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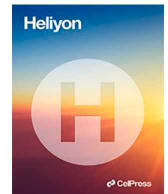

Research article

Network pharmacology, bioinformatics, and experimental validation to identify the role of *Hedyotis diffusa* willd against gastric cancer through the activation of the endoplasmic reticulum stress

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ABSTRACT

Background: Globally, gastric cancer (GC) is recognized as the third leading cause of cancer-related deaths and the fifth most prevalent malignant disease. Multiple studies have indicated that *Hedyotis diffusa* Willd, in pinyin, called Bai Hua She Cao (BHSSC), a traditional Chinese medicine (TCM) is an herbal remedy for cancer treatment. However, the specific mechanisms underlying its anti-tumor properties and mode of action are still unclear.

Methods: To determine the role of BHSSC in GC, candidate target genes were selected from The Encyclopedia of Traditional Chinese Medicine (ETCM) and analyzed using network pharmacology, bioinformatics, and experimental validation. Differentially expressed genes (DEGs) associated with gastric cancer were obtained from RNA sequencing (RNA-seq) data sourced from The Cancer Genome Atlas-Stomach adenocarcinoma (TCGA-STAD). The Reactome Pathway was examined using Analysis Tools, while KEGG pathways were analyzed using KOBAS. Gene Ontology (GO) evaluations were performed using WebGestalt and DAVID. The relationships between proteins were investigated using the STRING database. Furthermore, cell viability, colony formation, and cell migration ability were conducted in gastric cancer cells, BGC-823 and MGC-803.

Results: Network pharmacology and bioinformatics analyses revealed a significant association between BHSSC and metabolic pathways. *In vitro* experiments demonstrated that BHSSC effectively suppressed gastric cancer cell proliferation and colony formation, inhibited cell migration, and activated the endoplasmic reticulum (ER) stress. Furthermore, it was found that enhancement of the expression of IRE1α and BIP is the mechanism by which BHSSC activates ER stress.

Conclusions: The findings suggest that BHSSC exerts its effects through modulation of metabolic pathways, leading to the suppression of cell proliferation, inhibition of cell migration, and activation of the endoplasmic reticulum. These results provide valuable insights into the mechanisms underlying the therapeutic effects of BHSSC in GC and support its potential as a novel treatment option.

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Abbreviations

GC gastric cancer
BHSSC *hedyotis diffusa* Willd; Bai Hua She She Cao
TCM traditional Chinese medicine
ETCM Encyclopedia of Traditional Chinese Medicine
RNA-seq RNA sequencing
DEG differentially expressed genes
TCGA-STAD The Cancer Genome Atlas-Stomach adenocarcinoma
GO Gene Ontology
ER endoplasmic reticulum
UPR unfolded protein response;
PMSF Phenylmethanesulfonyl fluoride
PBS Phosphate Buffered Saline
RPMI Roswell Park Memorial Institute
CCK-8 Cell Counting Kit-8
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PPI Protein–protein interaction
CC cellular component
MF molecular function
BP biological process
NTA Network Topology-based Analysis
IRE1 inositol-requiring enzyme 1
ATF6 activating transcription factor 6
PERK double-stranded RNA-activated protein kinase-like ER kinase.

1. Introduction

Gastric cancer (GC), the third leading cause of cancer-related deaths worldwide, poses a significant threat to society and the general public. The development of GC is influenced by various factors, including advanced age, excessive salt consumption, *Helicobacter pylori* infection, and a history of inadequate fruit and vegetable intake [1]. The primary approach for treating early-stage stomach cancer is through endoscopic resection. For non-early-stage GC, surgical intervention is typically required, which involves performing a D2 lymphadenectomy. This procedure entails the removal of lymph nodes near the stomach mesentery and the branches of the celiac artery. To mitigate postoperative side effects, improve physical health, and enhance survival, patients with postoperative GC may attempt traditional Chinese medicine adjuvant therapies [2,3].

In traditional Chinese medicine, *Hedyotis diffusa* Willd (Family Rubiaceae) is commonly known as Bai Hua She She Cao (BHSSC) in pinyin. According to contemporary pharmacological research, BHSSC possesses a wide range of pharmacological properties, including anti-inflammatory [4], hepatoprotective effect [5,6], antiviral efficacy [7], antioxidant [8] and neuroprotective [9], anti-tumor [10], and other biological activities.

Various genetic and environmental factors can impair the cell's ability to fold and modify proteins in the endoplasmic reticulum (ER), leading to ER stress and the buildup of misfolded proteins in this organelle. In response, the unfolded protein response (UPR) signaling system is activated, triggering a series of transcriptional and translational events to restore ER homeostasis in the presence of a significant load of misfolded proteins in the ER. However, cells eventually start to self-destruct if ER stress levels are consistently high. Diseases like diabetes, cancer, and neurodegeneration might be triggered by ER stress and UPR signaling [11–13]. It has been demonstrated that ER stress and UPR are essential for the pathogenesis, development, and therapeutic response of many malignancies [14]. Studies have demonstrated the essential role of ER stress and UPR in the pathogenesis, development, and therapeutic response of many malignancies, making the targeting of ER stress and UPR signaling an attractive therapeutic strategy for GC [11,15–17].

BHSSC has a long history of clinical use as an anti-tumor drug [18–20]. The study of BHSSC 's anticancer abilities is currently an increasingly popular subject. However, the mechanisms behind its anti-tumor cell activity and mode of action remain unclear. Therefore, this study intended to determine whether its putative mode of action influences ER stress and UPR signaling in its anti-GC effects.

2. Methods and materials

2.1. Drug target genes analysis

The candidate target genes of BHSSC were from The Encyclopedia of Traditional Chinese Medicine (ETCM, http://www.tcmip.cn/ETCM/index.php/Home/Index/yc_details.html?id=795). The differential expressed genes (DEG) of gastric cancer were downloaded from the Cancer Genome Atlas-Stomach adenocarcinoma (TCGA-STAD, https://portal.gdc.cancer.gov/exploration) and analyzed by R

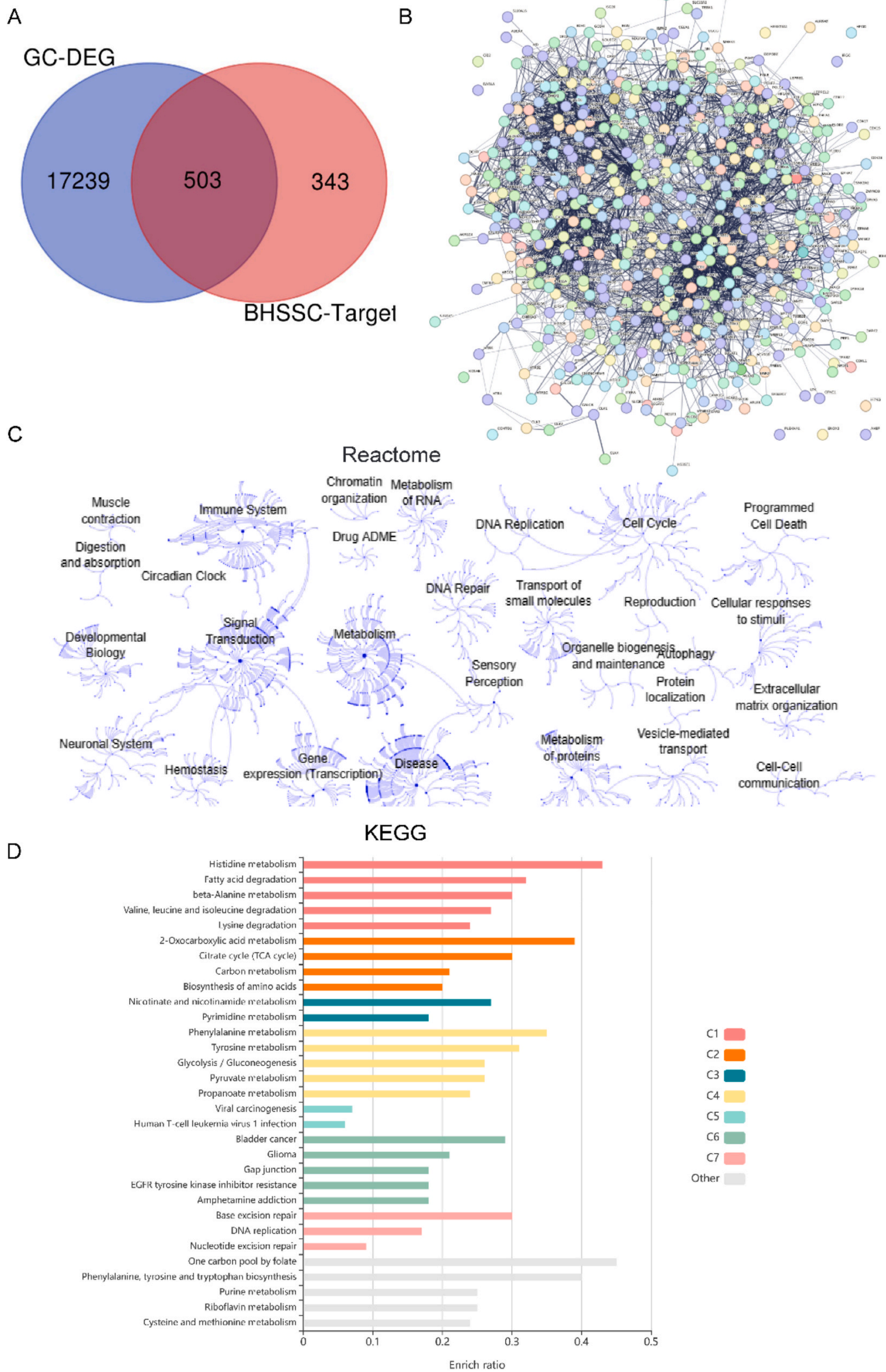

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Fig. 1. The potential targets of *Hedyotis diffusa* Willd, in pinyin, called Bai Hua She Cao (BHSSC) in Gastirc cancer (GC) *via* reactome and KEGG analysis. 503 drug-disease target genes were extracted by GC targets from TCGA-STAD and BHSSC targets from The Encyclopedia of Traditional Chinese Medicine (ETCM). Venn was drawn by the Venn online tool. Protein–protein interaction (PPI) was conducted by the STRING database in 2023. The Reactome Pathway was analyzed using Analysis Tools. KEGG pathway analysis was conducted using KOBAS. A. Venn of 503 drug-disease target genes. B. PPI of 503 drug-disease target genes. C. Reactome pathway analysis of 503 drug-disease target genes. D. KEGG pathway analysis of 503 drug-disease target genes.

Foundation with a fold change cut-off of 1.3 and P-value <0.05. Venn Diagram was drawn online (https://bioinformatics.psb.ugent.be/webtools/Venn/). The protein–protein association network was analyzed by the STRING database in 2023 (https://cn.string-db.org/cgi/input?sessionId=b4apxqd43I6Z&input_page_show_search=on) [21]. The Reactome Pathway was analyzed using Analysis Tools (https://reactome.org/) [22]. KEGG pathway analysis was conducted using KOBAS (http://kobas.cbi.pku.edu.cn/) [23].

2.2. Gene ontology (GO) analysis

GO analysis was conducted by WebGestalt (www.webgestalt.org) and DAVID (https://david.ncifcrf.gov). Network Topology-based Analysis (NTA) based on TCGA-RNASeq-STAD is conducted by WebGestalt.

2.3. The preparation of BHSSC water extracts

BHSSC water extracts crude powder (Sanyuan Longsheng biotechnology co. LTD, Batch No: LXBHSSC20200819) was prepared through 10-fold water boiled for 1.5 h twice and then spray dried. 1g BHSSC Extracts crude powder was dissolved in 10 mL purified water, filtered with 0.22 μM membrane and stored at −20 °C before use.

2.4. Cell culture

Gastric cancer BGC-823 cells and MGC-803 cells (Shanghai Cell Bank of the Chinese Academy of Sciences Shanghai Institute of Cell Biology, Shanghai, China) were cultured with Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher Scientific, USA) containing 10% Fetal Bovine Serum (Gibco, Thermo Fisher Scientific, USA).

2.5. Cell viability

Cell viability was conducted by CCK-8 assay [24]. BGC-823 cells and MGC-803 cells were seeded with 6 $x10^{3}$ cells/well in a 96-well plate and different doses of BHSSC (0–50 mg/mL) were added and incubated for 48 h. After discarding the medium containing different doses of BHSSC, 10% of the total volume of the CCK8 reagent (10 μL CCK8 solutions into 90 μL medium) was added to the medium and incubated for 2 h at 37 °C. The absorbance of OD_{450} nm was measured using a microplate (Thermo Scientific™ Multiskan Sky, Thermo Fisher Scientific, USA).

2.6. Cell colony formation

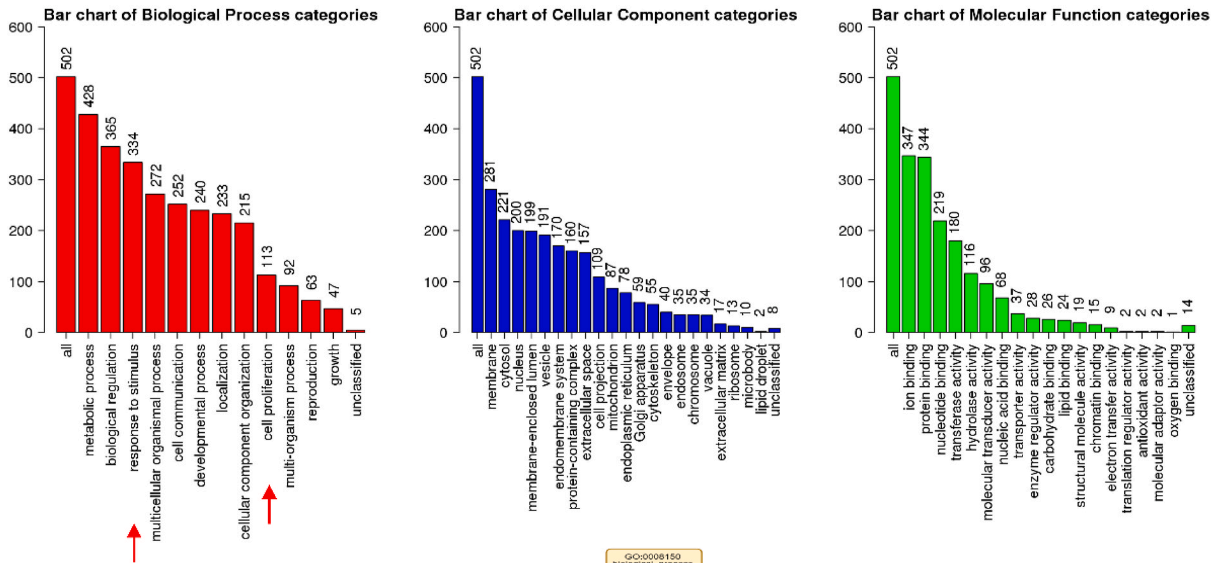
BGC-823 cells and MGC-803 cells were seeded with 500 cells/well in a 12-well plate and then treated with BHSSC (0, 3.125, 6.25 mg/mL) for 48 h. Next, the cell culture medium was replaced, and subsequently refreshed every three days. On the tenth day, the cell culture medium was discarded, the cells were washed with 1 mL phosphate-buffered saline (PBS, Gibco, Thermo Fisher Scientific, USA), fixed with 0.5 mL methanol for 15 min, and stained with 0.5 mL 0.1% crystal violet for 30 min. The liquid was then discarded and washed three times with water and photographed and analyzed for statistics using Image J software (National Institutes of Health, USA).

2.7. Wound healing assay

A wound healing assay was conducted to detect cell migration [25]. BGC-823 cells and MGC-803 cells were cultured with 5 $x10^{5}$ cells/well in a 24-well plate and after reaching 100% confluency, cells were scratched into a wound with a 10 μL tip and washed with PBS three times and photographed t 0 h. Then cells were treated with RPMI 1640 medium containing BHSSC (0, 3.125, 6.25 mg/mL) for 24 h then washed with PBS three times and photographed at 24 h. Wound width was measured using Image J software (National Institutes of Health, USA). The relative ratio of wound healing rates was analyzed and wounds were shown to observe cell migration.

2.8. Western-blot

BGC-823 cells and MGC-803 cells were plated in a 6-well plate with 5 $x10^{5}$ cells/well. Then cells were treated with BHSSC (0, 3.125, 6.25 mg/mL) for 48 h, and proteins were collected with RIPA-containing 1 mM Phenylmethanesulfonyl fluoride (PMSF, Beyotime, China) and 5 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate and 1 mM sodium orthovanadate (Phosphatase inhibitor cocktail A, Beyotime, China). Protein concentration was measured by BCA Protein Assay kit. 40 μg



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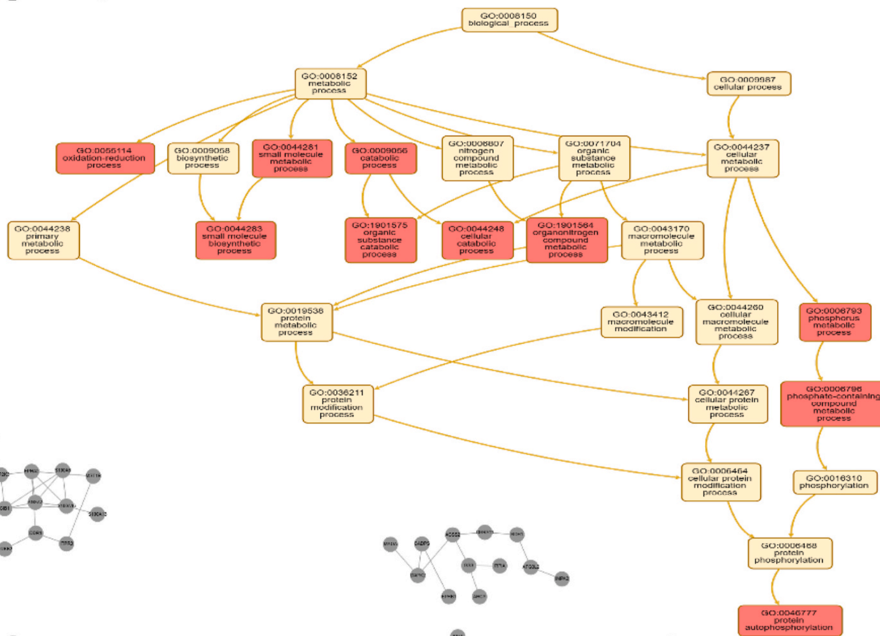

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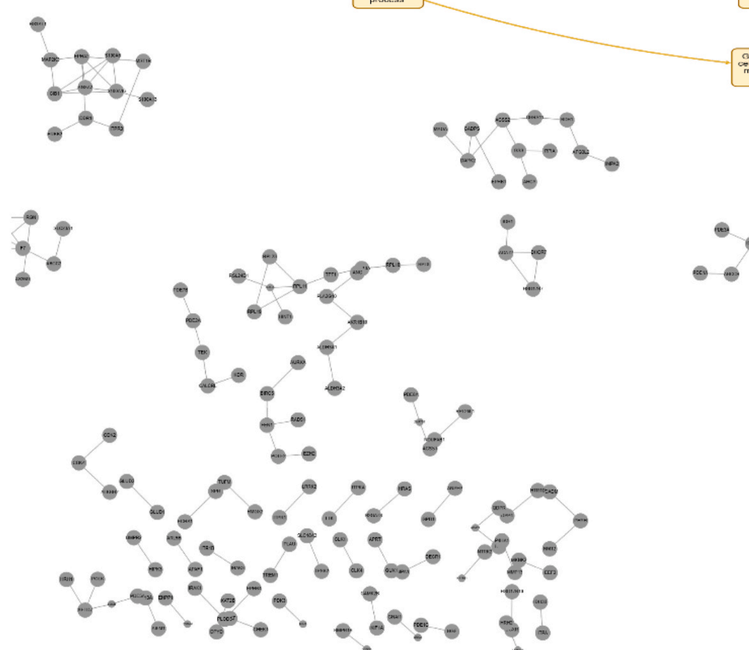

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Fig. 2. The potential targets of *Hedyotis diffusa* Willd, in pinyin, called Bai Hua She Cao (BHSSC) in gastric cancer (GC) were analyzed through Gene Ontology (GO) analysis. Drug-disease target genes were extracted by GC targets from TCGA-STAD and BHSSC targets from The Encyclopedia of Traditional Chinese Medicine (ETCM). GO analysis was conducted by WebGestalt. Network Topology-based Analysis (NTA) based on TCGA-RNA Seq-STAD is conducted by WebGestalt. A. The process of GO analysis, including BP, CC, and MF. B. The network diagram of GO analysis. C. Network Topology-based Analysis. CC represents cellular component; MF represents molecular function; BP represents biological process.

denatured proteins were loaded into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. SDS-PAGE electrophoresis was performed at 80V for 30 min, 120V for 1 h, and then transferred into the PVDF membrane at 250 mA for 2 h, next incubated with 5% skimmed milk powder for 1 h. After incubating the primary antibody IRE1α (14C10) Rabbit mAb 3294 (1:2000, Cell Signal Technology), BiP (C50B12) Rabbit mAb 3177 (1:2000, Cell Signal Technology) and GAPDH Mouse Monoclonal Antibody (1:2000, Beyotime) overnight at 4 °C and the other day incubating the secondary antibody Anti-rabbit IgG, HRP-linked Antibody 7074 or Anti-mouse IgG, HRP-linked Antibody 7076 (1:5000, Cell Signal Technology) for 1 h, the protein expression was detected using BeyoECL Plus (Beyotime, China) in a Visualizer (ChemiScope 6200, Clinx Science Instruments). The grayscale value was analyzed using Image J.

2.9. Statistical analysis

Results from three independent experiments were graphed by GraphPad 8.0 and analyzed using a *t*-test or ANOVA. A significant difference was shown with $^{**}P < 0.01$ or $^{*}P < 0.05$.

3. Results

3.1. The potential targets of BHSSC in GC via reactome and KEGG analysis

From Fig. 1A, there are 503 target genes of BHSSC in GC. A protein-protein association network through STRING was constructed by analyzing these 503 target genes (Fig. 1B). To identify these genes' functions, reactome and KEGG pathway analyses were also conducted using Analysis Tools and KOBAS respectively (Fig. 1C and 1D). The reactome pathway showed that the role of BHSSC in GC involves transcription, metabolism, signal transduction, immune system, cell cycle, DNA repair, DNA replication, cellular responses to stimuli, programmed cell death and etc. KEGG pathway revealed metabolism is the main contributor to its role, such as histidine metabolism, fatty acid degradation, beta-alanine metabolism, lysine degradation, 2-oxocarboxylic acid metabolism, citrate cycle, carbon metabolism, phenylalanine metabolism, tyrosine metabolism, glycolysis and so on.

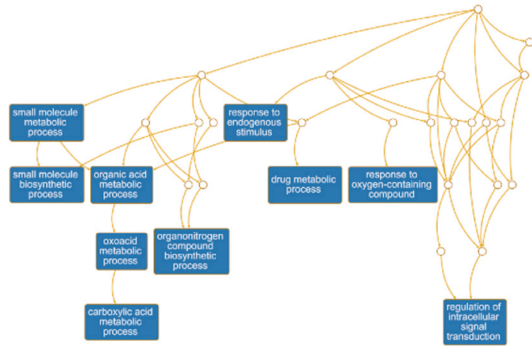
3.2. The potential targets of BHSSC in GC via GO analysis

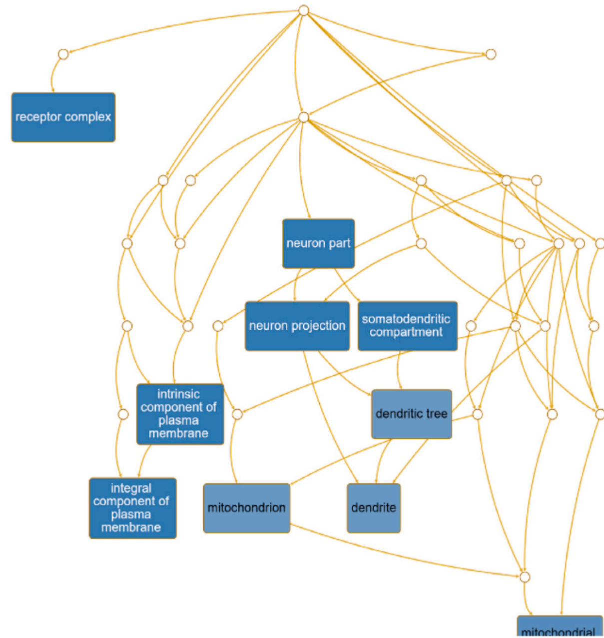
In order to clarify the functions of genes, GO is divided into three parts: cellular component (CC), molecular function (MF), and biological process (BP). By using the GO database, we can get information about what our target genes are mainly related to at the CC, MF, and BP levels. However, in WebGestalt, the interesting genes list contains 503 user IDs in which 502 user IDs are unambiguously mapped to 502 unique Entrez gene IDs and 1 user ID cannot be mapped to any Entrez gene ID. Thus, based on WebGestalt, 502 genes were analyzed. As shown in Fig. 2A and B, GO analysis showed that the BP of these genes was related to metabolic processes, response to stimulus, cell communication, cell proliferation, reproduction, and growth. The CC mainly consists of membrane, cytosol, nucleus, membrane-enclosed lumen, vesicle, endomembrane system, protein-containing complex, extracellular space, cell projection, mitochondrion, endoplasmic reticulum, and Golgi apparatus. The MF is mainly associated with ion binding, protein binding, nucleotide binding, transferase activity, hydrolase activity, molecular transducer activity and etc. Then, Network Topology-based Analysis (NTA) based on TCGA-RNASeq-STAD was constructed as depicted in Fig. 2C. In addition, to better understand the complexity of gene functions and interactions, providing guidance and insights for further research, the top 10 functions of the GO network diagram were also constructed as shown in Fig. 3.

Since WebGestalt can only identify and analyze the functions of 502 DEGs, we selected DAVID for analysis in order to clarify the functions of 503 differential genes. As shown in Fig. 4, the results of CC and MF are similar to WebGestalt, while the results of BP are more specific. BP analysis found that it was mainly related to signal transduction, protein phosphorylation, protein autophosphorylation, peptidyl-tyrosine phosphorylation, regulation of cell proliferation, transmembrane receptor protein tyrosine kinase signaling pathway, G-protein coupled receptor signaling pathway, and cell migration.

3.3. BHSSC suppresses cell proliferation and colony formation of GC cells in vitro

To validate the results of the network pharmacological GO analysis, we performed an experimental verification. Predictive analysis revealed that the role of BHSSC in GC may be associated with cell proliferation. The experimental results showed that BHSSC significantly inhibited the proliferation of gastric cancer cells, and the values of its IC_{50} in BGC-823 and MGC-803 for 48 h were 13.41 mg/mL, and 6.537 mg/mL individually (Fig. 5A). Additionally, we conducted a cell viability assessment of BHSSC on normal gastric cells GES-1 for a duration of 48 h. Results revealed that BHSSC (at concentrations ranging from 0 to 50 mg/mL) did not exhibit any significant effects on GES-1 cells, as demonstrated in Fig. S1. To further verify its activity, we performed colony formation experiments.





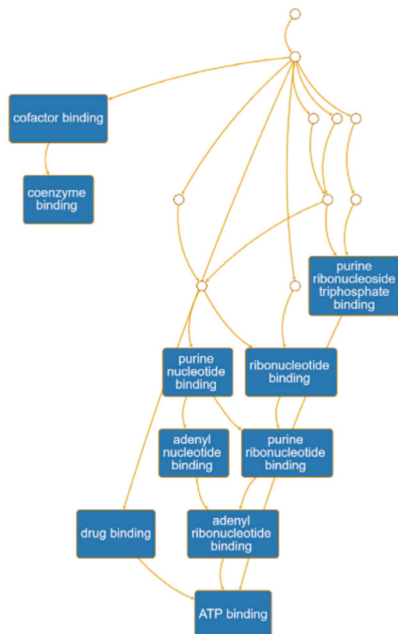


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Fig. 3. The potential targets of *Hedyotis diffusa* Willd, in pinyin, called Bai Hua She Cao (BHSSC) in gastric cancer (GC) were done *via* Gene Ontology (GO) analysis. GO analysis was conducted by WebGestalt (www.webgestalt.org). The network diagram of GO-BP, GO-CC, and GO-MF. BP represents biological process; CC represents cellular component; MF represents molecular function.

The results revealed that after 3.125 mg/mL and 6.25 mg/mL BHSSC treatment for 48 h, the ability of gastric cancer cell colony formation was significantly inhibited as shown in Fig. 5B and 5C.

3.4. BHSSC inhibits the cell migration of GC cells in vitro

GO analysis demonstrated that BHSSC exerts anti-tumor effects in correlation with cell migration; therefore, wound healing assays were used to verify its effects. The experimental results suggested that BHSSC dramatically represses gastric cancer cells' ability to migrate as presented in Fig. 6A and 6B and 6C. It revealed that the inhibition of cell migration is an integral component of BHSSC anti-tumor effects.

3.5. BHSSC induces the activation of ER stress in GC cells in vitro

According to the results of the GO-BP and Reactome pathway, the role of BHSSC in gastric cancer may be to activate ER stress by modulating the cellular response to stimuli. Herein, the key regulators of ER stress were detected by Western blot. IRE1α, also known as inositol-requiring enzyme 1 alpha, is a key player in the unfolded protein response (UPR) pathway, a cellular stress response associated with endoplasmic reticulum (ER) stress. When unfolded proteins accumulate in the ER, IRE1α becomes activated to restore ER homeostasis. BIP, which acts as a vital regulator of the ER stress response or immunoglobulin heavy-chain binding protein, is an ER-resident chaperone protein critical for protein folding and ER stress regulation. From the results in Fig. 7, it was discovered that BHSSC enhanced the expression of IRE1α and BIP in GC cells, which proved that BHSSC can indeed affect the ER stress pathway to exert anti-tumor effects.

4. Discussion

The principles and regulatory mechanisms of traditional Chinese medicine (TCM) offer a wealth of information that cannot be adequately captured by the single-target and single-component research approach typically used in Western medicine. To address this limitation, the emergence of network pharmacology technology has facilitated a shift towards comprehensive analysis and systemic regulation, enabling a more holistic understanding of the mechanisms underlying the therapeutic benefits of TCM. This advancement has resulted in a transition from the traditional single-target approach to an integrated network analysis, linking drug-target-disease interactions to explore the multifaceted mechanisms of drug action. Furthermore, bioinformatics tools have played a crucial role in identifying disease targets and functional proteins that exert central regulatory roles in various diseases.

As a traditional Chinese medicine, BHSSC can be used to treat multiple cancers, such as liver cancer [26,27], colorectal cancer [10], lung cancer [28,29], cervical cancer [30]. It has demonstrated its capacity to influence host immunological responses, tumor cell proliferation, cell death, angiogenesis, anti-inflammatory and antioxidant systems, as well as autophagy [31,32]. Recent research proved the role of BHSSC in human gastric cancer MNK-45 cells via reducing the potential of the mitochondrial membrane and inducing apoptosis [33]. A previous study based on network pharmacology predicted that BHSSC anti-gastric cancer mechanism may be mainly through apoptosis, cell cycle, differentiation, proliferation, migration, invasion, and angiogenesis, which is similar to our findings [34]. Our research utilized network pharmacology, bioinformatics, and experimental validation to predict and affirm the inhibitory effects of BHSSC on the proliferation and migration of gastric cancer cells. Importantly, our findings demonstrated that BHSSC triggers endoplasmic reticulum (ER) stress, which was corroborated by the enrichment of ER stress-related gene ontology (GO) terms in our analysis.

Importantly, using drug targets identified by network pharmacology analysis and GC targets identified through bioinformatic screening, we hypothesized that BHSSC's activity in GC is primarily influenced by metabolism and ER stress for the first time. The use of bioinformatics to extract GC samples from the TCGA database and screen for differential genes as potential targets for GC may be the cause of the discrepancy in prediction results. Furthermore, the database is updated very frequently. Therefore, the screening results must be experimentally validated, and we did this to confirm that BHSSC does indeed cause ER stress.

The relationship between ER stress and cancer has been increasingly recognized, with several studies identifying ER stress as a potential target for gastric cancer treatment. Studies have found that herbal medicine for GC may be associated with ER stress pathway activation [15,35]. Ferroptosis and ER stress could be brought on by Fuzheng Nizeng Decoction [36]. Small molecules from traditional Chinese medicine are able to activate ER stress to exert anti-tumor effects. It was found that dehydrofucoidan, the active ingredient of an herb called Juncus effusus, inhibited the growth and tumorigenicity of GC cells by selectively inducing a potent tumor-suppressive ER stress response and a modest apoptotic response [37]. By controlling the IRE1 pathway, according to Qian Gu et al. Wogonoside increases apoptosis and ER stress in human gastric cancer cells [38]. By inducing ER stress, baicalein aids in the death of GC cells [39]. Asiaticoside accelerated ER stress and slowed the spread of GC [40]. ER stress could be induced by isoquercitrin [41]. Reticulon 2 encourages gastric cancer spread by triggering ERK signaling through ER Ca (2+) efflux [42].

The appropriate level of ER stress and UPR should be maintained; else, disease could occur [43]. Three key sensors control UPR: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and double-stranded, RNA-activated protein kinase-like

GO-BP

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
GOTERM_BP_DIRECT	signal transduction	RT		73	14.3	2.6E-10	8.0E-8
GOTERM_BP_DIRECT	protein phosphorylation	RT		69	13.6	3.1E-30	2.6E-27
GOTERM_BP_DIRECT	protein autophosphorylation	RT		52	10.2	1.3E-38	2.2E-35
GOTERM_BP_DIRECT	peptidyl-tyrosine phosphorylation	RT		49	9.6	7.3E-41	2.5E-37
GOTERM_BP_DIRECT	negative regulation of apoptotic process	RT		37	7.3	1.2E-7	1.9E-5
GOTERM_BP_DIRECT	positive regulation of cell proliferation	RT		37	7.3	3.1E-7	4.8E-5
GOTERM_BP_DIRECT	transmembrane receptor protein tyrosine kinase signaling pathway	RT		35	6.9	2.4E-24	1.6E-21
GOTERM_BP_DIRECT	positive regulation of kinase activity	RT		33	6.5	5.1E-32	5.7E-29
GOTERM_BP_DIRECT	peptidyl-serine phosphorylation	RT		30	5.9	6.1E-15	2.9E-12
GOTERM_BP_DIRECT	intracellular signal transduction	RT		28	5.5	5.9E-5	4.4E-3
GOTERM_BP_DIRECT	positive regulation of protein phosphorylation	RT		26	5.1	3.5E-10	8.9E-8
GOTERM_BP_DIRECT	chemical synaptic transmission	RT		26	5.1	1.5E-8	2.9E-6
GOTERM_BP_DIRECT	apoptotic process	RT		26	5.1	1.7E-2	2.5E-1
GOTERM_BP_DIRECT	G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	RT		25	4.9	8.5E-22	4.8E-19
GOTERM_BP_DIRECT	multicellular organism development	RT		25	4.9	4.4E-13	1.8E-10
GOTERM_BP_DIRECT	positive regulation of cell migration	RT		24	4.7	4.4E-7	6.5E-5
GOTERM_BP_DIRECT	positive regulation of MAPK cascade	RT		23	4.5	2.6E-10	8.0E-8
GOTERM_BP_DIRECT	response to xenobiotic stimulus	RT		23	4.5	8.6E-7	1.2E-4
GOTERM_BP_DIRECT	nervous system development	RT		23	4.5	1.3E-3	4.9E-2
GOTERM_BP_DIRECT	positive regulation of gene expression	RT		22	4.3	2.9E-2	3.5E-1

GO-CC

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
GOTERM_CC_DIRECT	cytosol	RT		231	45.4	2.9E-20	7.6E-18
GOTERM_CC_DIRECT	cytoplasm	RT		211	41.5	2.2E-12	1.9E-10
GOTERM_CC_DIRECT	plasma membrane	RT		189	37.1	9.7E-9	5.0E-7
GOTERM_CC_DIRECT	nucleoplasm	RT		124	24.4	2.4E-3	3.6E-2
GOTERM_CC_DIRECT	extracellular exosome	RT		120	23.6	4.6E-17	8.0E-15
GOTERM_CC_DIRECT	membrane	RT		119	23.4	1.7E-3	2.9E-2
GOTERM_CC_DIRECT	integral component of plasma membrane	RT		89	17.5	1.0E-15	1.1E-13
GOTERM_CC_DIRECT	mitochondrion	RT		79	15.5	2.8E-11	1.8E-9
GOTERM_CC_DIRECT	extracellular region	RT		67	13.2	2.5E-2	1.9E-1
GOTERM_CC_DIRECT	extracellular space	RT		62	12.2	2.8E-2	2.1E-1
GOTERM_CC_DIRECT	endoplasmic reticulum	RT		56	11.0	1.5E-6	6.3E-5
GOTERM_CC_DIRECT	mitochondrial matrix	RT		44	8.6	1.4E-16	1.8E-14
GOTERM_CC_DIRECT	intracellular membrane-bounded organelle	RT		44	8.6	1.3E-4	3.2E-3
GOTERM_CC_DIRECT	Golgi apparatus	RT		43	8.4	4.9E-3	5.7E-2
GOTERM_CC_DIRECT	receptor complex	RT		42	8.3	1.6E-24	8.1E-22
GOTERM_CC_DIRECT	endoplasmic reticulum membrane	RT		40	7.9	1.5E-2	1.3E-1
GOTERM_CC_DIRECT	neuron projection	RT		36	7.1	7.2E-12	5.4E-10
GOTERM_CC_DIRECT	dendrite	RT		36	7.1	1.4E-9	7.8E-8
GOTERM_CC_DIRECT	synapse	RT		35	6.9	3.8E-7	1.8E-5
GOTERM_CC_DIRECT	perinuclear region of cytoplasm	RT		35	6.9	3.3E-4	6.8E-3
GOTERM_CC_DIRECT	cell surface	RT		32	6.3	2.5E-4	5.4E-3
GOTERM_CC_DIRECT	macromolecular complex	RT		29	5.7	5.9E-3	6.2E-2
GOTERM_CC_DIRECT	glutamatergic synapse	RT		26	5.1	3.2E-5	1.1E-3
GOTERM_CC_DIRECT	neuronal cell body	RT		24	4.7	4.2E-5	1.4E-3
GOTERM_CC_DIRECT	axon	RT		23	4.5	6.7E-5	2.0E-3

GO-MF

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
GOTERM_MF_DIRECT	protein binding	RT		369	72.5	3.0E-3	4.5E-2
GOTERM_MF_DIRECT	ATP binding	RT		154	30.3	6.3E-49	3.2E-46
GOTERM_MF_DIRECT	identical protein binding	RT		97	19.1	1.4E-12	1.4E-10
GOTERM_MF_DIRECT	metal ion binding	RT		95	18.7	3.5E-3	5.3E-2
GOTERM_MF_DIRECT	protein serine/threonine/tyrosine kinase activity	RT		94	18.5	8.6E-57	8.8E-54
GOTERM_MF_DIRECT	protein kinase activity	RT		69	13.6	1.2E-36	4.0E-34
GOTERM_MF_DIRECT	protein serine/threonine kinase activity	RT		67	13.2	2.0E-33	3.5E-31
GOTERM_MF_DIRECT	protein homodimerization activity	RT		55	10.8	1.4E-11	1.1E-9
GOTERM_MF_DIRECT	calcium ion binding	RT		51	10.0	3.5E-9	1.8E-7
GOTERM_MF_DIRECT	protein tyrosine kinase activity	RT		41	8.1	2.0E-34	4.2E-32
GOTERM_MF_DIRECT	zinc ion binding	RT		39	7.7	2.3E-3	3.7E-2
GOTERM_MF_DIRECT	transmembrane receptor protein tyrosine kinase activity	RT		32	6.3	2.1E-35	5.4E-33
GOTERM_MF_DIRECT	oxidoreductase activity	RT		27	5.3	3.5E-10	2.0E-8
GOTERM_MF_DIRECT	magnesium ion binding	RT		26	5.1	4.0E-9	1.9E-7
GOTERM_MF_DIRECT	GTP binding	RT		25	4.9	2.5E-4	6.3E-3
GOTERM_MF_DIRECT	kinase activity	RT		23	4.5	1.5E-7	6.8E-6
GOTERM_MF_DIRECT	GTPase activity	RT		22	4.3	7.0E-4	1.4E-2
GOTERM_MF_DIRECT	protein kinase binding	RT		21	4.1	6.6E-2	4.6E-1
GOTERM_MF_DIRECT	calmodulin binding	RT		19	3.7	1.1E-5	3.7E-4
GOTERM_MF_DIRECT	receptor binding	RT		19	3.7	2.8E-2	2.9E-1
GOTERM_MF_DIRECT	neurotransmitter receptor activity	RT		18	3.5	6.0E-11	4.1E-9

(caption on next page)

Fig. 4. The potential targets of *Hedyotis diffusa* Willd, in pinyin, called Bai Hua She Cao (BHSSC) in gastric cancer (GC) were conducted *via* Gene Ontology (GO) analysis. GO analysis was conducted by DAVID (https://david.ncifcrf.gov). The detailed results of GO-BP, GO-CC, and GO-MF. BP represents biological process; CC represents cellular component; MF represents molecular function.

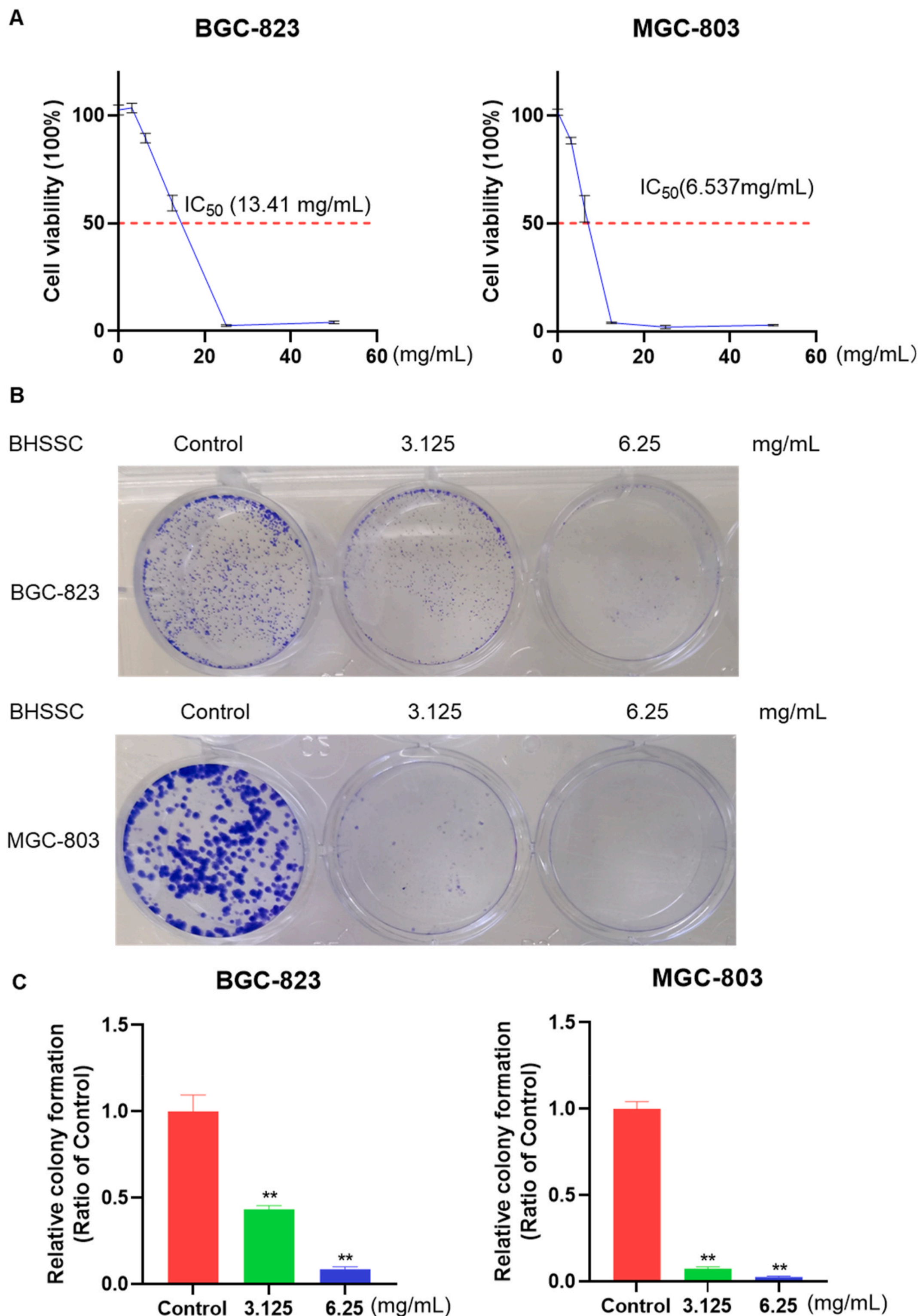

Fig. 5. *Hedyotis diffusa* Willd, in pinyin, called Bai Hua She Cao (BHSSC) suppresses cell proliferation and colony formation of gastric cancer cells *in vitro*. BGC-823 and MGC-803 were treated with BHSSC for 48 h. A. Cell viability was measured. B. Cell colony formation was detected. C. The results of relative colony formation of B. A significant difference was shown with $^{**}P < 0.01$ or $^{*}P < 0.05$.

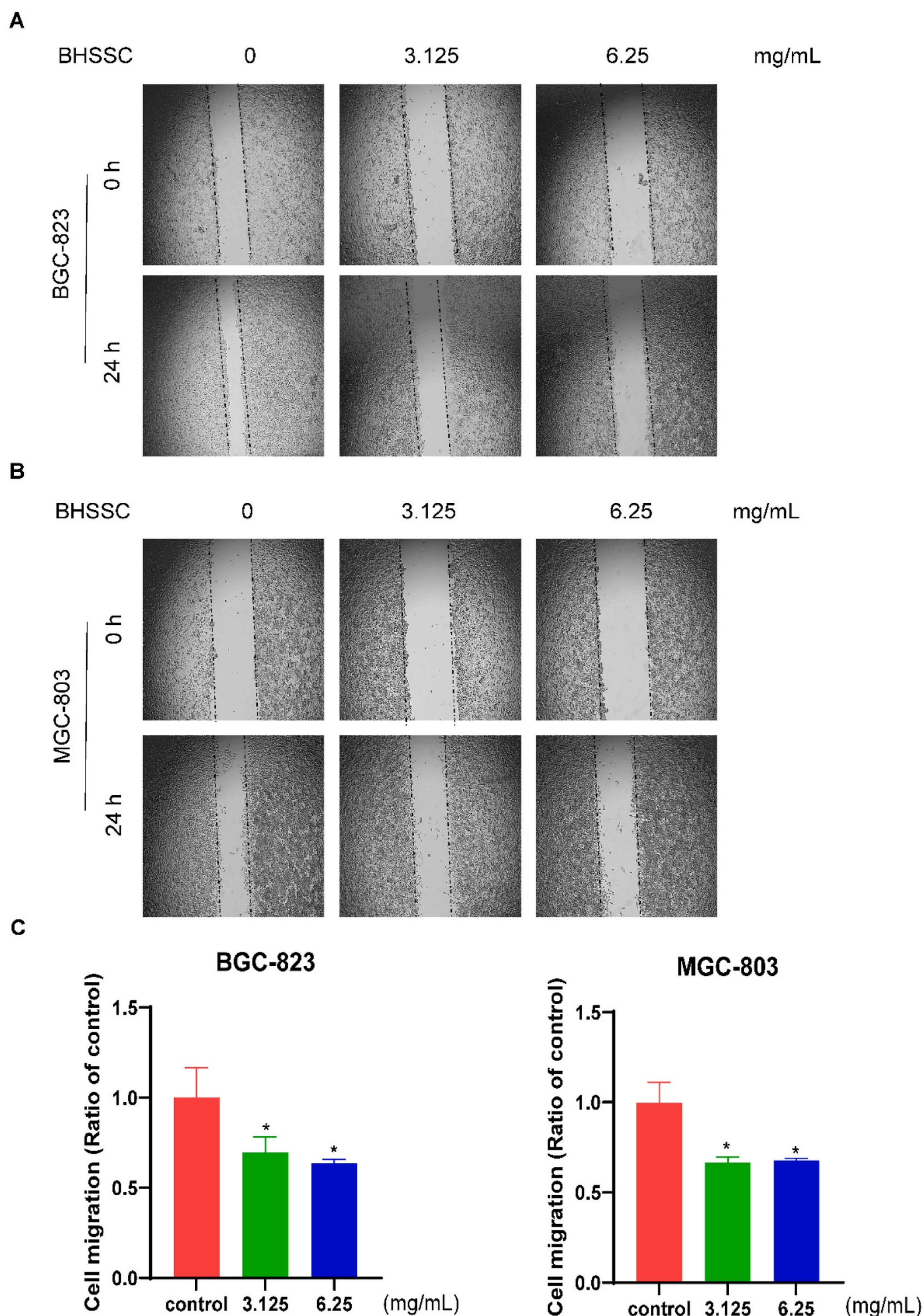

Fig. 6. *Hedyotis diffusa* Willd, in pinyin, called Bai Hua She Cao (BHSSC) inhibits the cell migration of gastric cancer cells *in vitro*. The wound healing assay was conducted after BHSSC treatment for 24 h. A. The wound healing of BGC-823 cells. B. The wound healing of MGC-803 cells. C. The results of cell migration of A and B. A significant difference was shown with * $P < 0.05$.

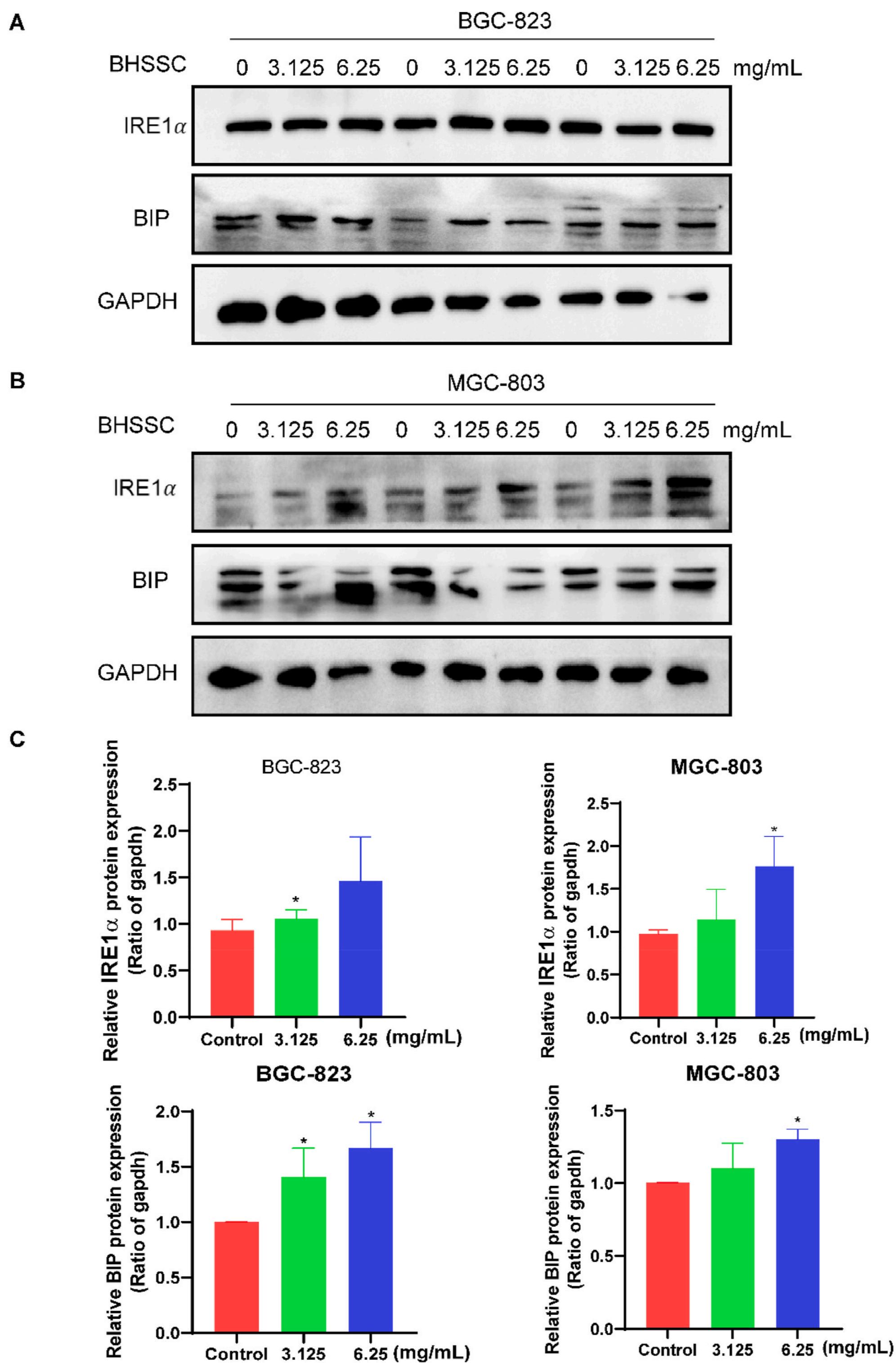

Fig. 7. *Hedyotis diffus*a Willd, in pinyin, called Bai Hua She Cao (BHSSC) induces the activation of endoplasmic reticulum (ER) stress in gastric cancer cells *in vitro*. BGC-823 and MGC-803 cells were treated with BHSSC for 48 h. The expressions of IRE1α and BIP protein were measured by Western blot. **A.** The expressions of IRE1α and BIP protein of BGC-823 cells **B.** The expressions of IRE1α and BIP protein of MGC-823 cells **C.** The relative protein expressions of A and B. A significant difference was shown with * $P < 0.05$.

ER kinase (PERK) [44]. Under physiological circumstances, all three of these sensors bind to the molecular chaperone Grp78/BiP, which actively promotes protein folding, the import of polypeptides, and the export of misfolded proteins to the ER-associated protein degradation [44,45]. This keeps all three sensors in their inactive conformation. Grp78/BiP separates from PERK, IRE1, and ATF6 as a result of the accumulation of misfolded proteins in the ER lumen, activating these proteins. The monomeric inactive conformation of IRE1 and PERK changes to an active oligomeric configuration [45]. On the cytoplasmic side (C-terminus), IRE1 has a ribo-nuclease domain and serine/threonine kinase activity. When ER stress is detected, IRE1 is activated by autophosphorylation, which causes the prematurely unspliced XBP1 mRNA form to be converted into the spliced XBP1form by the removal of a 26-nucleotide intron [46]. To transactivate UPR target genes, such as those whose products are involved in protein folding, secretion, and degradation [47]. XBP1-s, a transcription factor that moves to the nucleus, binds to the specific promoter elements, such as the ER stress response element and UPR elements. The evidence for IRE1's regulation of several cell physiologies, such as metabolism, immunity, cell differentiation, and apoptosis, is growing [12,13,48,49].

Furthermore, we aim to address the rationale behind concentration selection in the experimental process. In our investigation, the IC50 values of BHSSC in BGC-823 and MGC-803 cells for 48 h were determined to be 13.41 mg/mL and 6.537 mg/mL, respectively. When examining cell migration, our main focus is on the specific influence of the drug on cell migration, rather than its impact on cell proliferation or survival. Choosing concentrations below the IC50 can reduce the cytotoxic effects of the drug on the cells, allowing for a more precise evaluation of BHSSC's impact on cell migration. This ensures that the observed cell migration is directly affected by BHSSC and is not influenced by non-specific effects associated with BHSSC's cytotoxic properties. Meanwhile, considering that serum-free drug treatment for 48 h may result in reduced cell viability, we choose a 24-h serum-free culture to minimize the exposure of cells to potential harmful effects, allowing for a better evaluation of the drug's impact on cell migration and healing ability while reducing the damage to the cells themselves. Additionally, by using lower concentrations, we aimed to minimize the risk of cell toxicity and maintain cell viability throughout the experiments. Therefore, we have opted to use concentrations lower than the IC50 value in our experiments.

Overall, in our study, the results demonstrated that BHSSC induces ER stress activation in GC cells *in vitro*. However, the specific mechanisms underlying this effect remain to be fully elucidated. Further research is warranted to comprehensively understand the interplay between BHSSC, ER stress, and its potential antitumor effects.

5. Conclusions

In conclusion, we conducted a comprehensive investigation of the pathways and targets involved in BHSSC's action using network pharmacology, bioinformatics prediction, and empirical validation. Our exploration and validation of the mechanism underlying BHSSC's anti-GC effect revealed its potential involvement in triggering ER stress and suppressing cell division and migration. The discovery of BHSSC's ability to induce ER stress is relatively recent, and it remains unknown whether BHSSC specifically targets the core proteins of the ER stress pathway. Further research is needed to determine whether BHSSC can disrupt the interaction of key targets in the ER pathway, thereby providing a robust experimental foundation for the clinical application of herbal medicine.

Ethics statement

Review and/or approval by an ethics committee and informed consent was not needed for this study because TCGA belongs to public databases. The patients involved in the database have obtained ethical approval. Users can download relevant data for free for research and publish relevant articles. Our study is based on open source data, so there are no ethical issues and other conflicts of interest.

Consent for publication

Not applicable.

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Data availability statement

All data to support the conclusions have been either provided in the article or supp. Material.

CRediT authorship contribution statement

Ling Ou: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mengyang Li:** Writing – review & editing, Validation, Resources, Formal analysis, Data curation. **Yan Hou:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Formal analysis, Data curation, Conceptualization.

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

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