target Platform [29], MalaCards [30], CTD [31], GeneCards, and text mining [32]. To ensure data reliability, only genes that appeared in more than three databases were retained as the core gene set for ageing. A comprehensive target spectrum of BZBS is essential to study its substantive basis and mechanism of action in the treatment of ageing. We collected targets from DrugBank [33], TTD [34], ChEMBL [35], the CTD database (https://ctdbase.org/), and PubChem, and standardised their names using UniProt [36–38]. Gene sets related to ageing and potential targets of BZBS were analysed to identify the functional targets of BZBS for preventing and treating ageing. The target data were then submitted to STRING (version 12.0; https://string-db.org/) for constructing the PPI network (confidence 0.7) [39]. The PPI network was visualised using Cytoscape v3.9.0 [40]. To explain the mechanisms of action of BZBS against ageing from a systematic perspective, we performed KEGG pathway enrichment analyses using Metascape (https://metascape.org) and the ClueGO plugin in Cytoscape [41].

2.2. Preparation of BZBS and its compounds

The BZBS stock solution was prepared in DMEM/F12 and diluted to the desired concentration with DMEM/F12 before the experiment.

2.3. Cell culture and treatment

Human umbilical cord stem cells were purchased from Beijing Jing-Meng Cell Biotechnology Co. Ltd. (Cat # UC1139). MSCs were cultured in mesenchymal stem cell medium (Cat # MSC1201B, Cat # MSC1201S; Beijing Jing-Meng Cell Biotechnology Co., Ltd.) supplemented with 1‰ streptomycin, penicillin, and gentamicin, at 37 °C in a 5% carbon dioxide incubator. The media was changed every two days. The methods for isolation, cultivation, and characterisation of MSCs are described in detail by Fathi et al. [42].

Cells in the logarithmic growth phase cells were seeded on plates at a density of 7×10^4–1×10^5/mL and cultured for 24 h before treatment. Three or more accessory wells were set up for each independent experiment for each group to ensure data reliability. D-Gal can significantly induce senescence in MSCs [43]. The use of D-gal to accelerate animal ageing has gradually been recognised as an effective model for studying the mechanisms of ageing [44]. Previous studies have demonstrated that D-gal can be used to model rapid cell senescence *in vitro* [45–47]. In this study, D-gal-induced MSCs were used to establish a rapid ageing model. MSCs were divided into Normal group (Control), Model group (D-galactose 20 mg/mL, 72h) (Model), Low dose Bazi Bushen capsule group (D-galactose 20 mg/mL + BZBS 10 μg/mL, 72h) (BZ-low), High dose Bazi Bushen capsule group (D-galactose 20 mg/mL + BZBS 20 μg/mL, 72h) (BZ-high), and NMN group (D-galactose 20 mg/mL+20 μM, 72h) (NMN). After adding CDK4 inhibitor (MCE, Cat #HY-50767A, USA), the group was divided into Normal group (Control), HY-50767A group (D-galactose 20 mg/mL + CDK4 inhibitors 1 μM, 72h), and BZ-high + HY-50767A group (D-galactose 20 mg/mL + BZBS 20 μg/mL + CDK4 inhibitors 1 μM, 72h).

2.4. Cell viability assay

Cell viability was evaluated using CCK-8 (MCE, Cat # HY-K0301, USA) [48]. 10 μL of CCK-8 solution was added to each well and incubated in a 37 °C incubator for 1–4 h. The absorbance was measured at 450 nm using a multifunctional microplate reader to calculate cell viability.

2.5. SA-β-gal staining

SA-β-gal staining [49] was performed using the SA-β-gal staining kit (Beyotime, Cat #C0602, China). The cells were then washed with PBS and incubated in a fixative solution for 15 min at room temperature. Then, the cells were washed with PBS and incubated in SA-β-gal staining solution at 37 °C overnight without CO_2. Images were captured using an inverted microscope (Axio Vert.A1, Carl Zeiss, Germany), and positive cells were quantified from four fields in each well.

2.6. Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from MSCs using the Eastep® Super Total RNA Extraction Kit (Promega, Cat # LS1040, China), and reverse transcription of the RNA sample to cDNA was carried out using Prime Script reagent Kit (Takara, Cat # RR047A, Japan). qPCR was

performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Cat # RR820A, Japan). Primers for each target mRNA were designed and are listed in Table 2. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of target genes, and GAPDH was used as an internal control [50].

2.7. Adipogenic and osteogenic differentiation

2.7.1. Oil red O staining

Oil Red O staining was performed to analyse adipogenesis [51]. After 72 h of D-gal and BZBS treatment, the ADP1/ADP2 adipogenic induction medium (Pricella, Cat # PD-019, China) was exchanged for adipogenic culture (ADP1 for 3 days, ADP2 for 1 d) and Oil Red O staining was performed after 14 days. The cells were fixed with 4% neutral formaldehyde solution at room temperature for 30 min, stained at room temperature for 30 min, and washed with PBS to remove the floating colour. Oil red staining was visualised using an inverted microscope.

2.7.2. ALP staining and alizarin red staining

Osteogenic differentiation culture was performed after 72 h of D-gal and BZBS intervention, and the cells were cultured in osteogenic induction differentiation medium (Pricella, Cat # PD-017, China) for 7 days. A BCIP/NBT Alkaline Phosphatase Colour Development Kit (Beyotime, Cat #C3206, China) was used for staining. The cells were incubated at room temperature for 5–30 min or longer (up to 24 h) and washed with PBS to terminate the colour reaction. Osteogenic differentiation was cultured for 21 days, and alizarin red was used for staining. The cells were fixed with a 4% neutral formaldehyde solution at room temperature for 30 min, stained at room temperature for 30 min, and washed with PBS to remove the floating colour. ALP and Alizarin Red staining were visualised using a microscope [52].

2.8. Cell cycle assay

The cell cycle was measured using FCM [53]. A Cell Cycle Staining Kit (MULTISCIENCES, Cat # CCS012, China) was used to analyse the cell cycle distribution. Cells were collected and fixed with 70% ethanol at 4 °C overnight. The cells were then washed with PBS and incubated in propidium iodide (PI)/RNase A staining solution at room temperature in the dark for 30 min. The cell cycle distribution was detected using a flow cytometer (BD FACS Aria III flow cytometer).

2.9. Western blot

MSCs treated under different conditions were collected and lysed with RIPA buffer containing protease inhibitors. After centrifugation (13,000 rpm, 30 min) at 4 °C, total protein concentrations of the supernatant were quantified by BCA protein assay kit (SEVEN, Cat # SW101-02, China). A total of 50 or 100 μg of denatured protein samples were separated by 4–20% SDS-PAGE gel and then transferred onto a PVDF membrane. After being blocked with blocking buffer (LI-COR, Cat # 927–70001, USA), the PVDF membrane was incubated with appropriate primary antibodies [CyclinD1 (1:1000), CDK4 (1:1000)] overnight at 4 °C. The membranes were washed three times and incubated with appropriate secondary antibodies (1:5000) at 37 °C for 1 h. Finally, immuno-positive bands were visualised and quantified with Odyssey-imaging systems (Nebraska, USA) and normalised with the corresponding β-actin (1:5000) as the internal control. The primary antibodies used for MSCs were anti-CDK4 (Abcam, Cat # ab108357, Britain), anti-Cyclin D1 (Abcam, Cat # ab134175, Britain), and anti-β-actin (Abcam, Cat # ab8227, Britain). The operational method can also be found in detail elsewhere [53].

Table 2
List of qPCR primer sequences.

Primer names	Primer sequences
p16	F: GGGTCGGGTAGAGGAGGTG
	R: GCTGCCCATCATCATGACCT
p21	F: GTCCTTGGGCTGCCTGTTTT
	R: GTGGGAAGGTAGAGCTTGGG
OCT4	F: CCTTCGCAAGCCCTCATTTC
	R: TAGCCAGCTCCGAGGATCAA
Nanog	F: GAATGAAATCTAAGAGGTGGCA
	R: CCTGGTGGTAGGAAGAGTAAAGG
SOX2	F: AGAACCCCAAGATGCACAAC
	R: GGGCAGCGTGTACTTATCCT
CDK4	F: GAGGCGACTGGAGGCTTTT
	R: GGATGTGGCACAGACGTCC
Cyclin D1	F: GGAGAACAAACAGATCATCC
	R: GAATGAAGCTTTCCCTTCTG
E2F1	F: CGCCATCCAGGAAAAGGTGT
	R: GATGCCCTCAAGGACGTTGG
GAPDH	F: AGAAGGCTGGGGCTCATTTG
	R: AGGGGCCATCCACAGTCTTC

2.10. Statistics

All statistical analyses were performed using IBM SPSS 26.0. Data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test for equality of variance). One-way analysis of variance (ANOVA) was used, and the least significant difference (LSD) method was used for pairwise comparisons between groups. The Kruskal-Wallis test was used for non-normally distributed data analysis. $P < 0.05$ was considered statistically significant. All graphs were generated using GraphPad Prism (V.8.01). Experimental data were expressed as mean ± standard deviation ($\overline{X} \pm S$).

3. Results

3.1. Network pharmacology analysis

Based on the pathogenic genes reported in the ageing literature and the therapeutic targets of approved drugs, a PPI molecular network of ageing-specific pathogenesis was constructed, and the underlying mechanism was explored. Notably, cellular senescence, cell cycle, p53 signalling pathway, and Senescence-Associated Secretory Phenotype (SASP) are potential key signalling pathways regulated by BZBS that exert ageing effects (Fig. 2A, Table 3, and Table S1). A total of 207 targets were regulated by BZBS, and 54 key targets were selected through network parameters, including CCND1(Cyclin D1), CDKN1A(p21), CDKN2A(p16), E2F1, and CDK4 (Fig. 2B–Table 4).

3.2. Effects of BZBS on cell viability, p21 and p16 expression, and SA-β-gal staining in D-gal-induced senescent MSCs

The experimental results are shown in Fig. 3. Compared to the control group, the cell viability in the model group was significantly decreased ($p < 0.01$) (Fig. 3A), p21 and p16 mRNA (Fig. 3B and C) and SA-β-gal-positive ratio (Fig. 3D and E) were markedly increased ($P < 0.05$, $P < 0.01$). Compared to the model group, the cell viability of the BZBS groups was significantly increased ($p < 0.01$), and p21 and p16 mRNA and SA-β-gal-positive ratio were decreased ($P < 0.05$, $P < 0.01$); p16 mRNA and SA-β-gal-positive ratio of NMN group were also reduced ($p < 0.01$). These results suggest that BZBS improves the viability of senescent MSCs, downregulates p21 and p16 expression, and reduces the number of senescent MSCs.

3.3. Effect of BZBS on the differentiation potential of senescent MSCs

The effect of BZBS on the adipogenic potential of senescent MSCs is shown in Fig. 4A. Compared to the control group, the number of lipid droplets formed by the adipogenic differentiation of MSCs in the model group decreased significantly, and the staining became lighter. Compared to the model group, the number of lipid droplets formed in the BZBS and NMN groups increased significantly, and the staining became darker (Fig. 4A).

The effect of BZBS on the osteogenic potential of senescent MSCs is shown in Fig. 4B–D. Compared to the control group, the ALP and alizarin red staining areas in the model group decreased significantly, the staining became lighter, and the expression of calcium nodules decreased. Compared to the model group, the staining area of ALP and alizarin red staining in the BZBS and NMN groups increased, the staining became darker, and the expression of calcium nodules increased (Fig. 4B–D). These findings suggested that BZBS enhanced both osteogenic and adipogenic differentiation.

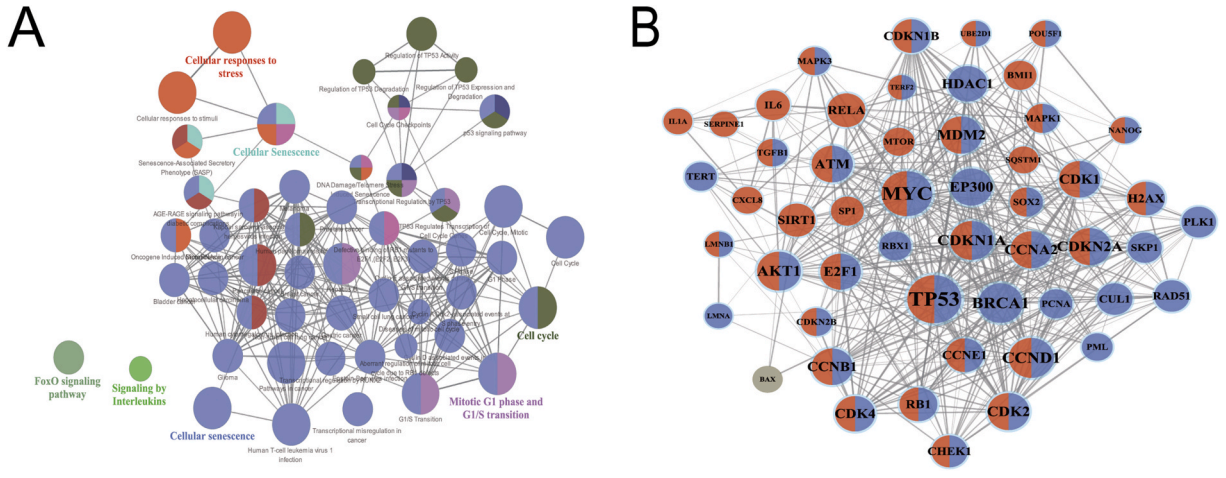

Fig. 2. The network mechanism of anti-ageing effects exerted by BZBS. (A) Enrichment analysis of KEGG pathway for targets regulated by BZBS exerting anti-ageing effects. **(B)** Key targets for the anti-ageing effects of BZBS.

Table 3
KEGG pathway enrichment analysis of targets involved in the anti-ageing effects exerted by BZBS (Top 10).

ID	Term	Term PValue
KEGG:04218	Cellular senescence	2.40743E-42
KEGG:04110	Cell cycle	6.37723E-39
R-HSA:1640170	Cell Cycle	2.71294E-33
R-HSA:2559583	Cellular Senescence	1.16843E-31
KEGG:05200	Pathways in cancer	6.67365E-31
R-HSA:2262752	Cellular responses to stress	7.70814E-30
R-HSA:8953897	Cellular responses to stimuli	1.42276E-29
KEGG:05166	Human T-cell leukaemia virus 1 infection	7.0644E-27
KEGG:05220	Chronic myeloid leukaemia	9.11453E-26
R-HSA:69,278	Cell Cycle, Mitotic	1.13862E-25

Table 4
Key targets for the anti-ageing effects of BZBS.

Name	Degree	Betweenness Centrality	Closeness Centrality
TP53	45	0.148473494	0.894736842
MYC	40	0.082818149	0.822580645
CCND1	31	0.037333763	0.718309859
BRCA1	30	0.056091746	0.708333333
AKT1	29	0.057198656	0.698630137
CDKN1A	29	0.041174705	0.698630137
MDM2	26	0.020931669	0.671052632
EP300	26	0.018948606	0.671052632
CDKN2A	26	0.013542006	0.671052632
CCNA2	26	0.012443273	0.662337662
CDK2	26	0.010284623	0.662337662
CCNB1	25	0.01224249	0.662337662
ATM	25	0.020584459	0.653846154
CDK1	25	0.016958246	0.653846154
E2F1	24	0.006594027	0.653846154
HDAC1	23	0.0138728	0.64556962
CDK4	23	0.008746474	0.6375
CDKN1B	20	0.006003036	0.614457831
RELA	20	0.034427293	0.62195122
H2AX	20	0.011413751	0.607142857
CCNE1	20	0.00599456	0.614457831
RB1	20	0.012287583	0.614457831
SIRT1	19	0.022322659	0.614457831
CHEK1	17	0.004297014	0.593023256
CUL1	16	0.005339689	0.573033708
RAD51	16	0.00432618	0.579545455
PLK1	15	0.001621685	0.573033708
SKP1	14	0.003773555	0.554347826
IL6	13	0.013450835	0.566666667
MAPK1	12	0.003361689	0.554347826
SOX2	12	0.002753501	0.542553191
BMI1	12	0.003771435	0.554347826
SP1	12	0.003009748	0.548387097
PCNA	12	0.000603861	0.542553191
TERT	11	0.006928212	0.542553191
RBX1	11	0.00404508	0.536842105
MAPK3	10	0.006610808	0.536842105
MTOR	10	0.003519078	0.548387097
CDKN2B	10	0.000630685	0.53125
TGFB1	9	0.00732846	0.53125
PML	9	0.000174711	0.536842105
CXCL8	8	0.004435775	0.53125
SQSTM1	8	0.002383251	0.536842105
POU5F1	7	0.000259145	0.51
NANOG	7	0.00020915	0.504950495
UBE2D1	6	0.001201368	0.485714286
IL1A	6	0.001068499	0.414634146
LMNB1	5	0.001314857	0.490384615
SERPINE1	5	0.001374833	0.451327434
TERF2	4	0.000255213	0.447368421
LMNA	4	0.000479303	0.451327434
BAX	3	0	0.495145631

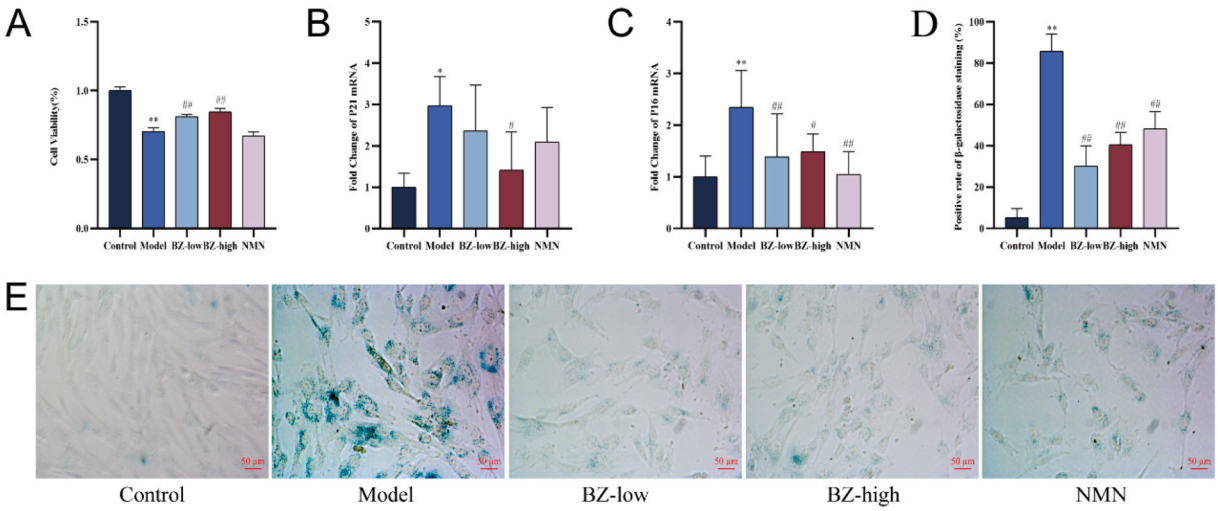

Fig. 3. Effects of BZBS on cell viability, p21 and p16 expression, and SA-β-gal staining in D-gal-induced senescent MSCs. (A) MSCs samples with no less than 1×10^6 cells/well were collected from each group. Cell viability was measured using the CCK-8 kit, as described in the Methods section. Effects of BZBS on cell viability of D-gal-induced MSCs (n = 6); **(B)** and **(C)** MSCs samples with no less than 1×10^6 cells/well were collected from each group. Total RNA was collected, and qPCR was performed according to the method described in the Methods section. Effect of BZBS on the expressions of p21 and p16 in senescent MSCs (n = 3, n = 6); **(D)** and **(E)** According to the method described in the Methods section, the MSCs were fixed at room temperature, then stained with SA-β-gal, and finally recorded using an inverted microscope. Effect of BZBS on SA-β-gal-positive ratio in senescent MSCs and quantitative analysis of SA-β-gal-positive cells. (n = 4; Magnification: 200x). The results represent the mean ± SD. vs Control, *p < 0.05, **p < 0.01; vs Model, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01.

3.4. Effect of BZBS on the expression of stemness-associated genes Nanog, OCT4, and SOX2 in senescent MSCs

Compared to the control group, the expression of *Nanog*, *OCT4*, and *SOX2* was significantly decreased in the model group (P < 0.05, P < 0.01). Furthermore, compared to the model group, *Nanog*, *OCT4*, and *SOX2* mRNA expression in the BZBS groups and NMN group was significantly increased (P < 0.05, P < 0.01) (Fig. 5A–C). These results indicated that BZBS maintained the expression of stemness-associated genes in D-gal-treated senescent MSCs.

3.5. Effect of BZBS on the distribution of cell cycle in senescent MSCs

The result of cell cycle analysis revealed that, compared to the control group, the proportion of MSCs in the G_0/G_1 phase in the model group (68.36 ± 1.12%) was significantly increased (P < 0.01). Compared to the model group, the proportion of G_0/G_1 phase in the BZBS groups (65.91 ± 1.85%, 64.08 ± 0.90%) was significantly reduced (P < 0.01) (Fig. 6A). These results indicated that BZBS promoted cell cycle progression and delayed cell senescence.

3.6. Effect of BZBS on the expression of cell cyclin-related factors CDK4, cyclin D1, and E2F1 in senescent MSCs

Compared to the control group, the expression of CDK4, Cyclin D1 and E2F1 in the model group was significantly decreased (P < 0.01). Compared to the model group, the expression of CDK4, Cyclin D1, and E2F1 in BZBS groups was significantly increased (P < 0.05, P < 0.01), and CDK4 and E2F1 mRNA and CDK4 and cyclin D1 proteins were increased in the NMN group (p < 0.05) (Fig. 6B–F). After treatment with a CDK4 inhibitor (HY-50767A), E2F1 mRNA of the HY-50767A group decreased compared to the control group (P < 0.01); however, there were no significant differences in E2F1 mRNA expression between the BZ-high + HY-50767A group and HY-50767A group (Fig. 6G). This demonstrated that BZBS promoted the expression of CDK4, Cyclin D1 and E2F1, which may be related to the Cyclin D1/CDK4/E2F1 signalling pathway.

4. Discussion

Stem cells are not immortal, and their function gradually declines with age [54]. Delaying stem cell senescence is one of the main methods used to resist tissue and body ageing and is an important means of reducing age-related diseases [55]. Network pharmacology predictions suggested that BZBS delays cellular senescence by regulating the cell cycle. This hypothesis was verified *in vitro*. Results showed that BZBS could significantly improve the cell viability of senescent MSCs, reduce the expression of β-galactosidase, down-regulate p16 and p21, increase differentiation ability, and upregulate *Nanog*, *OCT4*, and *SOX2* genes, significantly decreasing the proportion of G_0/G_1 phase senescent MSCs and increasing the expression of CDK4, Cyclin D1 and E2F1.

Network pharmacology techniques have been widely used to study the mechanism of TCM compound prescriptions and can

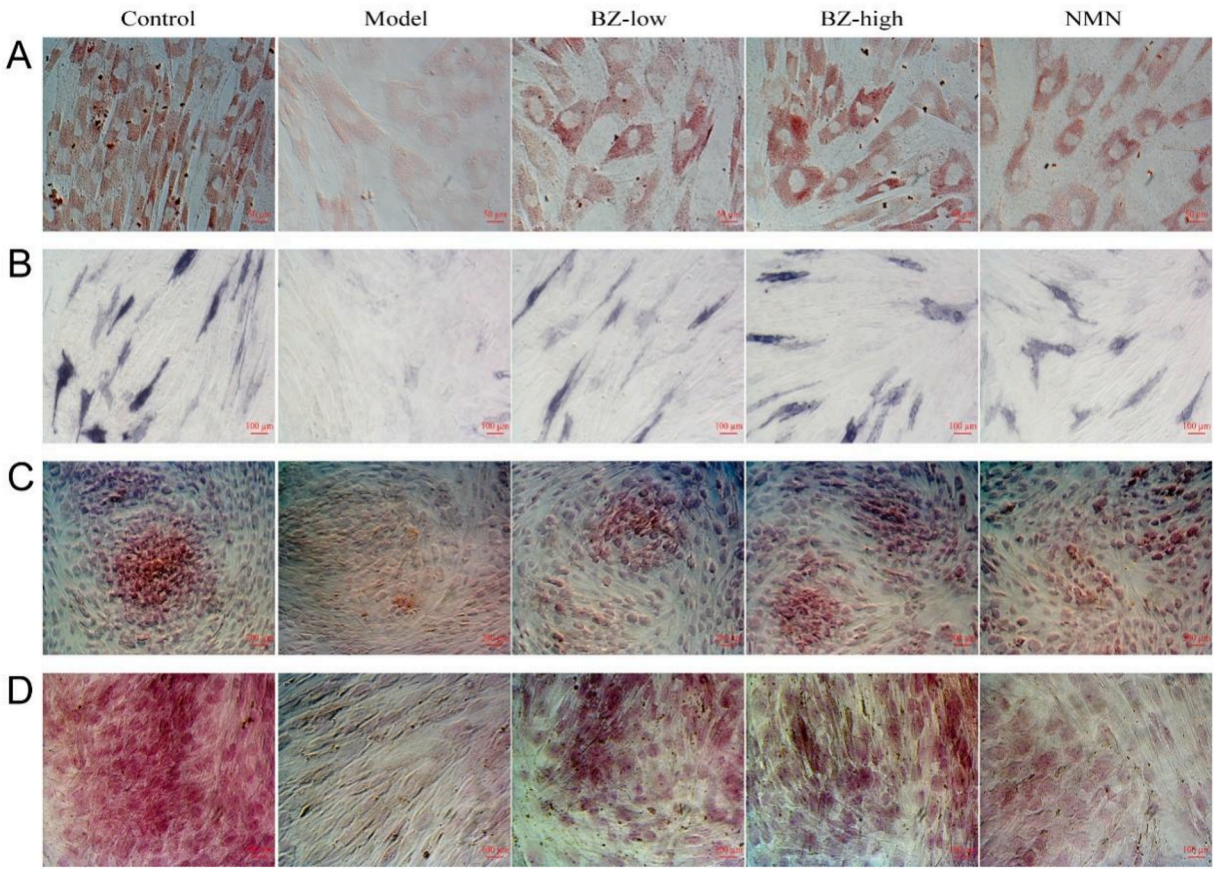

Fig. 4. Effect of BZBS on the differentiation potential of senescent MSCs. Each group of MSCs was fixed at room temperature and stained according to the method described in the Methods section. **(A)** Effect of BZBS on adipogenic potential in senescent MSCs. (Oil red O staining, Magnification: 200x). **(B), (C),** and **(D)** Effect of BZBS on osteogenic potential in senescent MSCs. (ALP staining, Magnification: 100×; Alizarin red staining, Magnification: 50x and 100x). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

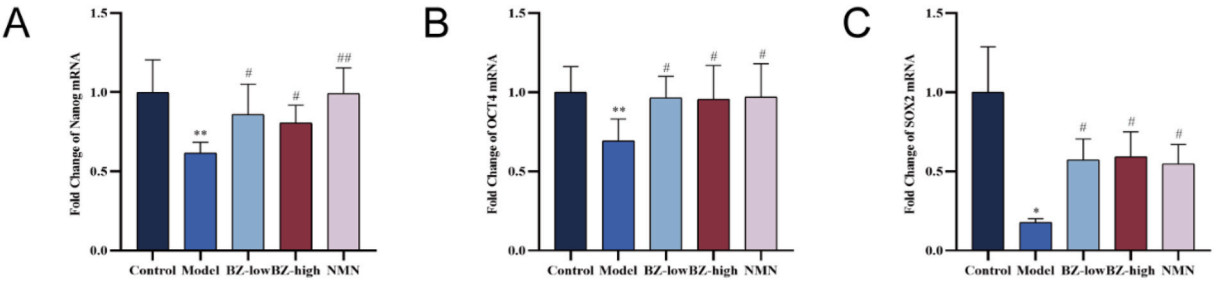

Fig. 5. Effect of BZBS on the expression of stemness-associated genes in senescent MSCs. MSCs samples with no less than 1×10^6 cells/well were collected from each group. Total RNA was collected and qPCR was performed according to the method described in the Methods section. **(A)**, **(B),** and **(C)** Effect of BZBS on the expression of *Nanog*, *OCT4*, and *SOX2* in senescent MSCs (n = 6, n = 6, n = 5). The results represent the mean ± SD. vs Control, *p < 0.05, **p < 0.01; vs Model, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01.

provide directional guidance for subsequent experiments [56–58]. In this study, through pathway enrichment analysis, we found that BZBS has a significant regulatory effect on cell cycle-related signalling pathways. In addition, the analysis of key targets in the PPI network suggested that BZBS may have potential regulatory effects on key cell cycle proteins such as CCND1 (Cyclin D1), CDKN1A (p21), CDKN2A (p16), E2F1, and CDK4, which may be related to the regulation of the Cyclin D1/CDK4/E2F1 pathway. Based on these results, preliminary verifications were conducted *in vitro*. The experimental results confirmed this prediction, indirectly reflecting the reliability of this method for studying the mechanism of action of TCM.

With increasing age and passage time, the proliferation and migration abilities of MSCs gradually decrease [59,60]. SA-β-gal

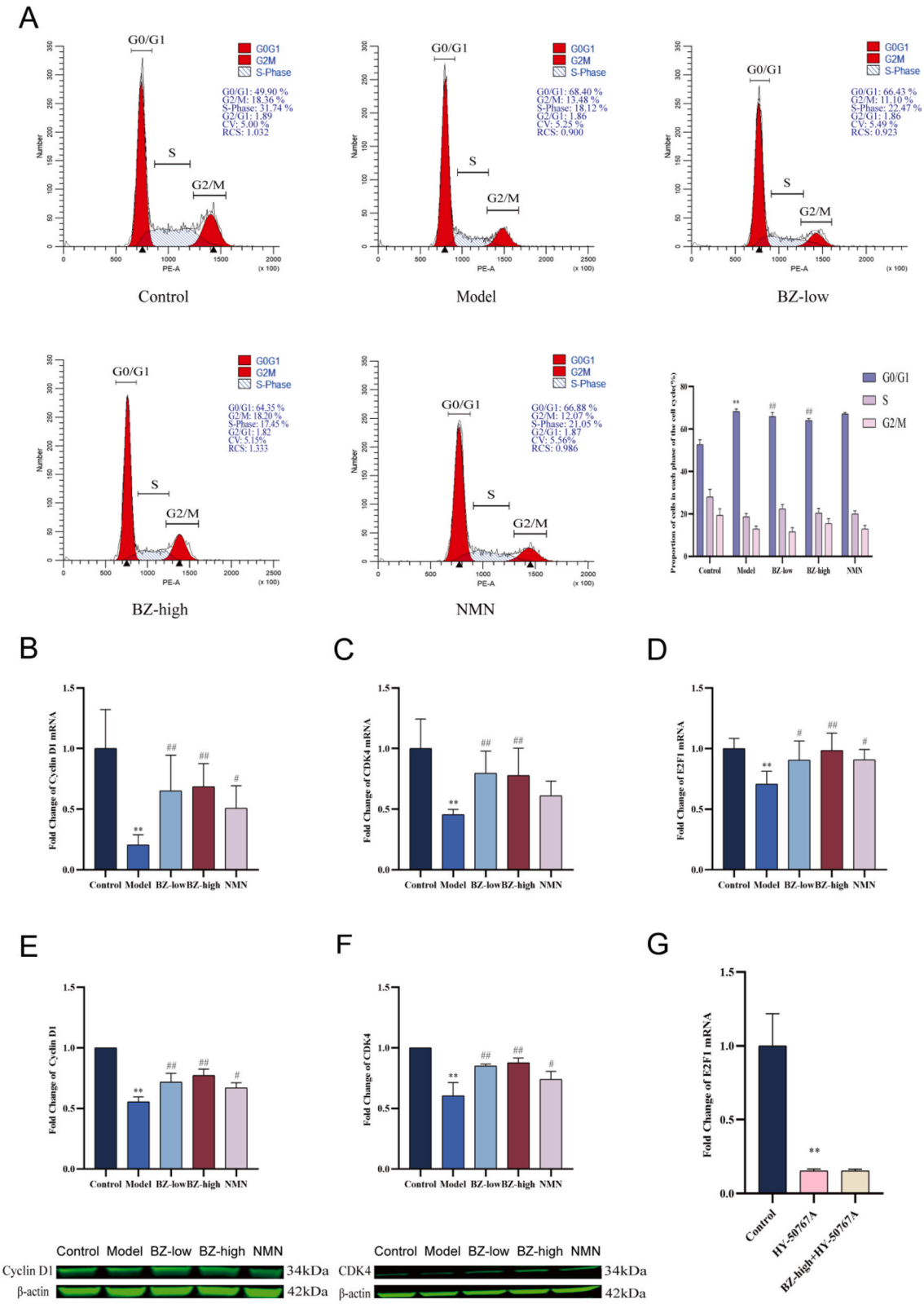

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Fig. 6. Effect of BZBS on the cell cycle of senescent MSCs. (A) FCM was used to detect the cell cycle. MSCs samples with no less than 2×10^6 cells/well were collected for PI staining in each group, and the cell cycle was detected according to the method described in the Methods section. Effect of BZBS on the distribution of cell cycle in senescent MSCs (n = 6); **(B)** and **(E)** Effect of BZBS on the expression of Cyclin D1 in senescent MSCs (n = 6, n = 3); **(C)** and **(F)** Effect of BZBS on the expression of CDK4 in senescent MSCs (n = 6, n = 3); **(D)** Effect of BZBS on the expression of E2F1 in senescent MSCs (n = 5); **(G)** Effect of BZBS on the expression of E2F1 in senescent MSCs after the treatment with CDK4 inhibitor (HY-50767A) (n = 6). The results represent the mean ± SD. vs Control, *p < 0.05, **p < 0.01; vs Model, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01.

activity, a proxy for the enhanced lysosomal content of senescent cells and a marker of lysosomal enzyme senescence, was significantly enhanced in senescent MSCs [61]. Previous studies have shown that β-galactosidase enzyme activity increases and staining deepens with age [42]. It was found that BZBS could improve cell viability and decrease the expression of senescence marker SA-β-gal in senescent MSCs. Moreover, MSCs can differentiate into cells of various germ layers, such as the bone, fat, and bone marrow matrix, under specific conditions [62]. However, the ability of MSCs to differentiate decreases or even disappears with ageing [63]. Previous studies have shown that betaine increases the differentiation ability of senescence MSCs [64]. In this study, BZBS enhanced the adipogenic and osteogenic differentiation capacities of senescent MSCs. *Oct4*, *Sox2*, and *Nanog* regulate the self-renewal and pluripotency of stem cells and are key factors in maintaining stem cells [65–69]. MSCs lose their pluripotency over time during culture, as shown by the varying degrees of decreased expression of *Oct4*, *Sox2*, and *Nanog* [70]. Total flavonoids of litchi seed (TFLS) decreased the expression of stem cell-related markers (*Oct4*, *Nanog*, and *Sox2*) and inhibited the activity of breast cancer stem cells (BCSCs) [71]. BZBS could upregulate the expression of *Oct4*, *Sox2*, and *Nanog* in senescent MSCs. These data suggest that BZBS improves phenotypes related to MSCs senescence. Meanwhile, the rationale of replenishing the kidney is essential to postpone MSCs ageing.

The cell cycle of MSCs is permanently arrested in the G_0 phase during cell senescence [72]; the cells are unable to enter the S phase, and mitosis fails [61]. Fathi et al. previously reported that co-culturing MSCs with Molt-4 can promote the senescence of Molt-4 and lead to cell arrest in the G_0/G_1 phase, significantly increasing the number of G_0/G_1 phase cells and regulate the cell cycle [53]. The data showed that BZBS reduced the proportion of G_0 stage senescent MSCs, consistent with the results from previous network pharmacology studies. The $p16^{Ink4a}$/Cyclin D1/CDK4/E2F1 signalling pathway is essential for irreversible growth arrest during MSCs senescence [73]. Several studies have shown that the expression of cell cycle arrest proteins p16 and p21 is upregulated during ageing and plays a crucial role in cell cycle regulation [74]. Decreased expression of p16 and p21 alleviates cellular senescence [75]. In the present study, BZBS inhibited p16 and p21 expression. Cyclin D1 and CDK4 play important roles in mammalian cell survival and proliferation, driving cells into the DNA synthesis (S) phase of the cell division cycle. The depletion of Cyclin D1 and CDK4 induces cellular senescence and inhibits cell cycle-dependent Cyclin D1-CDK4 complex formation [76]. The cyclin D1-CDK4 complex can mediate the partial phosphorylation of retinoblastoma protein (RB) and affect the release and activation of E2F1 transcription factors to regulate the cell cycle [77]. During MSCs senescence, RB is in a state of low phosphorylation, which inhibits the expression of the E2F1 factor closely related to the S phase and leads to the permanent arrest of the cell cycle [78]. Studies [79] have shown that mycotoxins can induce cell senescence, increase the expression of p16 and p21, and decrease the expression of Cyclin D1 and CDK4, leading to cell-cycle arrest in senescent cells. BZBS significantly upregulated Cyclin D1, CDK4, and transcription factor E2F1. Further studies showed that BZBS did not improve the expression of E2F1 after administration of a CDK4 inhibitor (HY-50767A). This suggests that BZBS could regulate the expression of E2F1 via the regulation of CDK4 expression. According to previous reports and the network pharmacology analysis in this study, BZBS may delay MSCs senescence by regulating the Cyclin D1/CDK4/E2F1 cell cycle-related signalling pathway.

However, it is worth noting that this study is based on the ingredient data in the herbs of the BZBS formula rather than the data obtained from the direct detection of BZBS in the whole formula. Although 14 components of the BZBS have been identified in previous studies [80], their main purpose was quality control. Therefore, in future research, the overall composition of the BZBS formula and the components absorbed by blood need to be identified [81,82]. This study only initially verified the *in vitro* effect of BZBS on delaying MSCs senescence and did not reflect the complex interactions and environment in living organisms. Our group will continue to explore and improve the *in vivo* experiment conditions.

5. Conclusions

Network pharmacology analysis suggested that BZBS may delay cell senescence through cell cycle interventions. Experimental validation showed that BZBS significantly improved the senescence phenotype, enhanced the proliferation and differentiation ability of senescent MSCs, maintained the stemness of senescent MSCs, and reduced the proportion of cells arrested in the G_0 phase, which may be related to the regulation of the Cyclin D1/CDK4/E2F1 cell cyclin-related signalling pathway. This study provided important experimental evidence for the role of BZBS in delaying MSCs senescence. More importantly, this study lays the foundation for further exploration of BZBS's anti-ageing effects on stem cells.

Ethical statement

The study used MSCs purchased from Beijing Jing-Meng Cell Biotechnology Co. Ltd. (Cat # UC1139; for research purposes only).

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Data availability statement

All data generated or analysed during this study are included in this published article (and its Supplementary Information files). Datasets generated or analysed during this study may be made available to interested researchers by the authors upon reasonable request.

CRediT authorship contribution statement

Yaping Zhang: Writing – original draft. **Tongxing Wang:** Software. **Yanfei Song:** Investigation. **Meng Chen:** Investigation. **Bin Hou:** Investigation. **Bing Yao:** Investigation. **Kun Ma:** Supervision. **Yahui Song:** Data curation. **Siwei Wang:** Data curation. **Dan Zhang:** Data curation. **Junqing Liang:** Supervision. **Cong Wei:** 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27646.

References

[1] C. Martins, et al., Metabolomics to study human aging: a review. mol med. https://doi.org/10.2174/1566524023666230407123727, 2023.
[2] C. Casey, M. Seidman, Healthy aging: strategies to slow the process, Otolaryngol. Clin. 55 (5) (2022) 899–907, https://doi.org/10.1016/j.otc.2022.06.016.
[3] G. Bjørklund, et al., Natural compounds and products from an anti-aging perspective, Molecules 27 (20) (2022), https://doi.org/10.3390/molecules27207084.
[4] C. López-Otín, et al., 186, Hallmarks of aging: An expanding universe. Cell (2023) 243–278, https://doi.org/10.1016/j.cell.2022.11.001.
[5] M. Al-Azab, et al., Aging of mesenchymal stem cell: machinery, markers, and strategies of fighting, Cell. Mol. Biol. Lett. (1) (2022) 27–69, https://doi.org/10.1186/s11658-022-00366-0.
[6] Z. Weng, et al., 11, Mesenchymal Stem/Stromal Cell Senescence: Hallmarks, Mechanisms, and Combating Strategies. Stem Cells Transl Med (2022) 356–371, https://doi.org/10.1093/stcltm/szac004.
[7] P. Salehinejad, M. Moshrefi, T. Eslaminejad, An overview on mesenchymal stem cells derived from extraembryonic tissues: supplement sources and isolation methods, 13, Stem Cells Cloning (2020) 57–65, https://doi.org/10.2147/sccaa.S248519.
[8] J. Liu, et al., Mesenchymal stem cells and their microenvironment, Stem Cell Res. Ther. (1) (2022) 13, https://doi.org/10.1186/s13287-022-02985-y, 429.
[9] X. Guo, et al., The role and mechanism of polysaccharides in anti-aging, Nutrients 14 (24) (2022), https://doi.org/10.3390/nu14245330.
[10] J. Li, S.G. Kim, J. Blenis, Rapamycin: one drug, many effects. Cell Metab 19 (3) (2014), https://doi.org/10.1016/j.cmet.2014.01.001, 373-9.
[11] A.A. Soukas, H. Hao, L. Wu, Metformin as Anti-Aging Therapy: Is It for Everyone? Trends Endocrinol Metab 30 (10) (2019), https://doi.org/10.1016/j.tem.2019.07.015, 745-755.
[12] D. Huang, et al., The role of collateral disease theory in the prevention and treatment of atherosclerosis in post-menopausal women: a narrative review, 9, Ann. Palliat. Med. (2020) 2314–2322, https://doi.org/10.21037/apm-20-1257, 4.
[13] D. Huang, et al., Bazi bushen capsule alleviates post-menopausal atherosclerosis via GPER1-dependent anti-inflammatory and anti-apoptotic effects, Front. Pharmacol. (2021) 12, https://doi.org/10.3389/fphar.2021.658998, 658998.
[14] T. Wang, et al., Repositioning of clinically approved drug Bazi Bushen capsule for treatment of Aizheimer's disease using network pharmacology approach and in vitro experimental validation, 9, Heliyon (2023) e17603, https://doi.org/10.1016/j.heliyon.2023.e17603, 7.
[15] C. Ji, et al., Bazi Bushen capsule attenuates cognitive deficits by inhibiting microglia activation and cellular senescence, Pharm. Biol. (1) (2022) 60, https://doi.org/10.1080/13880209.2022.2131839, 2025-2039.
[16] L. Li, et al., BaZiBuShen alleviates altered testicular morphology and spermatogenesis and modulates Sirt6/P53 and Sirt6/NF-κB pathways in aging mice induced by D-galactose and NaNO(2), J. Ethnopharmacol. (2021) 271, https://doi.org/10.1016/j.jep.2021.113810, 113810.
[17] D. Huang, et al., Chinese medicine Bazi Bushen capsule improves lipid metabolism in ovariectomized female ApoE-/- mice, 9, Ann. Palliat. Med. (3) (2020) 1073–1083, https://doi.org/10.21037/apm-20-906.
[18] D. Wang, et al., Estrogen-Like effect of Bazi bushen capsule in ovariectomized rats. J vis exp, 194, https://doi.org/10.3791/64884, 2023.
[19] L. Li, et al., BaZiBuShen alleviates cognitive deficits and regulates Sirt6/NRF2/HO-1 and Sirt6/P53-PGC-1α-TERT signaling pathways in aging mice, 282, J. Ethnopharmacol. (2022) 114653, https://doi.org/10.1016/j.jep.2021.114653.
[20] A.L. Hopkins, Network pharmacology, Nat. Biotechnol. (2007) 25, https://doi.org/10.1038/nbt1007-1110, 1110-1.
[21] A.L. Hopkins, Network pharmacology: the next paradigm in drug discovery. Nat Chem Biol 4 (11) (2008), https://doi.org/10.1038/nchembio.118, 682-90.

[22] L. Zhao, et al., Network pharmacology, a promising approach to reveal the pharmacology mechanism of Chinese medicine formula, J. Ethnopharmacol. (2023) 309, https://doi.org/10.1016/j.jep.2023.116306, 116306.
[23] Z.Y. Wang, et al., Mechanism of action of Daqinjiao decoction in treating cerebral small vessel disease explored using network pharmacology and molecular docking technology, Phytomedicine (2023) 108, https://doi.org/10.1016/j.phymed.2022.154538, 154538.
[24] S. Yu, et al., Network pharmacology and experimental verification to explore the anti-migraine mechanism of Yufeng Ningxin Tablet, J. Ethnopharmacol. (2023) 310, https://doi.org/10.1016/j.jep.2023.116384, 116384.
[25] X. Wang, et al., Network pharmacology and LC-MS approachs to explore the active compounds and mechanisms of Yuanjiang decoction for treating bradyarrhythmia, Comput. Biol. Med. (2023) 152, https://doi.org/10.1016/j.compbiomed.2022.106435, 106435.
[26] D. Gong, et al., Network pharmacology approach and experimental verification of Dan-Shen Decoction in the treatment of ischemic heart disease, 61, Pharm. Biol. (2023) 69–79, https://doi.org/10.1080/13880209.2022.2152059.
[27] X. Li, et al., Network pharmacology approaches for research of Traditional Chinese Medicines, Chin. J. Nat. Med. 21 (5) (2023) 323–332, https://doi.org/10.1016/s1875-5364(23, 60429–7.
[28] J. Piñero, et al., The DisGeNET knowledge platform for disease genomics: 2019 update, Nucleic Acids Res. (2020) 48, https://doi.org/10.1093/nar/gkz1021. D845-d855.
[29] D. Carvalho-Silva, et al., Open Targets Platform: new developments and updates two years on, 47, Nucleic Acids Res. (2019), https://doi.org/10.1093/nar/gky1133. D1056-D1065.
[30] N. Rappaport, et al., MalaCards: an amalgamated human disease compendium with diverse clinical and genetic annotation and structured search, Nucleic Acids Res. 45 (2017), https://doi.org/10.1093/nar/gkw1012. D877-D887.
[31] A.P. Davis, et al., The comparative toxicogenomics database: update 2019, Nucleic Acids Res. (2019) 47, https://doi.org/10.1093/nar/gky868. D948-d954.
[32] M. Safran, et al., GeneCards Version 3: the human gene integrator, 2010, Database (2010), https://doi.org/10.1093/database/baq020. baq020.
[33] D.S. Wishart, et al., DrugBank 5.0: a major update to the DrugBank database for 2018, Nucleic Acids Res. 46 (D1) (2018), https://doi.org/10.1093/nar/gkx1037. D1074-d1082.
[34] Y. Wang, et al., Therapeutic target database 2020: enriched resource for facilitating research and early development of targeted therapeutics, Nucleic Acids Res. 48 (D1) (2020) D1031–d1041, https://doi.org/10.1093/nar/gkz981.
[35] A. Gaulton, et al., The ChEMBL database in 2017, 45, Nucleic Acids Res. (2017), https://doi.org/10.1093/nar/gkw1074. D945-d954.
[36] S. Kim, et al., PubChem substance and compound databases, Nucleic Acids Res. (2016) 44, https://doi.org/10.1093/nar/gkv951. D1202-13.
[37] A.P. Davis, et al., Comparative toxicogenomics database (CTD): update 2023. Nucleic acids res. https://doi.org/10.1093/nar/gkac833, 2022.
[38] C. UniProt, UniProt: a worldwide hub of protein knowledge, Nucleic Acids Res. 47 (D1) (2019) D506–D515, https://doi.org/10.1093/nar/gky1049.
[39] D. Szklarczyk, et al., The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible, Nucleic Acids Res. 45 (D1) (2017) D362–D368, https://doi.org/10.1093/nar/gkw937.
[40] P. Shannon, et al., Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (11) (2003), https://doi.org/10.1101/gr.1239303, 2498-504.
[41] G. Bindea, et al., ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks, Bioinformatics 25 (8) (2009), https://doi.org/10.1093/bioinformatics/btp101, 1091-3.
[42] E. Fathi, I. Vietor, Mesenchymal stem cells promote caspase expression in molt-4 leukemia cells via GSK-3α/B and ERK1/2 signaling pathways as a therapeutic strategy, Curr. Gene Ther. 21 (1) (2021) 81–88, https://doi.org/10.2174/1566523220666201005111126.
[43] D. Zhang, et al., Coenzyme Q10 inhibits the aging of mesenchymal stem cells induced by D-galactose through Akt/mTOR signaling, Oxid. Med. Cell. Longev. (2015) 2015, https://doi.org/10.1155/2015/867293, 867293.
[44] S. Haider, et al., A high dose of short term exogenous D-galactose administration in young male rats produces symptoms simulating the natural aging process, Life Sci. (2015) 124, https://doi.org/10.1016/j.lfs.2015.01.016, 110-9.
[45] X.Y. Zhao, et al., The effect of overexpression of PGC-1α on the mtDNA4834 common deletion in a rat cochlear marginal cell senescence model, Hear. Res. (2013) 13–24, https://doi.org/10.1016/j.heares.2012.11.007, 296.
[46] Y. Shen, et al., Glutamine synthetase plays a role in D-galactose-induced astrocyte aging in vitro and in vivo, Exp. Gerontol. (2014) 58, https://doi.org/10.1016/j.exger.2014.08.006, 166-73.
[47] B. Guo, et al., D-Galactose-induced oxidative stress and mitochondrial dysfunction in the cochlear basilar membrane: an in vitro aging model, Biogerontology (2020) 21, https://doi.org/10.1007/s10522-020-09859-x, 311-323.
[48] E. Oner, S.G. Gray, S.P. Finn, Cell viability assay with 3D prostate tumor spheroids, Methods Mol. Biol. (2023) 263–275, https://doi.org/10.1007/978-1-0716-3056-3_17, 2645.
[49] Y. Zhang, et al., MicroRNA-19b-3p dysfunction of mesenchymal stem cell-derived exosomes from patients with abdominal aortic aneurysm impairs therapeutic efficacy, J. Nanobiotechnol. 21 (1) (2023) 135, https://doi.org/10.1186/s12951-023-01894-3.
[50] M.J. Livingston, et al., Tubular cells produce FGF2 via autophagy after acute kidney injury leading to fibroblast activation and renal fibrosis, Autophagy 19 (1) (2023) 256–277, https://doi.org/10.1080/15548627.2022.2072054.
[51] J. Zhang, et al., TRIM59 promotes steatosis and ferroptosis in non-alcoholic fatty liver disease via enhancing GPX4 ubiquitination, Hum. Cell 36 (1) (2023) 209–222, https://doi.org/10.1007/s13577-022-00820-3.
[52] M. Ren, et al., Melatonin repairs osteoporotic bone defects in iron-overloaded rats through PI3K/AKT/GSK-3β/P70S6k signaling pathway, Oxid. Med. Cell. Longev. (2023) 2023, https://doi.org/10.1155/2023/7718155, 7718155.
[53] E. Fathi, et al., Adipose tissue-mesenchymal stem cells caused to change the methylation status of hTERT gene promoter CpG islands of molt-4 leukemia cells as cell-based therapy, Curr. Mol. Med. 23 (3) (2023), https://doi.org/10.2174/1566524022666220118103136, 266-274.
[54] K.L. Rudolph, Stem cell aging. Mech Ageing Dev (2021) 193, https://doi.org/10.1016/j.mad.2020.111394, 111394.
[55] T. Yue, D. Li, Senescent stem cell dysfunction and age-related diseases, Stem Cell. Dev. 32 (19–20) (2023) 581–591, https://doi.org/10.1089/scd.2023.0065.
[56] H. Zhang, et al., Network pharmacology and experimental validation to reveal the pharmacological mechanisms of liuwei dihuang decoction against intervertebral disc degeneration, Drug Des. Dev. Ther. 15 (2021) 4911–4924, https://doi.org/10.2147/dddt.S338439.
[57] L. An, et al., Integrating network pharmacology and experimental validation to investigate the effects and mechanism of Astragalus flavonoids against hepatic fibrosis, Front. Pharmacol. (2020) 11, https://doi.org/10.3389/fphar.2020.618262, 618262.
[58] Q. Li, et al., Integrating network pharmacology and pharmacological validation to explore the effect of Shi Wei Ru Xiang powder on suppressing hyperuricemia, J. Ethnopharmacol. (2022) 298, https://doi.org/10.1016/j.jep.2022.115679, 115679.
[59] D.R. Wagner, et al., Dysfunctional stem and progenitor cells impair fracture healing with age, World J. Stem Cell. 11 (6) (2019) 281–296, https://doi.org/10.4252/wjsc.v11, i6.281.
[60] M.A. Elsied, et al., Walnut kernel oil and defatted extracts enhance mesenchymal stem cell stemness and delay senescence, Molecules 28 (17) (2023), https://doi.org/10.3390/molecules28176281.
[61] A. Hernandez-Segura, J. Nehme, M. Demaria, Hallmarks of cellular senescence, Trends Cell Biol. 28 (6) (2018) 436–453, https://doi.org/10.1016/j.tcb.2018.02.001.
[62] M.F. Pittenger, et al., Multilineage potential of adult human mesenchymal stem cells, Science 284 (5411) (1999), https://doi.org/10.1126/science.284.5411.143, 143-7.
[63] W. Kuang, et al., Functional and molecular changes of MSCs in aging, Curr. Stem Cell Res. Ther. 10 (5) (2015), https://doi.org/10.2174/1574888x10666150211162933, 384-91.
[64] Y. Jing, et al., Betaine regulates adipogenic and osteogenic differentiation of hAD-MSCs, Mol. Biol. Rep. 50 (6) (2023), https://doi.org/10.1007/s11033-023-08404-6, 5081-5089.
[65] N. Ivanova, et al., Dissecting self-renewal in stem cells with RNA interference, Nature 442 (2006), https://doi.org/10.1038/nature04915, 533-8.

[66] A. Sarkar, K. Hochedlinger, The sox family of transcription factors: versatile regulators of stem and progenitor cell fate, Cell Stem Cell 12 (1) (2013), https://doi.org/10.1016/j.stem.2012.12.007, 15-30.
[67] K. Takahashi, et al., Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (5) (2007), https://doi.org/10.1016/j.cell.2007.11.019, 861-72.
[68] W. Zhang, et al., Insights into the Nanog gene: a propeller for stemness in primitive stem cells, Int. J. Biol. Sci. 12 (11) (2016), https://doi.org/10.7150/ijbs.16349, 1372-1381.
[69] K.M. Loh, B. Lim, A precarious balance: pluripotency factors as lineage specifiers, Cell Stem Cell 8 (4) (2011), https://doi.org/10.1016/j.stem.2011.03.013, 363-9.
[70] K. Dzobo, et al., Fibroblast-Derived extracellular matrix induces chondrogenic differentiation in human adipose-derived mesenchymal stromal/stem cells in vitro, Int. J. Mol. Sci. 17 (8) (2016), https://doi.org/10.3390/ijms17081259.
[71] Y. Liao, et al., Total flavonoids of Litchi seed attenuate stem cell-like properties in breast cancer by regulating Notch3 signaling pathway, J. Ethnopharmacol. (2023) 305, https://doi.org/10.1016/j.jep.2023.116133, 116133.
[72] W. Huang, et al., Cellular senescence: the good, the bad and the unknown, Nat. Rev. Nephrol. 18 (10) (2022), https://doi.org/10.1038/s41581-022-00601-z, 611-627.
[73] M.J. Regulski, Cellular senescence: what, why, and how. Wounds 29 (2017) 168–174.
[74] P.R. Coryell, et al., Autophagy regulates the localization and degradation of p16(INK4a), Aging Cell 19 (7) (2020) e13171, https://doi.org/10.1111/acel.13171.
[75] T.D. Saccon, et al., Senolytic combination of dasatinib and quercetin alleviates intestinal senescence and inflammation and modulates the gut microbiome in aged mice, J Gerontol A Biol Sci Med Sci 76 (11) (2021), https://doi.org/10.1093/gerona/glab002, 1895-1905.
[76] F. Sun, et al., Ara-c induces cell cycle G1/S arrest by inducing upregulation of the INK4 family gene or directly inhibiting the formation of the cell cycle-dependent complex CDK4/cyclin D1, Cell Cycle 18 (18) (2019), https://doi.org/10.1080/15384101.2019.1644913, 2293-2306.
[77] C. Romagosa, et al., p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors, Oncogene 30 (18) (2011), https://doi.org/10.1038/onc.2010.614, 2087-97.
[78] B.G. Childs, et al., Senescent cells: an emerging target for diseases of ageing, Nat. Rev. Drug Discov. 16 (10) (2017), https://doi.org/10.1038/nrd.2017.116, 718-735.
[79] L. You, et al., Mycotoxins and cellular senescence: the impact of oxidative stress, hypoxia, and immunosuppression, Arch. Toxicol. 97 (2) (2023), https://doi.org/10.1007/s00204-022-03423-x, 393-404.
[80] G.S. Song, et al., [Determination of 14 components in bazibushen capsule by UPLC-ESI-MS/MS]. Yao xue xue bao 51 (2016) 792–796.
[81] Y. He, et al., Identification of active ingredients of huangqi guizhi wuwu decoction for promoting nerve function recovery after ischemic stroke using HT22 live-cell-based affinity chromatography combined with HPLC-MS/MS, Drug Des. Dev. Ther. 15 (2021) 5165–5178, https://doi.org/10.2147/dddt.S333418.
[82] Y. Rong, et al., LC-high-resolution-MS/MS analysis of chemical compounds in rat plasma after oral administration of Nao-Mai-Tong and its individual herbs, Biomed. Chromatogr. 31 (2017), https://doi.org/10.1002/bmc.3920.