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Original Article

Material basis revelation of anti-hepatoma effect of Huachansu (Cinobufacini) through down-regulation of thymidylate synthase

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ABSTRACT

Objective: Hepatocellular carcinoma (HCC) is a leading cause of mortality worldwide. Huachansu (Cinobufacini) is active extract isolated from the dry skin of *Bufo Bufo gargarizans*. It has now been widely used in clinical treatment of cancer, this study is to clarify the material basis of down-regulation of thymidylate synthase (TYMS) induced by Huachansu.

Methods: Our study utilized UPLC-MS/MS to identify major bioactive components from Huachansu. Cell Counting Kit 8 (CCK-8) assay and clone formation assay were used to examine the cell viability of tumor cells. TYMS and γ-H2AX level were detected by using quantitative real-time RT-PCR and/or western blotting. Small interfering RNA (siRNA) transfection was used to explore whether inhibition of TYMS could enhance the suppressive effect of Huachansu on cell growth of HCC cells.

Results: In our study, firstly, we identify 21 major bioactive components from Huachansu. CCK-8 assay results showed that Huachansu and its bioactive bufadienolides (Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin) significantly inhibited the proliferation of HepG2 and SK-HEP-1 cells in a dose- and time-dependent manner. Further molecular mechanistic investigation demonstrates that Huachansu significantly suppresses thymidylate synthase (TYMS), the enzyme which provides the sole de novo source of thymidylate for DNA synthesis. The inhibition of TYMS could lead to cell-cycle block and DNA damage of HCC cells. Furthermore, we identified that Huachansu markedly increased γ-H2AX expression, which indicated the presence of DNA damage. Moreover, we confirmed that transfection of cells with small interfering RNA specific to TYMS could increase the suppressive effects of Huachansu on the HCC cells proliferation. Quantitative RT-PCR analysis showed that Huachansu treatment had no effect on the transcription level of TYMS. Furthermore, proteasomal inhibitor MG132 could block TYMS inhibition induced by Huachansu, and concomitant administration of protein synthesis inhibitor cycloheximide (CHX) with Huachansu could further suppress the protein level of TYMS, indicating that Huachansu promotes proteasome-dependent degradation of TYMS in liver cancer cells. More importantly, the bioactive bufadienolides of Huachansu such as Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin could also significantly restrain the protein level of TYMS, revealing the material basis of inhibition of TYMS exposed to Huachansu. 5-Fluorouracil (5-FU) is a TYMS inhibitor, we also evaluate the effects of the combined treatment of Huachansu with 5-FU, the results show that interactions between Huachansu and 5-FU are synergistic or antagonistic. Thus, in clinical, attention should be paid to the dosage of Huachansu in combination with 5-FU.

Conclusion: Huachansu inhibits the growth and induces DNA damage of human HCC cells through proteasome-dependent degradation of TYMS, bioactive bufadienolides are the material basis of down-regulation of TYMS induced by Huachansu.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, GLOBOCAN study shows that liver cancer is the leading third cause of cancer death (Calderaro, Ziol, Paradis & Zucman-Rossi, 2019; Sung et al., 2021). HCC usually develops from chronic liver disease, which is mainly associated with hepatitis B (HBV) or C (HCV) virus infection, alcoholic liver disease or nonalcoholic fatty liver disease (NAFLD) (Calderaro et al., 2019; Sayiner, Golabi & Younossi, 2019; Yang et al., 2019). HCC's overall prognosis remains frustrating, except for those patients diagnosed at an early stage and received potentially curative therapies, such as surgical resec-

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tion and radiofrequency ablation (Rebouissou & Nault, 2020; Kanwal & Singal, 2019; Zhou, Shang, Yu & Tian, 2018). Thus, it is of considerable clinical significance for the development of therapeutic drugs for patients with HCC.

Traditional Chinese medicine (TCM) has a well-established history of good curative effect and low toxicity. In recent years, TCM has gained recognition as a novel type of chemotherapy adjuvant owing to its ability to enhance chemotherapy's effectiveness and impair its side effects. TCM is gaining interest among researchers as an innovative source of anticancer drugs after the approval of three new TCM-based drugs in 2007, ixabepilone, trabectedin, and temsirolimus (Gao et al., 2021).

Huachansu (Cinobufacini) is active extract isolated from the TCM dry skin of *Bufo Bufo gargarizans* Cantor (Qi et al., 2010). Chinese State Food and Drug Administration (SFDA) has approved Huachansu Capsules (Z20050846) as the drug for the treatment of cancer including HCC in 2005. Previous research has identified that Huachansu exerts anti-tumor effects by a variety of mechanisms of action (Liu et al., 2022), such as sensitization of tumor necrosis factor related apoptosis-inducing ligand (TRAIL)-mediated apoptosis, reversal of chemotherapy drug resistance, suppression of cellular invasion, induction of autophagic cell death and epithelial–mesenchymal transition (Cheng et al., 2019). Meanwhile, clinical studies have shown that the patients who take Huachansu do not exhibit cardiovascular, mucocutaneous, and gastrointestinal side-effects (Ni et al., 2019).

Thymidylate synthase (TYMS) is a folate-dependent enzyme, and catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate to 2′-deoxythymidine-5′-monophosphate. Moreover, this biochemical process supplies the only intracellular de novo source of 2′-deoxythymidine-5′-triphosphate for DNA synthesis. TYMS inhibition could cause the rapid depletion of thymidine monophosphate (TMP) pools and induce perturbation of the molecular level of deoxynucleotide triphosphate (dNTP), eventually lead to cell-cycle arrest and DNA damage (Schmitz & Chu, 2011; Wilson, Danenberg, Johnston, Lenz & Ladner, 2014; Chu, Callender, Farrell & Schmitz, 2003). In addition, TYMS is overexpressed in numerous tumour types, exhibits oncogene-like activity when overexpressed in mammalian cells, and an increased abundance of TYMS in cancer cells is associated with poor prognosis and treatment response. TYMS is an important drug target in the treatment of cancer (Chu et al., 2003), and, as one of the TYMS inhibitors, 5-fluorouracil (5-FU) has been diffusely used in clinic for cancer treatment.

In this study, we first identify major bioactive components from Huachansu by UPLC-MS/MS, and further molecular mechanistic investigation demonstrates that Huachansu inhibits the growth and induces DNA damage through the proteasomal degradation of TYMS in human hepatoma cells, more importantly, we uncover the material basis of inhibition of TYMS induced by Huachansu, in detail, we reveal that several different bufadienolides from Huachansu could obviously suppress the protein level of TYMS. In addition, we also evaluate the interactions between Huachansu and TYMS inhibitor 5-FU, the results indicate that the effects of the combined treatment of these two drugs are synergistic or antagonistic, suggesting that more attention should be paid to the dosage when these two drugs are combined in clinic.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA, and penicillin–streptomycin mixture were obtained from Corning Life Sciences (Steuben County, New York, USA). Cell Counting Kit 8 (CCK-8) was acquired from Bairuiji (Beijing, China). 1% crystal violet stain solution was acquired from Solarbio (Beijing, China). Antibodies against β-actin (sc-47778) were obtained from Santa Cruz (Santa Cruz, CA, USA). Antibodies against TYMS (ab108995) were obtained from Abcam (Cambridge, UK). Antibodies against γ-H2AX (Cat#7631), anti-rabbit (Cat#7074) and mouse (Cat#7076) IgG-HRP antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Huachansu was acquired from Shanxi Eastantai Pharmaceutical Co., Ltd. (product batch number: OD04). Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin were acquired from Chengdu Biopurify Phytochemicals Co., Ltd.

2.2. Cell culture

The HCC cell line HepG2 and SK-HEP-1 was acquired from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin, and maintained in humidified atmosphere with 5% CO_2 at 37 °C.

2.3. UPLC-MS/MS analysis of active components of Huachansu

The extraction method of Huachansu was as previously described (Wu et al., 2022). The Huachansu (50 mg) was added to 1.5 mL of 80% ethanol. The mixture was processed with vortex for 10 min and centrifugation at 4 000 g for 5 min, then filtered with a 0.22 μm filter. Cinobufagin (1 mg) was dissolved in 1.5 mL of 70% methanol, and then filtered with a 0.22 μm filter. An aliquot (2 μL) of the filtered supernatant was injected to a Q-Exactive Focus Orbitrap MS (Thermo Electron, Bremen, Germany) connected to the UPLC (Thermo Fisher Scientific, California, USA) via an ESI source. A Waters UPLC BEH C_{18} column (Waters Corporation, Milford, USA) (100 mm × 2.1 mm, 1.7 μm) was used and the column temperature was 40 °C. The flow rate was 0.2 mL/min, and the mobile phase consisted of 0.1% formic acid aqueous solution (A) and acetonitrile (B) with the gradient set was as follows: 0–3.0 min, 97%–97% A; 3.0–5.0 min, 97%–95% A; 5.0–7.0 min, 95%–90% A; 7–11 min, 90%–70% A; 11–13 min, 70%–70% A; 13–15 min, 70%–62% A; 15–18 min, 62%–50% A; 18–20 min, 50%–40% A; 20–24 min, 40%–0% A; 24–27 min, 10%–10% A; 27–28 min, 10%–97% A; 28–33 min, 97%–97% A. Mass detection was operated in the positive ion mode with the following conditions: the capillary temperature was 320 °C; the auxiliary gas heater temperature was 350 °C; electrospray ionization was 3 200 V. High resolution mass spectrum was obtained at full scan in a mass range of m/z 100–1 500 at a resolution of 70 000 examined by Orbitrap analyzer (Thermo Fisher Scientific, California, USA).

2.4. Cell viability assay

First of all, cells were seeded in 96-well plates (3.5×10^3 cells/well). After 24 h, cells were added with different concentrations of Huachansu for culturing 24 h and 48 h. Cell viability was evaluated using CCK-8 assay as previously described (Wu et al., 2022).

2.5. Colony formation assay

Briefly, cells were seeded in 6-well plates at 1 500 cells/well for 24 h before adding with indicated concentrations of Huachansu.

The culture medium was changed every 3 d, after treatment for 7 d, removed the old medium, colonies were fixed with 10% formaldehyde for 15 min, then stained with 0.5% crystal violet for 15 min. At last, cells were washed three times with PBS, then a digital camera was used to photograph the cell colonies.

2.6. Quantitative real-time-PCR (qRT-PCR)

We used qRT-PCR to analyze the mRNA level of TYMS in HepG2 and SK-HEP-1 cells. Total RNA was isolated with TRIzol Reagent (OMEGA, Norcross, USA) following the manufacturer's instructions. PrimeScript RT Reagent Kit (TaKaRa, Dalian, Chinese) was used to perform reverse transcription to synthesize the cDNA. qRT-PCR was performed by using TransStart Top Green PCR SuperMix (TransGen Biotech, Beijing, Chinese) following the manufacturer's instructions. The primer sequences are as follows: Human actin forward 5′-GGGACCTGACTGACTACCTC-3′, reverse 5′-TCA TACTCCTGCTTGCTGAT-3′; Human TYMS forward 5′-GAATCACATC GAGCCACTGAAA-3′, reverse 5′- CAGCCCAACCCCTAAAGACTGA-3′.

2.7. siRNA and transfection

Transfection of synthesized siRNAs was performed by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Small interfering RNAs (siRNA) specifically targeting TYMS were purchased from GenePharma (Shanghai, China). The siRNA sequences are as follows: siRNA-NC, 5′-UUCUCCGAACGUGUCACGUTT-3′; siRNA-TYMS, 5′-CAAUCCGCAUCCAACUAUUTT-3′.

2.8. Western blotting

The proteins extracted from cells were homogenized in a lysis buffer, then the detection of the protein expression was performed by western blotting assay as described in our previous paper (Wu et al., 2022).

2.9. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5.0 software. The data was expressed as means ± SEM. The data are representative of three independent experiments, and each was performed at least in triplicate. To determine the statistical significance of differences, a student's t test was used. $P < 0.05$ was indicative of statistically significant.

3. Results

3.1. Identification of bioactive components from Huachansu

As shown in Fig. 1 and Table 1, chemical profile of Huachansu was detected, and 21 bioactive components were identified from Huachansu. 17 of the compounds were identified as bufadienolides, two of the compounds were identified as nucleosides, one compound was identified as indole alkaloid, and one compound was identified as peptide.

3.2. Huachansu inhibits proliferation of human hepatoma cells

To investigate the proliferation effects of Huachansu on human hepatoma cells *in vitro*, HepG2 and SK-HEP-1 cells were cultured with various concentrations of Huachansu for 24–48 h, respectively. Then the cell viability of cancer cells was detected by the CCK-8 assay. The results suggested that Huachansu effectively inhibited cell viability in a dose- and time-dependent manner (Fig. 2A). Meanwhile, as compared to the control group, Huachansu inhibited the colony formation of cancer cells (Fig. 2B).

3.3. Bioactive components of Huachansu inhibit growth of human hepatoma cells

Next, the anti-proliferation effects of the identified bioactive components of Huachansu (Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin) on liver cancer cells were detected by CCK-8 assay. The cells were cultured with various concentrations of the different identified bioactive components of Huachansu for 48 h, respectively. The result showed that Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin could significantly inhibit the growth of HepG2 and SK-HEP-1 cells (Fig. 3A–B).

3.4. Huachansu inhibits TYMS expression in human hepatoma cells

TYMS is a key enzyme that catalyzes the conversion of dUMP to dTMP and participates in DNA precursor synthesis and repair, which is not only a crucial gene in cellular proliferation but also a significant target in cancer chemotherapy (Skibola et al., 2002; Mandola Mv, 2003). Some of the TYMS inhibitors have been used in the clinic for cancer treatment, such as 5-FU or other fluoropyrimidines (capecitabine and tegafur). We investigated whether Huachansu has effects on TYMS expression in tumor cells, the results suggested that exposure to Huachansu resulted in the significantly down-regulation of TYMS (Fig. 4A–B).

3.5. Huachansu induces DNA damage in human hepatoma cells

Many studies have shown that TYMS inhibition induces an imbalance among the levels of dUTP and dTTP, eventually causes a consequent reduction in the efficiency of DNA synthesis, and induces DNA damage (Takezawa et al., 2010; Schmitz & Chu, 2011). γ-H2AX assay is used to monitor DNA damage of cells (Ivashkevich, Redon, Nakamura, Martin & Martin, 2012). We explored the effects of Huachansu treatment on the expression of γ-H2AX. As shown in Fig. 4A–B, γ-H2AX protein expression was markedly enhanced in cancer cells treated with Huachansu, indicating the occurrence of DNA damage.

3.6. Bioactive components of Huachansu inhibit TYMS expression in human hepatoma cells

In our previous study, we identified that cinobufagin could obviously inhibit the protein level of TYMS (Yang et al., 2022), in present study, we explored the effects of several different bufadienolides from Huachansu identified by UPLC-MS/MS on the protein level of TYMS. As shown in Fig. 5A–G, a significant reduction of TYMS was detected after exposure to Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, or Resibufogenin in cancer cells. These results uncovered the material basis of inhibition of TYMS induced by Huachansu.

3.7. Huachansu inhibits proliferation and leads to DNA damage of human hepatoma cells through down-regulation of TYMS expression

Our previous study has shown that depletion of TYMS can inhibit the proliferation and induce DNA damage in HCC cells (Yang

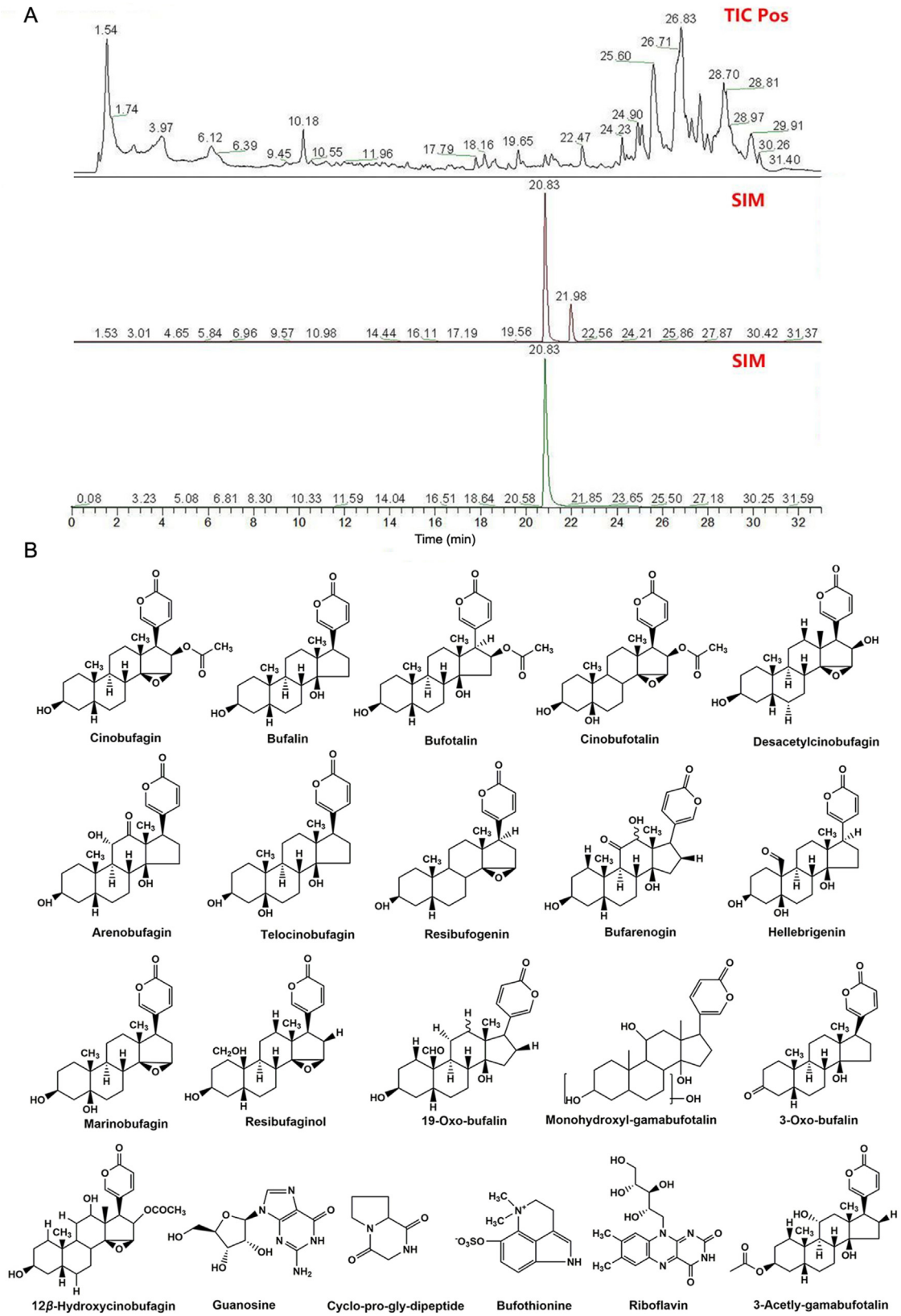

Fig. 1. UPLC-MS/MS analyses of Huachansu. (A) Total-ion chromatograms (TIC) of Huachansu, select-ion chromatograms (SIC) of cinobufagin in Huachansu, and cinobufagin reference in ESI$^+$ mode. (B) Structural formulas of 21 bioactive components identified from Huachansu.

et al., 2022). In this study, we proved that knockdown of TYMS increased the inhibitory effects of Huachansu on cell growth of HepG2 and SK-HEP-1 cells through using CCK-8 assay and colony formation assay (Fig. 6B–C), revealing that Huachansu inhibits the proliferation and leads to DNA damage partially through suppression of TYMS in HCC cells.

Table 1
Structural formulas of 21 bioactive components identified from Huachansu.

Peak No.	t_R (min)	ESI Mode	Formula	Theoretical mass (m/z)	Experimental mass (m/z)	Error (ppm)	MS^n	Indentification	References
1	20.83	$[M+H]^+$	$C_{26}H_{34}O_6$	443.242 81	443.242 13	−1.546	347.199 7, 251.179 5 , 183.080 4, 157.064 2, 145.101, 143.085 6	Cinobufagin	Juan et al., 2017; Hu, Yu, Yang, Zhu & Fong, 2011
2	19.87	$[M+H]^+$	$C_{24}H_{34}O_4$	387.252 98	387.252 50	−1.255	369.240 8, 351.231 7, 333.221 7, 255.210 2, 157.101 5, 143.085 2, 131.085 8	Bufalin/isomer	Han, 2018
3	18.16	$[M+H]^+$	$C_{26}H_{36}O_6$	445.258 46	445.257 78	−1.539	367.226 5, 349.215, 213.162 9, 143.085 5	Bufotalin	Han, 2018
4	16.05	$[M+H]^+$	$C_{26}H_{34}O_7$	459.237 72	459.237 15	−1.263	363.196 1, 145.101 3, 131.085 7, 91.054 7, 105.070 1	Cinobufotalin	Juan et al., 2017; Hu et al., 2011
5	18.08	$[M+H]^+$	$C_{24}H_{32}O_5$	401.232 25	401.231 66	−1.472	365.211 8, 215.798, 181.100 8, 159.116 9, 145.101 2, 143.085 5, 131.085 5, 119.085 8, 105.070 2, 91.054 7	Desacetylcinobufagin	Wu et al., 2020; Cao, Wang, Wang & Cui, 2009
6	15.43	$[M+H]^+$	$C_{24}H_{32}O_6$	417.227 16	417.226 29	−2.098	335.200 9, 363.194 6, 195.116 3, 183.116 8, 169.101 1, 159.080 4, 143.085 4, 129.069 7	Arenobufagin	Juan et al., 2017
7	16.65	$[M+H]^+$	$C_{24}H_{34}O_5$	403.247 90	403.247 41	−1.217	385.233 6, 367.225 4, 349.214 8, 339.231 0, 253.194 4, 215.178 9,199.148 7,197.131 9, 195.117 2, 173.132 3, 169.100 7, 159.116 6, 143.085 3	Telocinobufagin/isomer	Han, 2018
8	21.65	$[M+H]^+$	$C_{24}H_{32}O_4$	385.237 33	385.236 88	−1.184	367.227, 349.216 2, 321.221 1, 253.195 2, 159.117 1, 157.101 3, 155.085 7, 143.085 8	Resibufogenin	Ye & Guo, 2005
9	14.88	$[M+H]^+$	$C_{24}H_{32}O_6$	417.227 16	417.226 56	−1.451	399.299 1, 335.199 3, 159.080 3, 143.085 1	Bufarenogin	Liu, Xiao, Xue, Zhang & Liang, 2010; Wu et al., 2020
10	15.57	$[M+H]^+$	$C_{24}H_{32}O_6$	417.227 16	417.226 47	−1.666	363.195 1, 195.116 7, 183.116 6, 169.101 5, 159.116 7, 143.085 2,	Hellebrigenin	Hu et al., 2011; Juan et al., 2017
11	16.45	$[M+H]^+$	$C_{24}H_{32}O_5$	401.232 25	401.231 78	−1.173	383.220 7, 365.210 8, 185.132 2, 159.116 7, 105.070 2, 147.117 1, 145.101 2, 131.085 4, 119.085 5, 91.054 6	Marinobufagin	Ye & Guo, 2005
12	17.13	$[M+H]^+$	$C_{24}H_{32}O_5$	401.232 25	401.231 84	−1.023	383.221 7, 365.209 6, 337.216 3, 159.116 6, 147.116 4, 145.100 8, 131.085 2, 119.085 5, 105.070 2, 91.054 9	Resibufaginol	Ye & Guo, 2005
13	18.61	$[M+H]^+$	$C_{24}H_{32}O_5$	401.232 25	401.231 63	−1.547	365.209 7, 337.218 2, 159.117 2, 145.100 8, 131.085 5, 119.085 8, 105.07, 91.054 7	19-Oxo-bufalin	Wu et al., 2020
14	17.28	$[M+H]^+$	$C_{24}H_{34}O_6$	419.242 81	419.242 22	−1.420	401.232 9, 365.210 6, 251.177 9, 147.081, 105.070 2	Monohydroxyl-gamabufotalin	Juan et al., 2017
15	17.79	$[M+H]^+$	$C_{24}H_{32}O_4$	385.237 33	385.236 88	−1.184	367.225 5, 349.213 3, 321.954 7, 255.059 7, 253.195 6, 159.116 6, 157.101 7, 143.086 3	3-Oxo-bufalin	Juan et al., 2017
16	18.63	$[M+H]^+$	$C_{26}H_{34}O_7$	459.237 72	459.236 97	−1.655	381.206 3, 363.194, 159.116 8, 145.101 2, 119.085 5, 105.070 1	12β-Hydroxycinobufagin	Ye & Guo, 2005; Cao et al., 2009
17	2.30	$[M+H]^+$	$C_{10}H_{13}N_5O_5$	284.098 54	284.098 94	−1.426	284.075, 152.056 5, 135.03	Guanosine	Wu et al., 2020
18	2.70	$[M+H]^+$	$C_7H_{10}N_2O_2$	155.081 50	155.081 30	−1.316	155.081 4, 127.086 5, 115.086 7, 99.092 1, 82.065 7	Cyclo-pro-gly-dipeptide	Wu et al., 2020
19	6.45	$[M-H]^-$	$C_{12}H_{14}N_2O_4S$	283.074 70	283.074 55	−0.545	203.117 8, 188.094 4, 160.099 5, 58.065 8	Bufothionine	Wu et al., 2020
20	11.65	$[M+H]^+$	$C_{17}H_{20}N_4O_6$	377.145 56	377.145 20	−0.957	377.145, 243.087 6, 71.013 4	Riboflavin	Wu et al., 2020
21	19.25	$[M+H]^+$	$C_{26}H_{36}O_6$	445.258 46	445.257 97	−1.112	367.226 5, 349.216 2, 267.138 4, 253.194 4, 213.164 2, 169.101 2, 143.085 7	3-Acetly-gamabufotalin	Juan et al., 2017; Wu et al., 2020

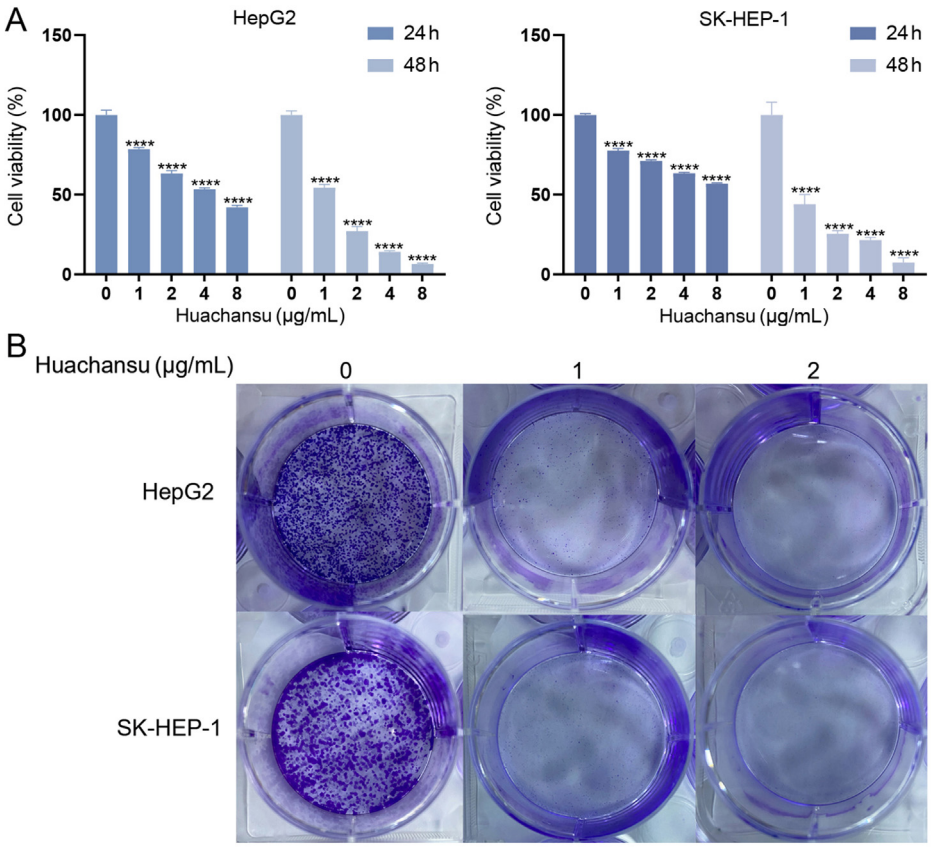

Fig. 2. Huachansu reduces cell viability of human hepatoma HepG2 and SK-HEP-1 cells. (A) HepG2 and SK-HEP-1 cells were cultured with various concentration of Huachansu for 24 h and 48 h, the cell viability was detected using the CCK-8 assay. (B) Representative images of the cell colony of tumor cells treated with or without Huachansu. Cancer cells were cultured with or without Huachansu for 7 d. $^{****}P < 0.000\ 1$ *vs* control group. All data are presented as the mean ± SEM. The data are representative of three independent experiments, and each was performed at least in triplicate.

3.8. Huachansu promotes proteasome-dependent degradation of TYMS in human hepatoma cells

For the purpose of clarifying the molecular mechanism of reduction of TYMS induced by Huachansu, we first used QRT-PCR to investigate the effect of Huachansu on TYMS mRNA level. As shown in Fig. 7A, the mRNA level of TYMS was not affected by Huachansu treatment, the results suggested that Huachansu treatment did not affect the transcription of TYMS. Therefore, we examined whether the stability of TYMS could be affected by Huachansu. Liver cancer cells were incubated with Huachansu with or without proteasome inhibitor MG132, and the immunoblotting data showed that MG132 completely recovered the Huachansu-induced reduction of TYMS (Fig. 7B). The protein synthesis inhibitor cycloheximide (CHX) was used to inhibit TYMS protein synthesis, and cells were treated with CHX with or without Huachansu, the results showed that CHX treatment alone could induce TYMS inhibition, and the treatment of Huachansu in combination with CHX could further inhibit the protein of TYMS (Fig. 7C). These results demonstrated that Huachansu promoted proteasome-dependent degradation of TYMS.

3.9. Growth inhibitory effects of Huachansu with 5–FU

5-FU is a TYMS inhibitor used for treatment of cancer (Schmitz & Chu, 2011). Our study demonstrated that Huachansu inhibited TYMS. Therefore, we investigated the effects of combination treatment of Huachansu and 5-FU on the growth of human hepatoma cells, we conducted the combination index (CI) to evaluate the combined effect of drugs, and CI <1, CI = 1, and CI > 1 indicate synergetic, additive, and antagonistic effect, respectively (Rodea-Palomares et al., 2010). As shown in Fig. 8A–B and Table 2, in HepG2 cells, lower concentration of Huachansu (0.5 μg/mL) with 5-FU (0.5 and 1 μg/mL) resulted in synergistic effects, whereas higher concentration of Huachansu (1 and 2 μg/mL) exhibited antagonistic effects with 5-FU. In SK-HEP-1 cells, lower concentration of 5-FU (0.25 μg/mL) with Huachansu produced synergistic effects, however most combinations of Huachansu and 5-FU (0.5 and 1 μg/mL Huachansu with 0.5 μg/mL of 5-FU; 0.5, 1, and 2 μg/mL Huachansu with 1 μg/mL of 5-FU) exhibited antagonistic effects. Our results indicated that combination of 5-FU and Huachansu exhibited different types of combination effects according to the different cell lines and drug concentrations, suggesting combination of 5-FU and Huachansu capsules or Huachansu injection in clinic should be accompanied by caution due to the possibility of antagonistic effects.

4. Discussion

HCC is the most common primary liver cancer, and it accounts for up to 90% of all primary hepatic malignancies (Grandhi et al., 2016). Most HCC patients are diagnosed at an advanced stage of the disease. Currently, the only targeted drug approved by FDA to treat advanced liver cancer is sorafenib (Dimri & Satyanarayana, 2020). Treatment of HCC with TCM or natural medicine has a long history, which could be commonly used as an adjunct to enhance the efficacy of chemotherapy and reduce the side effects of cancer chemotherapies (Cheng et al., 2019). Hua-

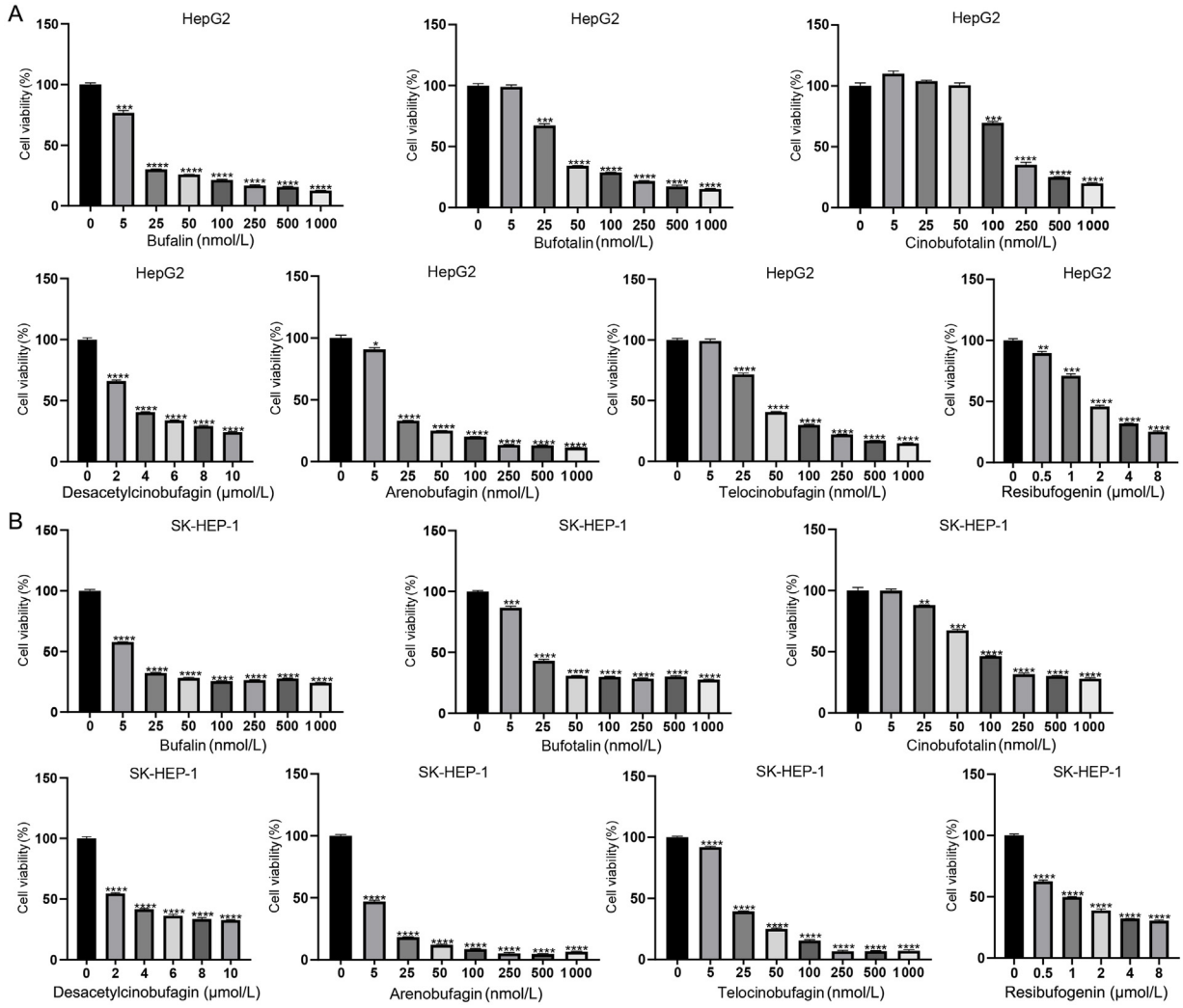

Fig. 3. Bufadienolides identified from Huachansu inhibit proliferation of human hepatoma HepG2 and SK-HEP-1 cells. Effects of Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin on the proliferation of (A) HepG2 and (B) SK-HEP-1 cells were evaluated using the CCK-8 assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.000\ 1$ *vs* control group. All data are presented as the mean ± SEM. The data are representative of three independent experiments, and each was performed at least in triplicate.

chansu is active extract isolated from the TCM dried toad skin of *Bufo bufo gargarizans* Cantor and *Bufo melanostictus* Schneider, which is used for the clinical treatment of cancer, such as liver, lung, and colorectal cancers (Meng et al., 2012). Previous research has identified that bufadienolides are the primary bioactive components of Huachansu, such as cinobufagin, bufalin, resibufogenin (Huang et al., 2020). In our study, we first identify 21 bioactive chemical constituents from Huachansu by UPLC/MS-MS, a large proportion of which belong to bufadienolides. Previous research has shown that Huachansu synergistically enhances the efficacy and reduces toxicity of the conventional chemotherapy (Meng et al., 2009). Published report has shown that Huachansu Capsule inhibits the growth of gastric cancer cells via inhibiting AKT signaling (Ni et al., 2019). Liu et al have demonstrated that Huachansu causes HCC cells apoptosis and DNA damage via suppression of TOPO I and TOPO II (Liu et al., 2015). Qi et al have proved that Huachansu triggers apoptosis of liver cancer cells via mitochondria- and Fas-mediated pathways (Qi et al., 2012). Huachansu has been reported to inhibit migration and invasion of colon cancer cells through inhibiting EMT and Wnt/β-catenin signaling pathways (Wang et al., 2020).

TYMS is frequently over-expressed in human malignant tumors (Wang et al., 2017), TYMS is considered to be a key enzyme that affects the prognosis of patients with various cancers, and TYMS level shows a negative correlation with the survival probability of cancer patients (Ajiki et al., 2006; Nii et al., 2009). Furthermore, our previous study has revealed that knockdown of TYMS inhibits the growth of HCC cells (Yang et al., 2022). We demonstrate that Huachansu and bufadienolides (Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin) identified from Huachansu by UPLC-MS/MS markedly inhibit the proliferation of HCC cells and inhibit the protein level of TYMS. Furthermore, we find that depletion of TYMS enhances the suppressive effects of Huachansu on the growth of HCC cells.

The DNA damage reaction causes cell cycle block and induces DNA repair. If the damaged lesions are successfully repaired, the cells will survive, and cells with more severe damage are induced to undergo apoptosis (Gasser & Raulet, 2006; Mj, 2015). Published report has shown that inhibition of TYMS leads to an imbalance between the level of dUTP and dTTP, as a result, induces a reduction in DNA synthesis efficiency. In addition, the dUTP-dTTP imbalance leads to misincorporation of dUTP into DNA and causes DNA

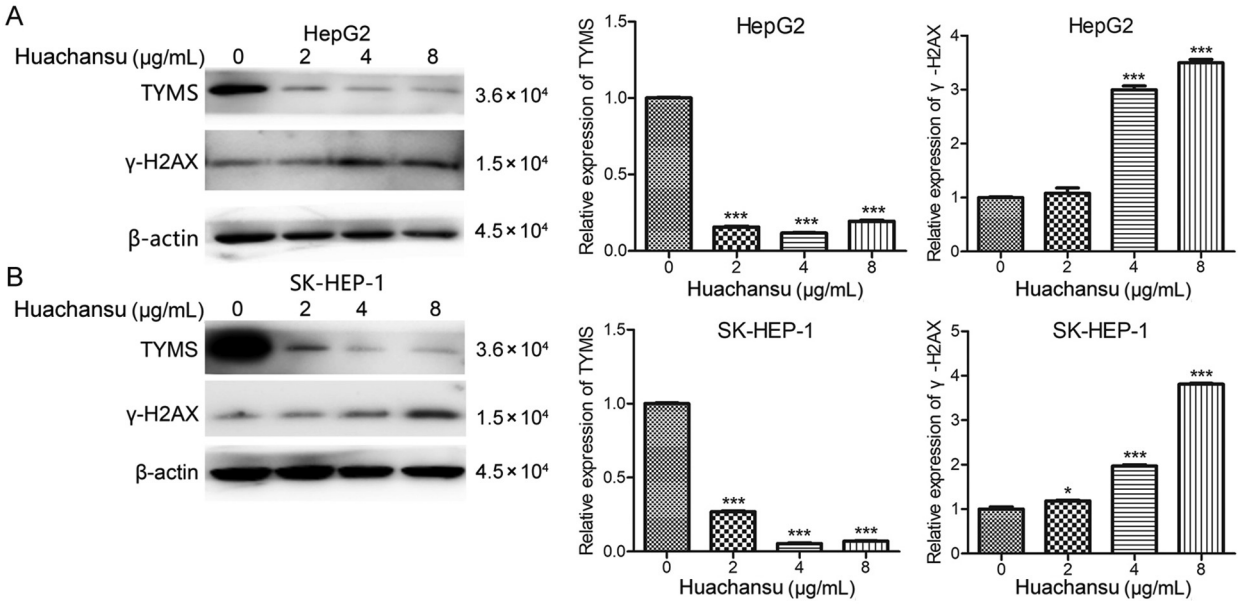

Fig. 4. Huachansu inhibits TYMS and promotes γ-H2AX expression in HepG2 and SK-HEP-1 cells. (A) HepG2 or (B) SK-HEP-1 cells were exposed to Huachansu for 24 h, the protein expression of TYMS or γ-H2AX was examined through western blotting. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* control group. All data are presented as the mean ± SEM. The data are representative of three independent experiments, and each was performed at least in triplicate.

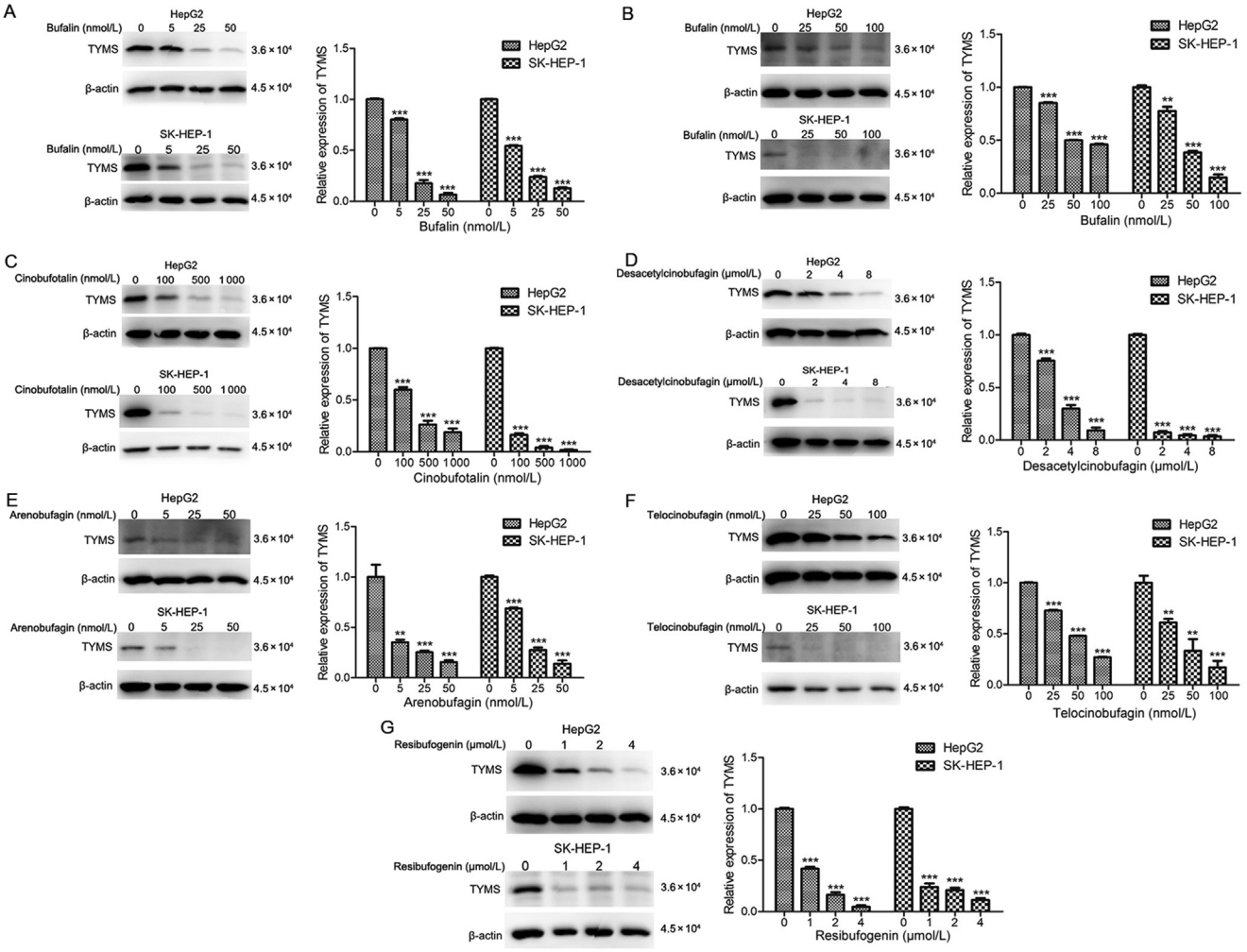

Fig. 5. Bufadienolides identified from Huachansu inhibit TYMS expression in HepG2 and SK-HEP-1 cells. TYMS protein level was determined through Western blotting after treatment with various concentrations of (A) Bufalin, (B) Bufotalin, (C) Cinobufotalin, (D) Desacetylcinobufagin, (E) Arenobufagin, (F) Telocinobufagin, or (G) Resibufogenin in HepG2 and SK-HEP-1 cells. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control group. All data are presented as the mean ± SEM. The data are representative of three independent experiments, and each was performed at least in triplicate.

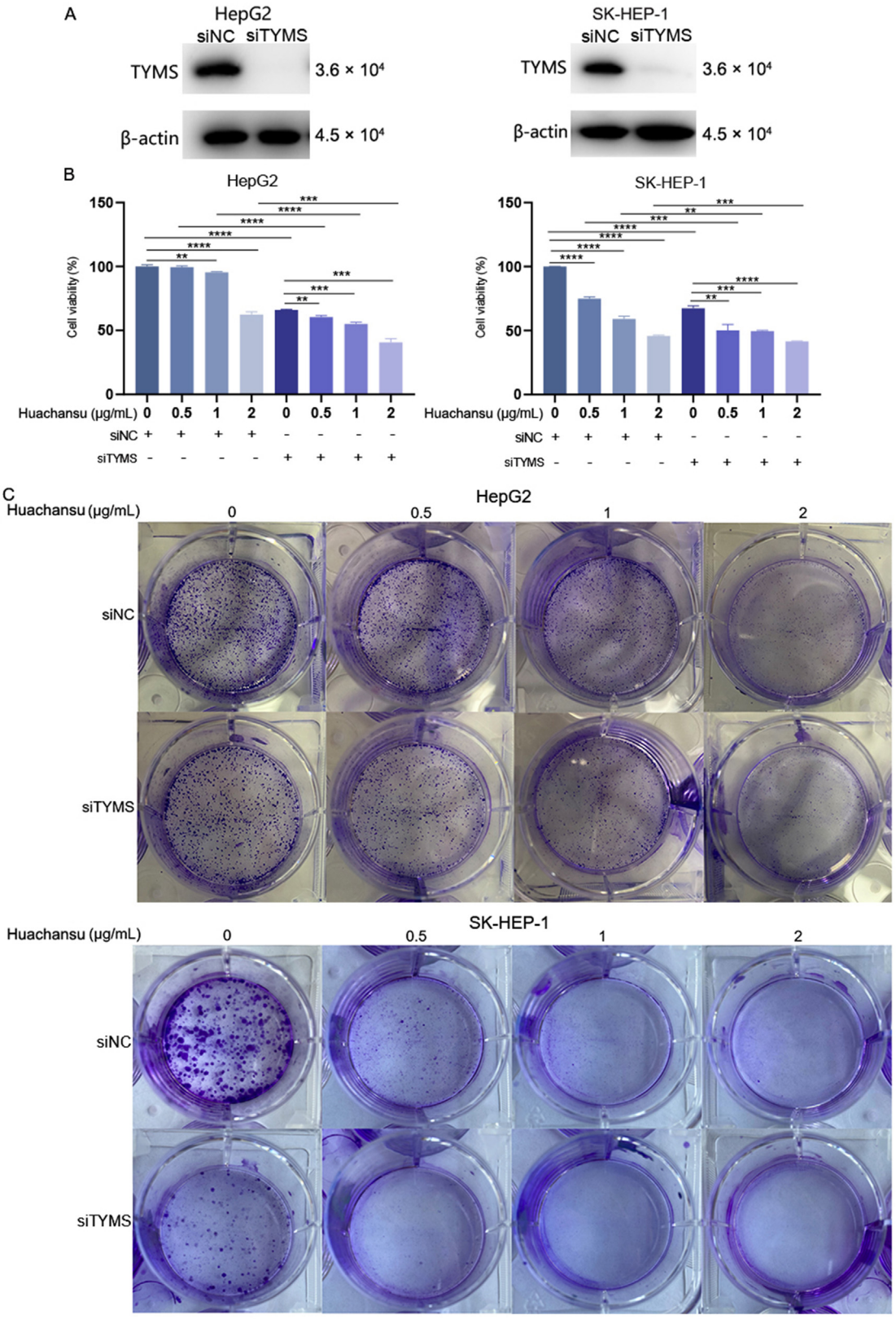

Fig. 6. Huachansu inhibits proliferation and induces DNA damage via down-regulation of TYMS in HepG2 and SK-HEP-1 cells. (A) Cells were transfected with TYMS siRNAs or control siRNAs for 48 h, then cell lysates were subjected to immunoblotting analysis for evaluation of TYMS protein level. (B-C) Cells transfected with TYMS siRNAs or control siRNAs were treated with or without Huachansu for 48 h, then the cell viability was examined by CCK-8 assay and colony formation assay. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.000\,1$, siNC (0.5, 1, 2 μg/mL) *vs* siNC (0 μg/mL) or siTYMS (0.5, 1, 2 μg/mL) *vs* siTYMS (0 μg/mL) or siNC (0, 0.5, 1, 2 μg/mL) *vs* siTYMS (0, 0.5, 1, 2 μg/mL), respectively. All data are presented as the mean ± SEM. The data are representative of three independent experiments, and each was performed at least in triplicate.

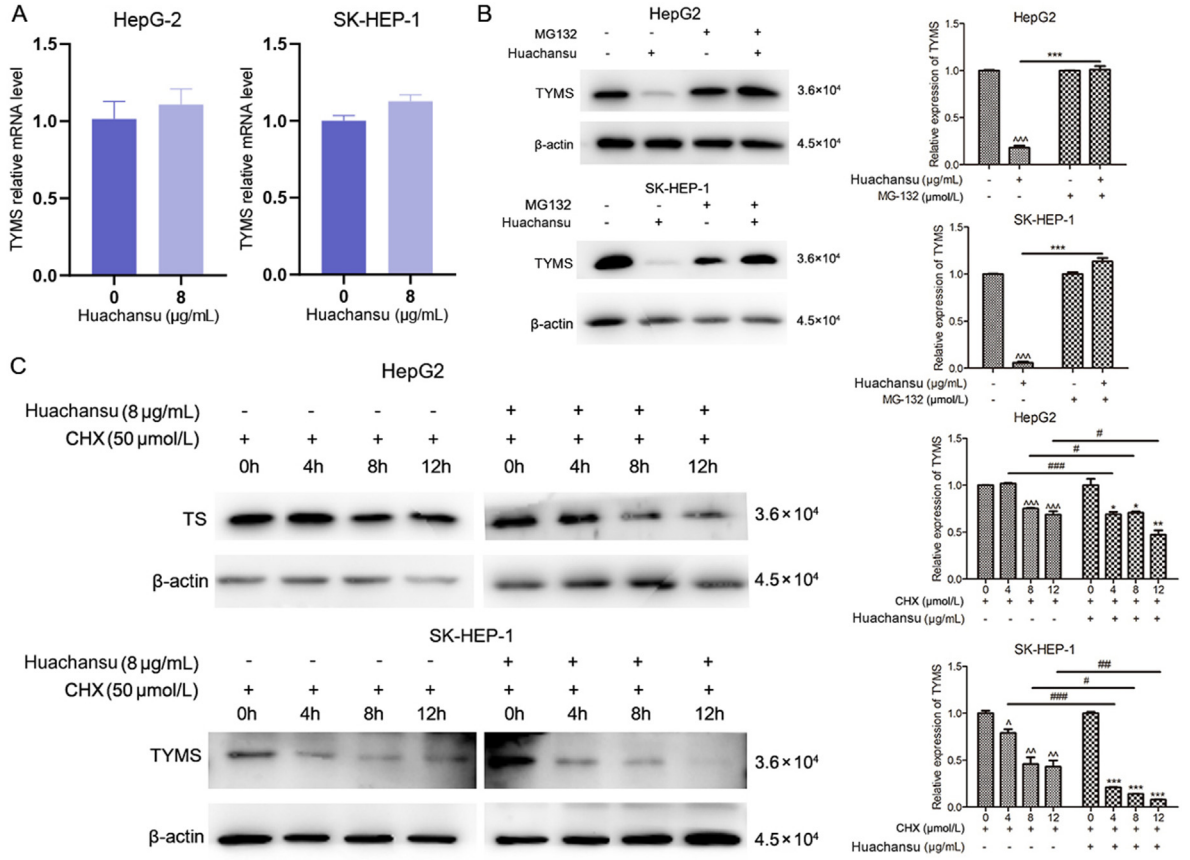

Fig. 7. Huachansu down-regulates TYMS through proteasomal degradation pathway in HepG2 and SK-HEP-1. (A) Evaluation of TYMS mRNA level by quantitative RT-PCR in HepG2 and SK-HEP-1 cells incubated with or without Huachansu. (B) HepG2 and SK-HEP-1 cells were cultured with Huachansu in the absence or presence of MG132 (20 μmol/L), TYMS protein level was detected by western blotting. All data are presented as the mean ± SEM. $^{\wedge\wedge\wedge}P < 0.001$ *vs* control group; $^{***}P < 0.001$, Huachansu group *vs* MG-132 + Huachansu group. (C) Cancer cells were incubated with CHX (50 μmol/L) in the absence or presence of Huachansu for different points of time followed by western blotting for the evaluation of TYMS protein level. All data are presented as the mean ± SEM. $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ *vs* CHX-0 h group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* CHX + Huachansu-0 h group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, CHX (0, 4, 8, 12 h) *vs* CHX + Huachansu (0, 4, 8, 12 h), respectively. The data are representative of three independent experiments, and each was performed at least in triplicate.

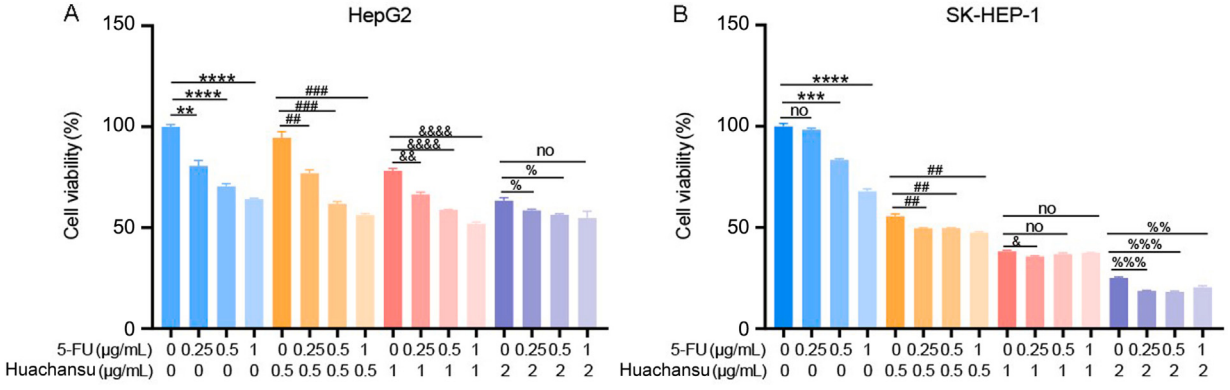

Fig. 8. Effects of combination of Huachansu and 5-FU in suppression of human hepatoma cells proliferation. (A) HepG2 and (B) SK-HEP-1 cells were treated with Huachansu in combination with 5-FU for 48 h, the cell viability was examined using the CCK-8 assay. *: 5-FU (0.25, 0.5, 1 μg/mL) *vs* control, respectively; #: 5-FU (0.25, 0.5, 1 μg/mL) + Huachansu (0.5 μg/mL) *vs* Huachansu (0.5 μg/mL), respectively; &: 5-FU (0.25, 0.5, 1 μg/mL) + Huachansu (1 μg/mL) *vs* Huachansu (1 μg/mL), respectively; %: 5-FU (0.25, 0.5, 1 μg/mL) + Huachansu (2 μg/mL) *vs* Huachansu (2 μg/mL), respectively. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.000\,1$; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&\&}P < 0.000\,1$; $^{\%}P < 0.05$, $^{\%\%}P < 0.01$, $^{\%\%\%}P < 0.001$. All data are presented as the mean ± SEM. The data are representative of three independent experiments, and each was performed at least in triplicate.

damage (Takezawa et al., 2010). Among the variety of types of DNA damage, the DNA double-strand break is the most deleterious. In addition, our previous study has shown that suppression of TYMS enhances the protein level of γ-H2AX, the marker of DNA damage (Yang et al., 2022). In our present study, we uncover that Huachansu leads to DNA damage of HCC cells, indicating that Huachansu induces DNA damage of HCC cells via down-regulation of TYMS.

Table 2
CI index of Huachansu in combination with 5-FU.

5-FU (μg/mL)	Huachansu (μg/mL)	CI	
		HepG2	SK-HEP-1
0.25	0.5	1.030	0.900
0.5	0.5	0.877	1.068
1	0.5	0.897	1.186
0.25	1	1.103	0.973
0.5	1	1.091	1.080
1	1	1.036	1.187
0.25	2	1.179	0.927
0.5	2	1.272	0.968
1	2	1.313	1.044

The proteasome is a large multi-subunit complex which consists of several dozen proteins, and it is responsible for the degradation of protein in cells (Takeuchi, Chen & Coffino, 2007). Degradation of protein within the cell is performed mainly through the 26S proteasome which functions in recognizing and degrading its target substrates, and, polyubiquitin chains covalently attach to the target substrate, which mediate the latter's recognition and delivery to the proteasome. However, several proteasomal substrates such as TYMS, ODC (ornithine decarboxylase), p53, c-Jun, and c-Fos are degraded without the need for ubiquitin modification. The degradation of TYMS requires both the 20S and 19S proteasome complexes (Erales & Coffino, 2014). Our present study shows that TYMS mRNA level is not affected by Huachansu, additionally, we demonstrate that proteasome inhibitor MG132 completely reverses the inhibition of TYMS in response to Huachansu, and Huachansu could enhance the suppression of TYMS induced by protein synthesis inhibitor CHX, indicating Huachansu promotes proteasome-dependent degradation of TYMS.

Published study has reported that compared to platinum-based chemotherapy (PBC) alone, Huachansu plus PBC improve the survival rate of cancer patients (Tan et al., 2021). Huachansu combined with gemcitabine-oxaliplatin could enhance clinical curative effect (Qin et al., 2008). 5-FU, as a TYMS inhibitor, is a first-line drug used for the treatment of several cancer types. We uncover Huachansu and eight bufadienolides (Cinobufagin, Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin) identified from Huachansu significantly inhibit TYMS, so we investigate the effects of combination treatment of Huachansu and 5-FU on the proliferation of HCC cells, we uncover that the effects of combination treatment of different doses of Huachansu and 5-FU exhibit antagonistic or synergistic. Thus, in clinic, attention should be paid to the dose of 5-FU and Huachansu when these two drugs are combined.

5. Conclusion

In summary, we identify 21 major bioactive components from Huachansu (Cinobufacini), and illustrate that Huachansu and bufadienolides identified from Huachansu inhibit the proliferation of HCC cells. Mechanism studies indicate that Huachansu inhibits proliferation and induces DNA damage of HCC cells through down-regulation of TYMS. In addition, Huachansu promotes proteasome-dependent degradation of TYMS in HCC cells. More importantly, we demonstrate that bufadienolides identified from Huachansu such as Bufalin, Bufotalin, Cinobufotalin, Desacetylcinobufagin, Arenobufagin, Telocinobufagin, and Resibufogenin could also significantly suppress the protein level of TYMS, uncovering the material basis of inhibition of TYMS induced by Huachansu. Furthermore, we demonstrate that the effects of combination treatment of 5-FU and Huachansu exhibit antagonistic or synergistic, implying more attention should be given to the combination of these two drugs in clinical practice. Our study provides a theoretical basis for the clinical application of Huachansu, and promotes the development of new anti-tumor drugs from TCM.

CRediT authorship contribution statement

Qi Wu: Project administration, Validation, Data curation, Formal analysis, Visualization, Writing – original draft. **Qimei Chen:** Data curation, Formal analysis, Writing – review & editing. **Jingyi Yang:** Formal analysis, Writing – review & editing. **Jiayu Zhang:** Conceptualization, Project administration, Validation, Writing – original draft, Writing – review & editing. **Ailin Yang:** Conceptualization, Data curation, Project administration, Validation, Writing – original draft, 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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