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Review Article

A Critical Assessment for Hualer, A Traditional Chinese Medicine, for Cancer Therapy: Medicinal Characteristics, Molecular Mechanisms of Action, *In-Vitro* and *In-Vivo* Anticancer Activities, and Future Research Directions

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Abstract

Huaier (*Trametes robiniophila* Murr) is a traditional Chinese medicine with a clinical application history of over 1,000 years. Its chemical components mainly include polysaccharides, sterols, and alkaloids. Huaier has been shown to demonstrate potent antitumor effects in a variety of cancer types, including breast cancer, colorectal cancer, gastric cancer, liver cancer, lung cancer, and others. In recent years, multiple *in-vitro* experiments have confirmed the good antitumor effect of Huaier and its mechanism of action, such as inhibiting proliferation, inducing apoptosis and oxidative stress, interfering with cell cycle arrest, inhibiting tumor metastasis and angiogenesis, inducing autophagy, and regulating immune function. In addition, multiple *in-vivo* studies and clinical trials have demonstrated the multidimensional antitumor potential of Huaier, such as slowing tumor progression, reversing drug resistance, improving chemotherapy drug sensitivity, and extending the survival time of cancer patients. In this article, the extraction methods of Huaier and its properties for the treatment of many cancers are reviewed. Moreover, the current molecular mechanisms of Huaier are summarized, revealing that it has great potential as an anticancer drug and providing strong theoretical support for related research. Furthermore, this review also provides suggestions for further research on the anticancer effects of Huaier, hoping to offer fresh perspectives for researchers in the realm of anticancer medicine.

Keywords: Anticancer mechanisms; Anticancer activity; Cancer therapy; Huaier.

Abbreviations: AEG1, astrocyte elevated gene 1; AKT, Protein kinase B; AKT, protein kinase B; AR-V7, AR splice variant 7; ASK1, apoptosis signal-regulating kinase 1; AU, adenine uridine; Akt, protein kinase B; Bax, Bcl2-associated X protein; Bcl2, B cell lymphoma 2; CBP, CREB binding protein; CDK2, cyclin-dependent kinase 2; CK1, casein kinase 1; CSNK2B, the regulatory subunit of casein kinase 2; DIXDC1, dishevelled-axin (DIX) domain-containing 1; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DvI, disaster victim identification; EMT, epithelial–mesenchymal transitio; EMT, epithelial–mesenchymal transition; ERK, extracellular signal-regulated kinase; ERα, estrogen receptor alpha; EZH2, enhancer of zeste homolog 2; FLIP, FADD-like inhibitor protein; GC, gastric cancer; GS3Kβ, glycogen synthase kinase-3 beta; Gsk-3, glycogen synthase kinase 3; HIF1α, hypoxia-inducible factor 1 alpha; HP, Huaier polysaccharide; IFN, interferon; IKB, ikappaB; IKK, ikappaB kinase; IL, interleukin; Ik-B, I-kappa-B-alpha; JAK2, Janus kinase 2; JNK, c-Jun N-terminal kinase; JUN, Jun proto-oncogene; LEF1, lymphoid-enhancing factor 1; LRP5/6, lipoprotein receptor-related protein 5/6; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MKK1, mitogen-activated protein kinase kinase 1; MMP, matrix metalloproteinase; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; MMPs, matrix metalloproteinases; MTDH, Metadherin; NAP1L1, nucleosome assembly protein 1-like 1; NF-kB, nuclear transcription factor-kappa B; NK, Natural Killer cell; P13K, phosphatidylinositol 3-kinase; PARP, poly (ADP-ribose) polymerase; PDK, 3-phosphoinositide-dependent protein kinase; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PI3K, phosphoinositide 3-kinase; PIP, prolactin-induced protein; PKR, protein kinase R; PTEN, phosphatase and tensin homolog; RTK, receptor Tyrosine Kinases; S6K, S6 kinase; STAT3, signal transducer and activator of transcription 3; TAK1, transforming growth factor beta-activated kinase 1; TCF1, T cell factor 1; TCM, traditional Chinese medicine; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; TRAF2, tumor necrosis factor receptor associated factor 2; TRAF6, tumor necrosis factor receptor associated factor 6; VEGF, vascular endothelial growth factor; VEGF, vascular endothelial growth factor.; XIAP, X-chromosome-linked inhibitor of apoptosis protein; YAP1, yes-associated protein 1; iNOS, inducible nitric oxide synthase; mTOR, mammalian target of rapamycin; p-STAT3, phosphorylated STAT3.

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Introduction

Cancer stands as a major disease impacting human health, emerging as the second most common cause of mortality globally. The existing array of cancer therapies encompasses surgical procedures, radiotherapy, chemotherapy, immunotherapy, and targeted therapy.[1,2] Undoubtedly, these conventional treatment methods

have significantly prolonged the survival period of cancer patients. However, challenges persist due to the risks associated with surgery, substantial side effects of radiation and chemotherapy, immunotherapy resistance, and poor prognoses.[3–5]

In recent years, traditional Chinese medicine (TCM) and its active components have been widely used in cancer treatment due to their multitarget effects and minimal side effects.[6,7] In contrast to Western medicine, which often targets individual molecules or pathways with purified compounds, TCM contains multiple herbs and ingredients with various effects. It possesses the ability to regulate various aspects of cancer cell behavior, including proliferation, apoptosis, adhesion, and migration. Additionally, it can inhibit tumor angiogenesis, modulate the host immune system, and effectively suppress tumor growth.[8–10] Multiple clinical studies have confirmed that TCM can reduce the risk of cancer in inflammatory patients, target signaling pathways, regulate immune suppression to suppress cancer progression, prolong progression-free survival, and improve patient quality of life.[11–14] As an adjuvant therapy, TCM not only improves the efficacy of conventional treatment, but it also exerts a synergistic effect by increasing the sensitivity of chemotherapy drugs, reversing drug resistance, and reducing the risk of postoperative tumor recurrence and metastasis.[15,16] Although the role of TCM in tumor treatment is gradually being recognized, it still faces some challenges. TCM contains complex components and multiple targets, so it still needs a lot of exploration to identify and analyze the active ingredients, screen the target of active ingredients, and elucidate the specific mechanism of action. In addition, due to the lack of clinical trial data support and the complexity of TCM treatment methods, it can only be used as an adjuvant therapy for various cancers.[17,18] Therefore, in-depth research and clinical validation are needed to clarify the anticancer mechanisms and efficacy of TCM.

Huaier (*Trametes robiniophila* Murr), a traditional Chinese herbal extract, has a rich history spanning over a thousand years. Huaier is brownish yellow with a special aroma, high water solubility, stable appearance, and low toxicity.[19] Its chemical constituents mainly include Huaier polysaccharides (HPs), sterols, and alkaloids, making it a valuable adjuvant therapy for various diseases.[20–22] Huaier was first reported in 659 AD by Li Ji *et al*. as "*Tang Bencao*", and it was used for hemostasis, the treatment of intestinal hemorrhoids.[23] According to TCM theory, Huaier is believed to nourish Yin, moisten dryness, and nourish the heart, thereby alleviating anxiety, insomnia, and related nervous system conditions. It is also thought to tonify qi, strengthen the spleen, and improve appetite loss and indigestion.[24] Additionally, it is suggested to moisten the lungs and relieve dry cough with minimal sputum.[25] In modern medicine, in the 1980s, Huaier was first used clinically for the treatment of advanced liver cancer. After a series of investigations, drug development, and clinical trials, Huaier granule was approved for the treatment of liver cancer in China in 1992. Because of its unique anticancer effect, it has been widely studied. In recent years, researchers have shown that Huaier exhibits antitumor effects across various cancers such as breast cancer, lung cancer, liver cancer, and colon cancer.[26–30] As shown in Figure 1, its anticancer effects are the result of a combination of multiple mechanisms, including tumor cell growth inhibition, promotion of cell apoptosis, and immune system regulation. In clinical practice, Huaier is commonly used as an adjunct therapy or combination therapy, which can significantly slow down the development of tumors, prolong the survival rate of patients, and reduce the recurrence rate of cancer.[31,32]

In recent years, increasing evidence has revealed the antitumor activity of Huaier, which also has shown great potential in clinical tumor treatment and prevention. This article summarizes the separation and extraction methods of Huaier, reviews the antitumor effects of Huaier and its derivatives, and further explores their molecular mechanisms and research significance, providing new insights for the development of new anticancer drugs and cancer treatment strategies.

Isolation and extraction of the active substances of Huaier

Huaier grows on the trunks of locust, black locust, and Catalpa, and it is the fruiting body of the Polyporaceae fungus *Trametes robiniophila*, mainly distributed in northern China. Its main components are polysaccharides, sterols, and alkaloids.[33] Among them, polysaccharides, primarily found in fungi, algae, and root tubers, are widely considered important components in TCM.[34,35] They are long-chain polymers formed by the linkage of similar or different monosaccharides through glycosidic bonds. The hydrolysis products of HPs contain six monosaccharides and eighteen amino acids. Polysaccharides have received widespread attention due to their low toxicity and various pharmacological and biological activities. Studies have shown that many polysaccharides can inhibit the proliferation of tumor cells both *in vitro* and *in vivo*.[36] Polysaccharides exert antitumor effects by inducing apoptosis and cell cycle arrest in tumor cells, promoting autophagic accumulation.[37] By stimulating nonspecific or specific immune responses, they can indirectly suppress tumors while also exerting regulatory effects on the immune system.[38–40] In recent years, studies on Huaier have predominantly centered on the antitumor aspects of Huaier particles or extracts. However, there are many Huaier extraction methods, such as water extraction, acid extraction, alkali extraction, salt precipitation, enzyme extraction, ultrasonic assisted extraction, supercritical fluid extraction, and so on. Different extraction methods will affect the extraction efficiency and biological activity of Huaier.[21,41,42]

The water extraction methods for Huaier can be divided Into ordinary water extraction and high-temperature/high-pressure water extraction, both of which yield different polysaccharide contents. Different extraction methods require Huaier to be divided into small pieces and air-dried until a constant weight is achieved. For ordinary water extraction, the defatted and dried samples are mixed with distilled water and placed in a 90°C water bath for 2 h. This process is repeated once, and the supernatants are combined after centrifugation. After vacuum concentration, four volumes of precooled anhydrous ethanol are added. The mixture is precipitated at 4°C and centrifuged after 24 h. The precipitate is then freeze-dried to obtain the HP extract.[35,43]

The high-temperature/high-pressure water extraction method can be further divided into the fractionation alcohol precipitation method and the deproteinization alcohol precipitation method. The main purpose of the alcohol precipitation method is to remove water-soluble impurities such as starch, gum, pectin, mucilage, protein, tannin, pigment, and inorganic salts from the water extract, while retaining the effective ingredients of the medicine. In the fractionation alcohol precipitation method, distilled water is added to the defatted and dried Huaier sample, which is then placed in a high-pressure sterilization pot at 121°C for 2 h. The mixture is filtered, and the supernatants from two filtrations are combined and centrifuged for 10 min. The supernatant is then concentrated to a viscous state. Ethanol is added to the concentrated supernatant in 1×, 2×, 3×, and 4× volumes, respectively, and allowed to stand at 4°C for 24 h. After centrifugation and removal of the supernatant,

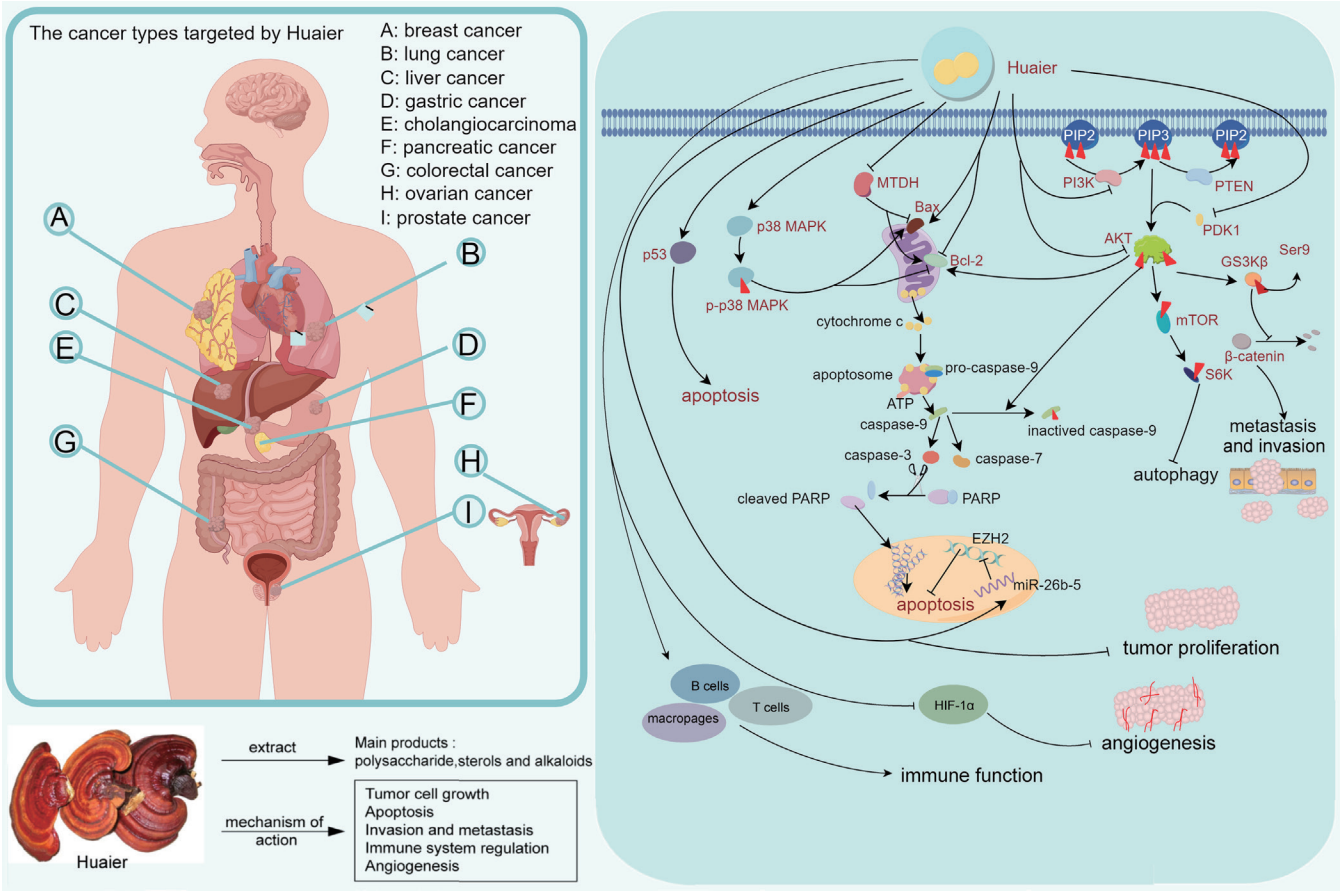

Fig. 1. The overview of Huaier includes the cancer types treated, the main compounds, and the mechanisms of action. AKT, protein kinase B; EZH2, enhancer of zeste homolog 2; GS3Kβ, glycogen synthase kinase-3 beta; HIF1α, hypoxia-inducible factor 1 alpha; MAPK, mitogen-activated protein kinase; MTDH, Metadherin; PARP, poly (ADP-ribose) polymerase; PDK, 3-phosphoinositide-dependent protein kinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP, prolactin-induced protein; PTEN, phosphatase and tensin homolog; S6K, S6 kinase; mTOR, mammalian target of rapamycin.

the precipitate is freeze-dried to obtain four fractions of alcohol-precipitated crude polysaccharides. In the deproteinization alcohol precipitation method, Sevag reagent is added to the HP concentrate and mixed and oscillated for 30 min. After standing, the aqueous phase is collected. This process is repeated two or three times. Furthermore, the supernatant from protein removal is treated with ethanol in the same manner as mentioned before, resulting in four fractions of deproteinized alcohol-precipitated crude polysaccharides.[44]

In order to have a high extraction efficiency, shorten the extraction time, and also give full play to the efficacy of Chinese medicine, the alcohol extraction method can be used for the extraction of Huaier. First, the Huaier is ground into a powder, then it is sifted and mixed with 20–40 mL of ethanol for every 1 g of Huaier powder. The mixture is then subjected to reflux extraction for 2–3 cycles, with each cycle lasting 2 h. After filtration, the extracted liquids are combined, and the ethanol is recovered by rotary evaporation. Subsequently, the combined extracted liquid is condensed into a dense paste. After freeze-drying, the extract of HPs is obtained. Finally, the obtained compounds can be identified by nuclear magnetic resonance spectroscopy, infrared spectroscopy, mass spectrometry, ultraviolet spectroscopy, and other spectroscopic methods, and a total of five compounds can be obtained, whose structural types are mainly glycosides and steroids. As shown in Figure 2, the chemical structure of Huaier ethanol extract is (a) denosine, (b) ergosta-7,22-dien-3B-0l, (c) ergosterol, (d) 3β-hydroxystigmast-5,22-dien-7-one, andI) daucosterol.[45]

The alkaline extraction method of Huaier is divided into the cold alkali method and hot alkali method. In the hot alkali method, distilled water, NaOH, and urea are added to the defatted and dried sample, stirred, and then subjected to constant temperature soaking at 65°C for 1 h. After centrifugation for 20 min, the pH is adjusted to neutral using HCl, followed by vacuum concentration. The same method as water extraction is used for precipitation with ethanol. The precipitate is freeze-dried, dissolved in water again, centrifuged for 20 min to collect the supernatant, and then precipitated with ethanol, as described above. The resulting precipitate is freeze-dried to obtain the water-soluble crude polysaccharides extracted using the hot alkali method. In the cold alkali method, distilled water, NaOH, and 25 g of urea are added to the defatted and dried sample, stirred, and then soaked in a 4°C refrigerator for 48 h. The subsequent steps are the same as the method described above.[46]

In the water extraction process, some of the active ingredients that are insoluble in water may be lost, and petroleum ether may be used to extract the Huaier. After natural air drying at 50°C and crushing, the Soxhlet extraction method is used with 85% ethanol, according to the solid-liquid ratio of 1:5 (g/mL), and the dry powder

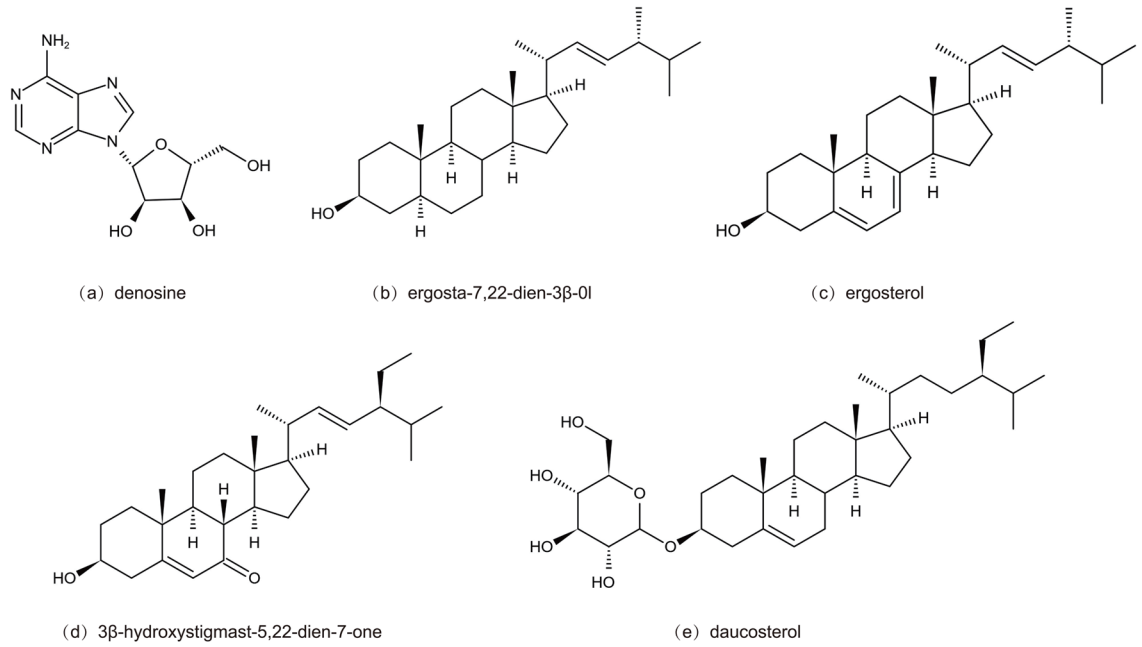

Fig. 2. The chemical formulas of components of the Huaier ethanol extract.

is reflux extracted for 2 h, filtered, and the supernatant is removed. The same amount of solvent is added to the filter residue, repeated three times, combined with the supernatant, and spun dry to obtain the extract of ethanol. Then, ultrasonic extraction is carried out, according to the mass-volume ratio of extract and petroleum ether of 1:5, and the supernatant is filtered for 20 min each time. After repeating three times, the supernatants are combined, and then the anhydrous petroleum ether is removed by spinning dry. The results of gas chromatography–mass spectrometry analysis show that the main component of the Huaier petroleum ether extract is methyl oleate. Other low-polar chemical components are mainly polyunsaturated fatty acids, mainly including methyl linoleate, monoethylhexyl phthalate, and methyl 2-hydroxy-tetracosanate.[47] Polyunsaturated fatty acids have been reported to have anticancer, anti-inflammatory, and oxidation activities;[48,49] therefore, Huaier petroleum ether extract is also expected to be developed as an anticancer drug after more in-depth research verification.

According to a study, as the concentration of Huaier extract increases, a significant decrease in cell density can be observed. Damaged cells show a condensed cytoplasm, protrusions on the membrane surface, shrinkage in volume, detachment from normal cells, a fragmented appearance, the presence of opaque granules within the cells, and a noticeable decrease in the number of intact cells, indicating that the Huaier extracts obtained by different methods can promote the apoptosis of K562 cells.[50] However, the crude polysaccharide content, the actual yield of crude polysaccharides, and the inhibitory rate on tumor cells vary among different extraction methods (Table 1).[36–42,47] The actual yield of crude polysaccharides in the cold alkali extraction method is much greater than that using the hot-alkali extraction method, but the highest extraction rate is achieved with the high-temperature and high-pressure extraction method, yielding HPs with a content of 34.59%. In the high-temperature/high-pressure fractionation extraction method, the polysaccharide content of the experimental group obtained by the deproteinizing operation is obviously greater than that of the product obtained by the nondeproteinizing method. The extraction method with the highest tumor inhibition rate is the hot alkali method.[44] When the extract with a concentration of 2 mg/mL is applied to K562 cells for 72 h, the inhibition rate reaches up to 92.28%.

In the two methods of high-temperature/high-pressure fractionation extraction and ethanol extraction, the polysaccharide content of the experimental group after deproteinization was significantly greater than that of the nondeproteinization group. Among them, the tumor inhibition rate of products obtained by the hot alkali method was the best.

Mechanism of the antitumor action of Huaier

Inhibition of the growth and proliferation of tumor cells

p53, a key regulator of cellular stress response, is one of the most commonly mutated genes in human cancer, and more than half of tumor cells have deviations in the p53 signaling pathway due to *TP53* gene mutations.[51] The mechanism of action of p53 protein, the expression product of the *TP53* gene, is mainly indirect in the cell cycle. p53 can promote the expression of p21 protein (an inhibitor of cyclin-dependent kinase 2; CDK2), thus inhibiting the transformation from the G1 to S phase in the cell cycle, preventing cells from entering the DNA synthesis phase, thereby restraining cell division and growth.[52,53] As an anticancer drug, Huaier regulates the p53 pathway to inhibit cancer cell proliferation.[54]

Some studies have shown that the aqueous extract of Huaier has an obvious inhibitory effect on the human breast cancer cell lines MCF-7 and MDA-MB-231. Interestingly, the cell cycle of MCF-7 was arrested by Huaier in the G0/G1 phase, thereby inhibiting proliferation,[55] but the cell cycle of MDA-MB-231 cells was not affected. However, the apoptosis rates of MCF-7 and MDA-MB-231 cells, both late and early, were significantly increased by the time and dose of Huaier. Additionally, the mechanism of cell cycle arrest and apoptosis was investigated. The results showed that with Huaier treatment, the expression of p53 and phosphoryl-

Table 1. Advantages and disadvantages of the extraction methods for Huaier polysaccharides

Extraction method	Advantages	Disadvantages	References
Water extraction	Simple procedure, shorter procedure time.	The yield of the extract was low. Loss of bioactive ingredients in Huaier.	36,37
High-temperature and high-pressure water extraction	The extraction yield was high.	Complex procedure, long procedure time. High protein removal rate led to decreased antioxidant activity of Huaier. The heat-sensitive bioactive components of water extraction products may be volatilized or destroyed by temperature.	38
Cold-alkali method	High extraction rate. The product has a high polysaccharide content.	The DPPH clearance rate of the obtained extract is low, and the antioxidant capacity is weak.	39
Hot-alkali method	The extraction yield was high. The clearance rate of DPPH is high, and the antioxidant ability is strong.	The polysaccharide content in the extract is low and requires a higher concentration to inhibit tumor growth.	39
Ethanol extraction	Compared with the Huaier water extract and other organic solvent extracts, alcohol extraction inhibited MKN45 cell proliferation at lower concentrations.	Organic compounds such as *n*-butanol can cause certain pollution to the environment and have higher costs.	40–42
Petroleum ether extraction	Extracts the active ingredient, which is insoluble in water.	The antibacterial and antioxidant capacity of the product was low.	47

DPPH, 2,2-diphenyl-1-picrylhydrazyl.

ated p53 was upregulated in a time- and dose-dependent manner. The findings indicate an increase in the expression levels of p53 and phosphorylated p53 upon treatment with Huaier, suggesting that Huaier can serve as an inducer to arrest the MCF-7 cell cycle through p53 activation. Furthermore, there is evidence suggesting a correlation between the *TP53* mutation status and the survival duration of cancer patients.[56] It has been found that Huaier inhibits the proliferation of cutaneous squamous cell carcinoma by suppressing the methylation levels of *TP53*.[57] With the increase in the concentration of the Huaier water extract and prolongation of the treatment time, human melanoma A875 cells also showed cell cycle arrest, with a significant increase in G2/M phase cells and a marked decrease in the proliferation rate.[58] Further investigation into the inhibitory mechanism revealed an elevation in the p53 protein expression within A875 cells.

The Wnt/β-catenin pathway is a crucial signaling pathway that plays a significant role in embryonic development by regulating cell growth and proliferation. In this pathway, the β-catenin protein binds to receptor kinases on the cell membrane, thereby modulating the Wnt signaling pathway and actively participating in various processes, including cell proliferation, differentiation, and migration.[59] Research has uncovered that Huaier extract possesses the ability to suppress the viability of MiaPaCa-2 and Panc-1 pancreatic cancer cells in a manner dependent on both the time and dose. *In-vitro* experiments have further demonstrated that Huaier exerts control over the proliferation of pancreatic cancer. Delving into the mechanism has unveiled that the inhibition of β-catenin expression is implicated, suggesting that Huaier modulates the growth and proliferation of pancreatic cancer cells through the Wnt/β-catenin pathway.[27]

In the intracellular signaling system, c-Jun N-terminal kinase (JNK)/p38 acts as a pathway for mitogen-activated protein kinase (MAPK), which is responsible for transmitting various signals from the outside of the cell to the inside of the nucleus. It regulates a plethora of crucial physiological processes, encompassing metabolism, survival, cell division, and death.[60] Several studies have indicated that Huaier can effectively inhibit the proliferation of cervical cancer tumors, possibly via the JNK/p38 signaling pathway.[61] After Huaier treatment, C33A cells were arrested in the G2/M phase in a dose-dependent manner. At the same time, the expression levels of extracellular signal-regulated kinase (ERK), JNK, and p38-MAPK were significantly affected by Huaier, suggesting that the activity of Huaier against cervical cancer may be realized by the MAPK signaling pathway.

Mitogen-activated protein kinase kinase (MEK), including MEK1 and MEK2, is a component of the MAPK pathway and serves as a downstream effector of RAS, which is capable of activating ERK.[62] Research has revealed the involvement of MEK in the induction of cell cycle-related proteins in various types of cancers. In approximately 31% of malignant tumors with RAS mutations, alterations in cell cycle-related genes have been observed.[63] By western blot analysis of cell cycle-associated proteins, it was found that Huaier extract induced cell cycle arrest in neuroblastoma cells at the G0/G1 stage.[64] The results demonstrated downregulation of cyclin D3 in all three neuroblastoma cell lines treated with Huaier extract; this protein is known as one of the downstream effectors of the ERK1/7 signaling pathway. Further investigations revealed changes in MEK/ERK-related proteins, indicating that Huaier extract suppresses cyclin D3 expression by inhibiting the MEK/ERK signaling pathway, leading to cell cycle arrest.

c-Myc, recognized as a proto-oncogene, encodes a protein that serves as a key regulator of human cancer cell behavior. Transcription of c-Myc not only governs cell proliferation but also orchestrates various biological processes, including the cell cycle.[65] An experimental study evaluated the anticancer activity of Huaier in inhibiting gastric cancer (GC) cell proliferation by inducing cell cycle arrest.[66] The suppression of proliferation in two GC cell lines (HGC27 and MGC803) exhibited a positive correlation with both the concentration and duration of exposure to the Huaier *n*-butanol extract, suggesting a time- and dose-dependent inhibition. Additionally, the extract inhibited the expression of c-Myc and Bmi1 in GC cells. Furthermore, transfection of a Bmi1 plasmid into the

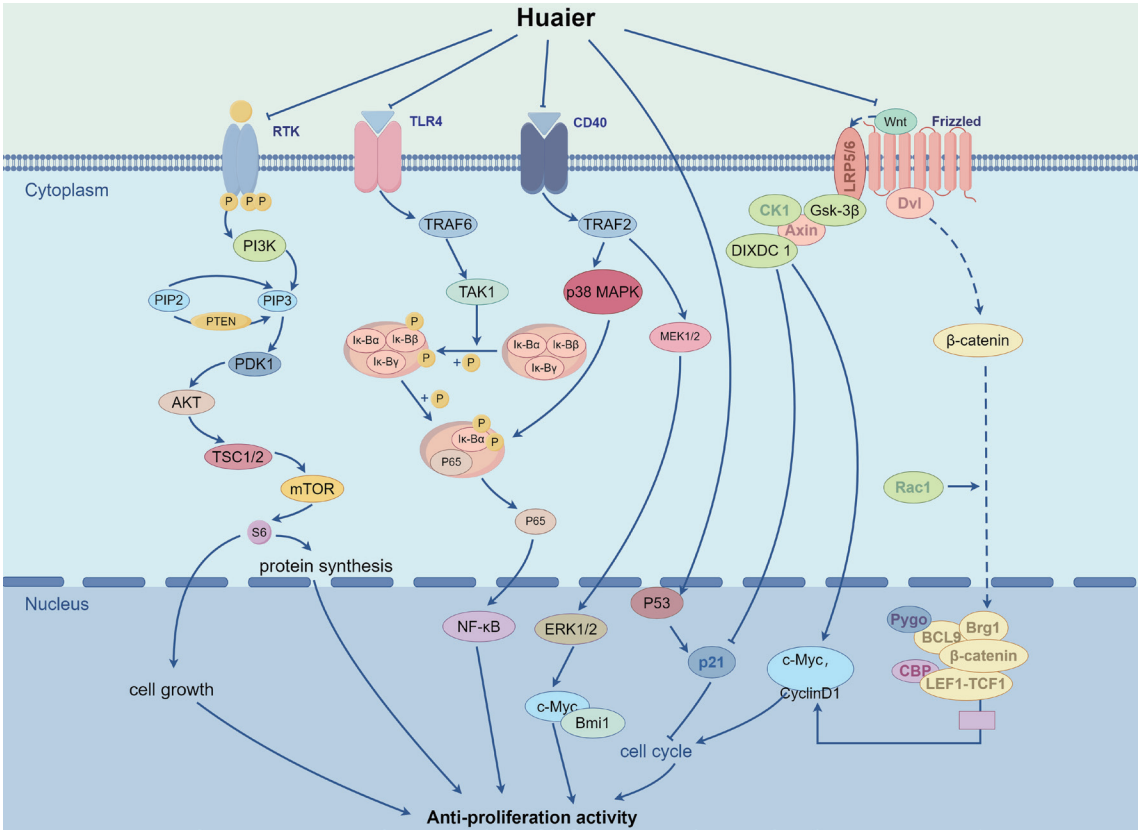

Fig. 3. Mechanism of Huaier inhibiting cell proliferation. AKT, protein kinase B; CBP, CREB binding protein; CK1, casein kinase 1; DIXDC1, dishevelled-axin (DIX) domain-containing 1; Dvl, disaster victim identification; Gsk-3, glycogen synthase kinase 3; Ik-B, I-kappa-B-alpha; LEF1, lymphoid-enhancing factor 1; LRP5/6, lipoprotein receptor-related protein 5/6; MAPK, mitogen-activated protein kinase; NF-kB, nuclear transcription factor-kappa B; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP, prolactin-induced protein; PTEN, phosphatase and tensin homolog; RTK, receptor Tyrosine Kinases; TAK1, transforming growth factor beta-activated kinase 1; TCF1, T cell factor 1; TLR4, toll-like receptor 4; TRAF2, tumor necrosis factor receptor associated factor 2; TRAF6, tumor necrosis factor receptor associated factor 6; mTOR, mammalian target of rapamycin.

GC cell line MGC803 resulted in an elevation of Bmi1 expression, correlating with increased invasion and migration capabilities of MGC803 cells. This led to a reversal of the inhibitory effect of the Huaier butanol extract on GC cells. These findings suggest that the Huaier butanol extract may impede the proliferation and metastasis of GC cells by targeting the c-Myc/Bmi1-mediated signaling pathway.

In summary, as illustrated in Figure 3, the inhibition of cell proliferation by Huaier is associated with various molecular pathways. These pathways include the influence of p53 protein, the Wnt/β-catenin pathway, the MAPK/p38 pathway, and the c-Myc/Bmi1 pathway (Table 2).[27,28,55,66–99]

Tumor cell apoptosis induction

Apoptosis, a genetically regulated process, refers to a programmed form of cell death that effectively eliminates damaged cells, including those with DNA damage or during development. Inducing apoptosis in tumor cells through pharmacological means is a crucial component of cancer therapy.[100] There are two main pathways involved in cellular apoptosis signaling: the extrinsic apoptotic pathway, also known as the death receptor-dependent pathway,[101] and the intrinsic apoptotic pathway, also known as the mitochondria-dependent pathway. These pathways represent key mechanisms by which cells regulate programmed cell death and are critical bridges in the complex balance of cell survival and death. It involves the convergence of intracellular signals at the mitochondrial level, triggered by various stress conditions, to facilitate cell apoptosis. Within the mitochondrial pathway, members of the B cell lymphoma 2 (Bcl2) family proteins, including both anti-apoptotic Bcl2 proteins and pro-apoptotic Bcl2-associated X protein (Bax) proteins, regulate apoptosis. Previous studies have shown that reducing the expression of Bcl2 and enhancing the expression of Bax can effectively promote cell apoptosis.[102] However, whether exogenous or endogenous, they will eventually trigger the activation of caspase 3, a key endonuclease, and then guide the cell toward apoptosis. Huaier has been demonstrated to elevate the ratio of Bax/Bcl2 and induce the expression of cleaved caspase 3, thereby facilitating cell apoptosis (Table 2).

The research findings demonstrate that Huaier effectively inhibits proliferation and induces apoptosis in various cancer cell lines. In lung cancer A549 and NCI-H1650 cells, Huaier elevates the Bax/Bcl2 ratio and induces cleaved caspase 3 expression, promoting apoptosis.[67] Furthermore, the extract of Huaier promotes apoptosis in NCI-H1299 cells by downregulating the expression of the anti-apoptotic protein Bcl2.[103] Similarly, it has been discovered that Huaier treatment has inhibitory effects on the proliferation of liver cancer cells, specifically Bel-7404, Bel-7402, and SMMC-7721.[68] Further investigation into the mechanism reveals

Table 2. Activity and mechanism of Huaier for tumor inhibition *in vivo* and *in vitro*

Cancer type	Mechanisms of action	*In-vitro* activity	*In-vitro* activity	Ref.
Breast cancer	Activates the p53 signaling pathway; Decreased ratio of Bcl2 to Bax leads to mitochondria-mediated apoptosis	Inhibits cell viability (IC_{50} = 4–8 mg/mL) and induces migration and invasion as well as induces cell cycle arrest and apoptosis	NR	55
Breast cancer	Reverses EMT by inducing autophagic degradation of Snail	Inhibits cell invasion, migration, and EMT	NR	75
Breast cancer	Inhibits the linc00339/miR-4656/CSNK2B signaling pathway	Inhibits cell viability	Inhibits tumor growth in an MDA-MB-231 xenograft mouse model with a 100-μL solution containing 50 mg of Huaier.	66
Breast cancer	Suppresses the infiltration of M2-polarized macrophages within the breast cancer microenvironment, modulates the polarization of TAMs, enhances macrophage phagocytic activity, and reduces macrophage-mediated angiogenesis.	Inhibits cell viability	Inhibits tumor growth in 4T1 mouse breast cancer model with a 150-μL solution containing 75 mg of Huaier extract	76
Breast cancer	Suppresses the ERα signaling pathway and induces apoptosis via the lncRNA H19/miR-675-5p/CBL signaling pathway	Inhibits cell viability and induces autophagy and apoptosis.	NR	77
Breast cancer	Inhibits the mTOR pathway	Inhibits cell viability	Inhibits tumor growth (20% of tumor volume at 8g/kg) in an ELT3 xenograft tumor model	78
Breast cancer	Inhibits the mTOR/S6K pathway	Inhibits cell viability (IC_{50} = 4 mg/mL) morphological changes and induces autophagy	Inhibits tumor growth in an MDA-MB-231 xenograft model with Huaier extract (100-μL solution containing 50 mg of Huaier extract)	79
Breast cancer	Inhibits the Hedgehog pathway	Inhibits cell viability (IC_{50} = 2.758 ± 0.441 mg/mL), eliminates $CD44^+/CD24^-$ cells and downregulates stemness gene signatures	NR	73
Breast cancer	Downregulates the ratio of Bax/Bcl2 and MTDH protein expression	Inhibits cell viability and induces apoptosis	NR	80
Breast cancer	Inhibits the estrogen receptor alpha-36 signaling pathway	Inhibits cell viability (IC_{50} for Mb436 and SUM159 cells were 205.12 ± 36.41 and 195.34 ± 27.62 μg/mL at 48 h, respectively) and downregulates the expression of stem-related genes	Inhibits tumor growth in a NOD/SCID mouse orthotopic xenograft model at 60 mg/kg	72
Cholangio-carcinoma	Modulates lncRNA TP73-AS1	Inhibits proliferation, migration, and invasion as well as induces apoptosis and oxidative stress	Inhibits the growth and metastasis of cholangiocarcinoma in a CCLP1 xenograft mouse model (3 g/kg Huaier dissolved in 200 μL of sterile saline)	71
Colorectal cancer	Inhibits Wnt/β-catenin pathway	Inhibits cell viability (IC_{50} = ⍰8 mg/mL)	NR	81
Colorectal cancer	Reduces STAT3 phosphorylation	Inhibits cell viability, induces migration and invasion, and induces apoptosis	Inhibits growth in an HCT116 xenograft tumor in nude mice at a concentration of 4 g/kg	82

(continued)

Table 2. *(continued)*

Cancer type	Mechanisms of action	*In-vitro* activity	*In-vitro* activity	Ref.
Gastric cancer	Promotes the suppression of the c-Myc-Bmi1 signaling pathway	Inhibits cell viability, induces migration and invasion, and induces cell cycle arrest	NR	66
Gastric cancer	Inhibits the expression of Twist	Inhibits cell metastasis	NR	83
Gastric cancer	Inhibits the PI3K/Akt signaling pathway	Inhibits cell viability (IC_{50} = 2.104 mg/mL for MKN45 cells and 3.579 mg/mL for SGC7901 cells) and induces cell cycle arrest and apoptosis	NR	70,84
Gastric cancer	Reduces the expression of syntenic and p-STAT3	Inhibits cell viability (IC_{50} values ranging from 107.5 to 141.1 μg/mL for 24 h, 53.4 to 87.7 μg/mL for 48 h, and 33.8 to 60.3 μg/mL for 72 h)	Inhibits growth in an MGC803-Luc cell xenograft mouse model at a dose of 100 mg/kg	85
Gastric cancer	Inhibits MEK/ERK signaling pathway.	Inhibits cell proliferation, invasion, and metastasis	Inhibits growth in an OE19 or SK-GT-4 xenograft tumor in nude mice (38.83% inhibition) at a dose of 100 mg/kg/day	86
Liver cancer	Induces the down-regulation of YAP1	Inhibits cell viability and induces migration, invasion, and apoptosis	NR	68
Liver cancer	Decreases Lamin B1 and elevated NOV	Inhibits cell viability, induces migration and invasion, and induces cell cycle arrest and apoptosis	NR	87
Liver cancer	Inhibits the JNK signaling pathway	Inhibits cell proliferative viability and induces cell cycle arrest and apoptosis	NR	88
Liver cancer	Suppresses angiogenesis	Inhibits cell proliferative viability and induces apoptosis	Inhibits tumor growth (33.3% for 0.5 mg/kg, 50.7% for 1 mg/kg, and 56.0% for 2 mg/kg) in the implanted SMMC-7721 xenograft rat model	74
Liver cancer	Inhibits the AEG1/EMT pathway	Inhibits cell viability and induces migration, invasion, and cell adhesion	NR	89
Liver cancer	Enhances the MAPK pathways and inhibits the Akt/mTOR signaling pathway	Inhibits cell growth, induces cell cycle arrest, and triggers cell cycle arrest and apoptosis	NR	90
Liver cancer	Inhibits the AU-rich element RNA-binding protein 1 signaling pathway	Inhibits cell growth, adhesion, migration, and motility	Inhibits tumor growth at a daily dose of 400 and 800 μg/kg in an orthotopic hepatocellular carcinoma metastasis tumor model	91
Liver cancer	Regulates the expression of p53	Inhibits cell growth	Inhibits tumor growth in a diethyl nitrosamine-induced model	92
Lung cancer	Inhibits MTDH, JAK2/STAT3 and MAPK signaling pathways	Inhibits cell viability (IC_{50} = 3.56 mg/mL), induces migration and invasion, and induces cell cycle arrest and apoptosis	Inhibits tumor growth with 50 mg of Huaier (in a total volume of 0.1 mL) in an A549 and NCI-H1650 xenograft mouse model	67

(continued)

Table 2. *(continued)*

Cancer type	Mechanisms of action	*In-vitro* activity	*In-vitro* activity	Ref.
Lung cancer	Suppresses the JNK/JUN/IL8 signaling pathway, consequently inhibiting IL8, and also impedes the Wnt/β-catenin signaling pathway	Inhibits cell viability proliferation and induces apoptosis	Inhibits tumor growth in an A549 xenograft mouse model at a dose of 400 mg	93
Lung cancer	Inhibits the PI3K/Akt/HIF1α pathway	Inhibits cell viability and induces migration and invasion	Inhibits tumor growth in an A549 xenograft mouse model with the administration of Huaier granule (2.5 g/kg)	84
Lung cancer	Targets epidermal growth factor receptor	Inhibits nonsmall cell lung cancer cell proliferation and promotes apoptosis	Inhibits tumor growth in an A549 xenograft mouse model at a dose of 50 mg of Huaier/100 μL of solution	94
Lung cancer	Strengthens let-7d-5p and targets NAP1L1	Inhibits cell viability and induces migration and invasion	Inhibits tumor growth in an A549 xenograft mouse model at a dose of 2.5 g/kg	95
Lung cancer	Inhibits the miR-26b-5p/EZH2 pathway	Suppresses cell proliferation and induces apoptosis	NR	69
Ovarian Cancer	Inhibits the Akt/GSK3β/β-catenin pathway	Inhibits cell viability, induces migration and invasion, and induces apoptosis	Inhibits growth in an SKOV3 xenograft tumor in nude mice at a dose of 4 g/kg	96
Pancreatic cancer	Inhibits the Wnt/β-catenin pathway	Inhibits cell viability (IC_{50} = 5 mg/mL) and induces migration and invasion as well as induces apoptosis and EMT	Inhibits tumor growth in a pancreatic cancer xenograft model with MiaPaCa-2 cells at 50 mg/100 μL	27
Pancreatic cancer	Promotes ferroptosis	Inhibits cell viability and increased autophagosome	Inhibits tumor growth in a Panc1 pancreatic subcutaneous tumor model at 2 g/kg	97
Prostate Cancer	Inhibits the AR/AR-V7 pathway	Inhibits prostate cancer cell viability and motility	Inhibits growth in a 22Rv1 xenograft mouse model at a Huaier extract dose of 50 mg in 100 μL of normal saline	98
Triple-negative breast cancer	Inhibits the CircCLASP1/PKR/eIF2α signaling pathway	Induces immunogenic cell death and enhances infiltration of immune cells	Inhibits tumor growth in a 4T1 mouse breast cancer model with 100 μL of solution containing 50 mg of Huaier by gavage every two days	28
Triple-negative breast cancer	Inhibits the nuclear factor kappa B/IκBα pathway	Inhibits cell viability (IC_{50} > 4 mg/mL) and induces cell cycle arrest	Inhibits tumor growth in an MCF-7 xenograft model with Huaier extract (250 mg/mL) by gavage once every two days	99

NR, not reported. EMT, epithelial–mesenchymal transitio; CSNK2B, the regulatory subunit of casein kinase 2; mTOR, mammalian target of rapamycin; S6K, S6 kinase; MTDH, Metadherin; STAT3, signal transducer and activator of transcription 3; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, protein kinase B; p-STAT3, phosphorylated STAT3; YAP1, yes-associated protein 1; MAPK, mitogen-activated protein kinase; AU, adenine uridine; JAK2, Janus kinase 2; HIF1α, hypoxia-inducible factor 1 alpha; JNK, c-Jun N-terminal kinase; JUN, Jun proto-oncogene; NAP1L1, nucleosome assembly protein 1-like 1; AR-V7, AR splice variant 7; PKR, protein kinase R.

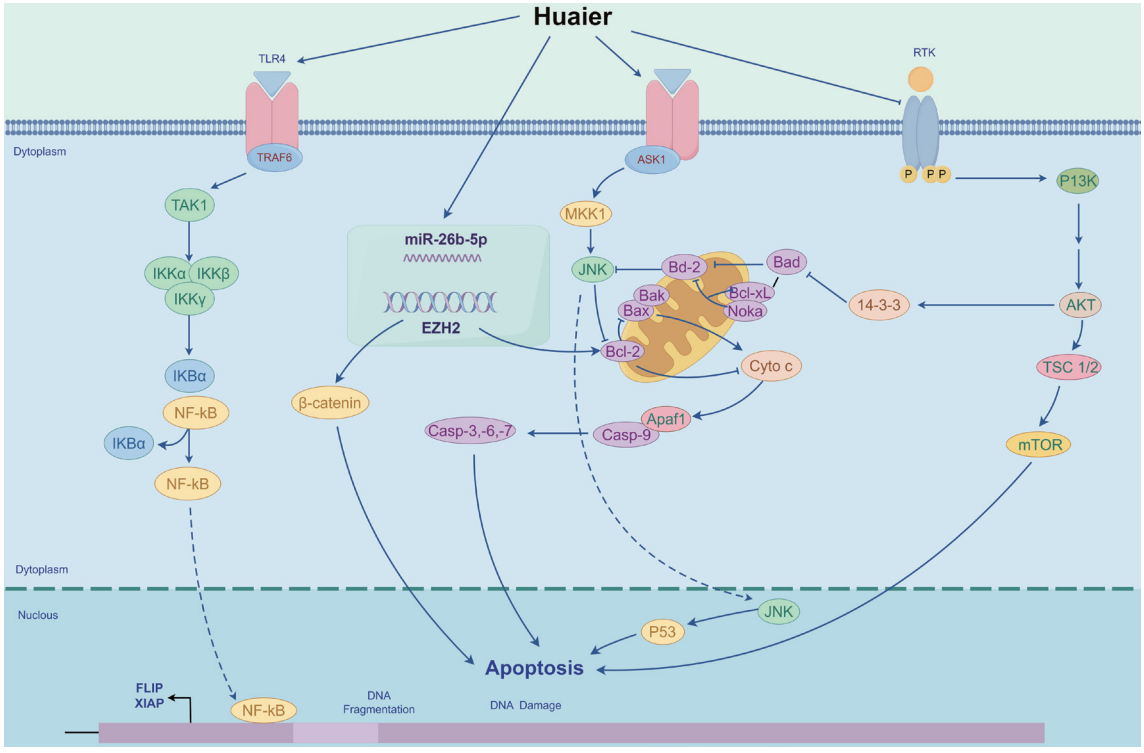

Fig. 4. Mechanism of Huaier inducing apoptosis of cancer cells. TLR4; TRAF6, tumor necrosis factor receptor associated factor 6; TAK1, transforming growth factor beta-activated kinase 1; IKK, ikappaB kinase; IKB, ikappaB; NF-kB, nuclear transcription factor-kappa B; XIAP, X-chromosome-linked inhibitor of apoptosis protein; FLIP, FADD-like inhibitor protein; EZH2, enhancer of zeste homolog 2; ASK1, apoptosis signal-regulating kinase 1; MKK1, mitogen-activated protein kinase kinase 1; JNK, c-Jun N-terminal kinase; RTK, receptor Tyrosine Kinases; P13K, phosphatidylinositol 3-kinase.

that Huaier treatment induces a dose-dependent decline in Bcl2 protein levels and an elevation in Bax protein levels. Moreover, it upregulates p53 expression and enhances apoptosis through caspase-3 activation in A875 cells.[58] Furthermore, the downregulation of Bcl2 expression and the upregulation of Bax further suggest that Huaier induces cell apoptosis through the mitochondrial pathway. Additionally, it has been reported that Huaier extract inhibited cell proliferation and induced apoptosis in the colorectal cancer cell lines HCT116 and HCT8.[27] Significant changes in the levels of apoptosis-related proteins, such as p53, Bax, Bcl2, procaspase 3, and cleaved caspase 3, were observed after the treatment of Huaier extract to HCT116 and HCT8 cells. In breast cancer and lung cancer, miR-26b-5p has low expression,[69] and its overexpression can facilitate tumor cell apoptosis. This effect was confirmed in the breast cancer cell lines MCF-7 and MDA-MB-231 through analyses involving propidium iodide-annexin-V staining and western blotting, which consistently demonstrated caspase 3 as the key mediator of apoptosis. The rhodamine 123 assay showed a decrease in the mitochondrial membrane potential, downregulation of Bcl2 expression, and upregulation of Bax expression, suggesting that the Huaier extract triggers cell apoptosis via the mitochondrial pathway (Fig. 4).

Numerous miRNAs have been identified as being either downregulated or upregulated in human cancers, serving as either oncogenic miRNAs or tumor suppressor miRNAs.[104] It has been demonstrated that dysregulated miRNAs might participate in the pathogenesis and progression of human cancers.[105,106] In experiments conducted on lung adenocarcinoma A549 cells, it was observed that exposure to Huaier led to the differential expression of 66 miRNAs, notably showing a significant increase in miR-26b-5p levels. Subsequent transfection of A549 cells with miR-26b-5p mimics effectively inhibited cell proliferation and induced apoptosis. Moreover, the effects induced by Huaier treatment on A549 cells were reversed upon transfection with an miR-26b-5p inhibitor. However, despite this reversal, it is noteworthy that Enhancer of zeste homolog 2 (EZH2) is upregulated and involved in cell proliferation and apoptosis in multiple cancers.[107] It has been confirmed as a target of miR-26b-5p.[108] Research findings suggest an inverse relationship between miR-26b-5p and EZH2, as evidenced by elevated EZH2 expression and decreased miR-26b-5p levels in nonsmall cell lung cancer cell lines.[69] Moreover, knockdown of EZH2 using shRNA resulted in the overexpression of miR-26b-5p following Huaier treatment, primarily attributed to the decreased expression of EZH2 and its associated proteins. These findings underscore the significant role of EZH2 dysregulation in the antitumor effects of the Huaier-miR-26b-5p pathway in lung cancer cells, suggesting that Huaier may inhibit proliferation and induce apoptosis through the miR-26b-5p-EZH2-mediated pathway.

The mammalian target of rapamycin (mTOR) is a well-conserved serine/threonine protein kinase belonging to the phosphatidylinositol 3-kinase-related kinase protein family.[109] Research has shown that mTOR-mediated signaling pathways, such as the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway,[110] mTOR/p70S6K pathway,[111] and AMPK/mTOR pathway,[112] play essential roles in cellular processes including cell proliferation, metabolism, autophagy, apoptosis, and migration.

Furthermore, these pathways participate in the development and progression of numerous human diseases and can regulate various biological functions within the body.[113] The PI3K pathway

is activated by various survival factors and can activate Akt, which is pivotal in transmitting survival signals. As depicted in Figure 4, the activation of the PI3K/Akt pathway can suppress the activity of pro-apoptotic elements belonging to the Bcl2 family, including Bad, Bax, caspase 9, GSK-3, and FoxO1.[114] In GC cell lines, as the concentration of Huaier increases, there is a corresponding decrease in the viability of MKN45 and SGC7901 cells, coupled with a dose-dependent increase in apoptosis rates. Consequently, western blotting was employed to evaluate the protein expression associated with the PI3K/Akt signaling pathway, given its role in inducing cell apoptosis. The results demonstrated a dose-dependent reduction in the expression levels of p-Akt1, PI3K, PDK1, p-PTEN, and Bcl2, accompanied by the upregulation of caspase 9 expression.[70]

Inhibition of the invasion and metastasis of tumor cells

Tumor metastasis is a complex and continuous process that involves multiple steps and factors, ultimately resulting in treatment failure and patient mortality in cancer. Approximately 90% of cancer-related fatalities are associated with the metastatic dissemination of primary tumor cells to distant sites, away from their original site of occurrence.[115,116] The migration of cancer cells to other parts of the body plays a significant role in the progression and spread of the disease, presenting a substantial obstacle in effectively treating and overseeing cancer patients. *In-vitro* cell invasion, migration, and scratching tests can be used to evaluate the ability of tumor cells to metastasize.

To confirm the impact of Huaier extract on cell migration and invasion, the findings from the wound healing assay demonstrated that treatment with Huaier markedly impeded the migration of pancreatic cancer MiaPaCa-2 and Panc-1 cells in comparison to the control group. Furthermore, the invasion potential of these cells was assessed using Transwell chambers coated with a matrix gel. Remarkably, exposure to Huaier extract resulted in a significant reduction in the invasive capacity of both MiaPaCa-2 and Panc-1 cells. Further analysis into the mechanisms involved revealed that treatment with Huaier extract resulted in a significant reduction in the protein expression levels of matrix metalloproteinase (MMP) 2 and MMP9, pivotal markers associated with migration and invasion, as confirmed by western blot analysis. These results strongly indicate that Huaier extract may possess potent antimetastatic properties by effectively inhibiting cell migration and invasion in pancreatic cancer cell lines, potentially through the modulation of MMP2 and MMP9 expression.[27]

Studies have shown that abnormal expression of CDKN2A is associated with cell migration ability in many types of cancers.[117] Reduced expression or loss of CDKN2A leads to dysregulation of cell cycle control, promoting cell proliferation and migration.[118] CDKN2A produces two main proteins: p16 (INK4a) acts as a CDK inhibitor, while p14 (ARF) serves as a stabilizer of p53.[119] p53 can directly suppress the activity of transcription factors, thereby interfering with the expression of a range of genes involved in cell migration and invasion.[120] Additionally, *TP53* can suppress key factors such as MMPs and MAPK that promote the processes of cell migration and invasion.[121] Huaier extract exhibits inhibitory effects on the migration of squamous cell carcinoma SCL-1 and A431 cells. Wound healing and Transwell invasion assays revealed that knocking down the *CDKN2A* or *TP53* genes could reverse the inhibitory effects of Huaier extract on SCL-1 and A431 cell migration. This finding indicates that Huaier extract suppresses the proliferation, migration, and invasion of squamous cell carcinoma cells by targeting the methylation levels of *CDKN2A* and *TP53*.

In both the wound healing assay and the Transwell assay, Huaier extract significantly suppressed the migratory and invasive potential of CCLP1 cells.[71] Previous studies have demonstrated elevated expression levels of TP73-AS1 in cholangiocarcinoma cells compared to human intrahepatic biliary epithelial cells.[122] In order to further investigate its function, TP73-AS1 siRNA transfected into CCLP1 cells could significantly inhibit cell viability and proliferation. Conversely, overexpression of TP73-AS1 reversed these effects. These findings suggest that Huaier extract exerts its inhibitory effects on cholangiocarcinoma cell proliferation, invasion, and metastasis by regulating the expression of TP73-AS1.

Some studies have demonstrated the involvement of c-Myc in various cellular processes such as cell cycle regulation, cytoskeleton remodeling, and cell adhesion. Moreover, the upregulation of cell migration and invasion has been closely linked to the c-Myc signaling pathway and its downstream targets.[123] Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells undergo a transition to acquire mesenchymal properties. This process plays a crucial role in embryogenesis, wound healing, and tissue regeneration.[124] In the context of the Huaier *n*-butanol extract, it has been observed that the invasion and metastasis of HGC27, MGC803, and AGS GC cells are significantly inhibited in a concentration-dependent manner. As the concentration of Huaier increases, there is a notable inhibition of c-Myc and its downstream targets such as Bmi1 and the EMT-associated protein vimentin within the c-Myc/Bmi1 signaling pathway.[66] These findings strongly suggest that Huaier exerts its inhibitory effects on the invasion and migration of GC cells by suppressing EMT (Table 2).

During EMT, epithelial cells undergo a process where they lose their adhesive connections with neighboring cells and adopt a mobile and invasive behavior.[125] This transformation involves a series of molecular alterations in cells, including the decrease in expression of epithelial markers like E-cadherin, and the increase in expression of mesenchymal markers such as N-cadherin and vimentin.[126] These changes lead to a loss of cell polarity and increased cell motility.[127] It was observed that the expression of astrocyte elevated gene 1 (AEG1) and N-cadherin was significantly reduced, while the expression of E-cadherin was enhanced in MHCC97-H liver cancer cells.[128] These findings strongly suggest that HPs possess the potential to suppress the metastasis of MHCC97-H cells by inhibiting the EMT process and the AEG1 signaling pathway. In conclusion, Hauier exhibits inhibitory effects on the invasion and migration of cancer cells through various mechanisms, as depicted in Figure 5. This suggests its potential as a therapeutic agent in combating cancer metastasis.

Inhibition of the formation of tumor stem cells

Tumor stem cells, constituting a subset of cells within a tumor, exhibit both self-renewal and differentiation capabilities, thereby exerting a pivotal influence on tumor initiation, progression, and resistance to therapy.[129] Some natural compounds have been found to have potential tumor stem cell-inhibiting activity, such as emodin, which inhibits the formation of tumor stem cells and suppresses breast cancer lung metastasis.[130] These natural compounds can target tumor stem cells by modulating signaling pathways, thus influencing the cell cycle, inducing apoptosis, and inhibiting the expression of stem cell-related genes.

Estrogen receptor alpha 36 (ERα36), as a subtype of ERα, is mainly expressed in the cytoplasm and cell membrane.[131] ERα36 inhibits genomic estrogen signal transduction and plays an important role in the growth, self-renewal, differentiation, and tumor implantation of breast cancer stem cells.[132] In order to detect the

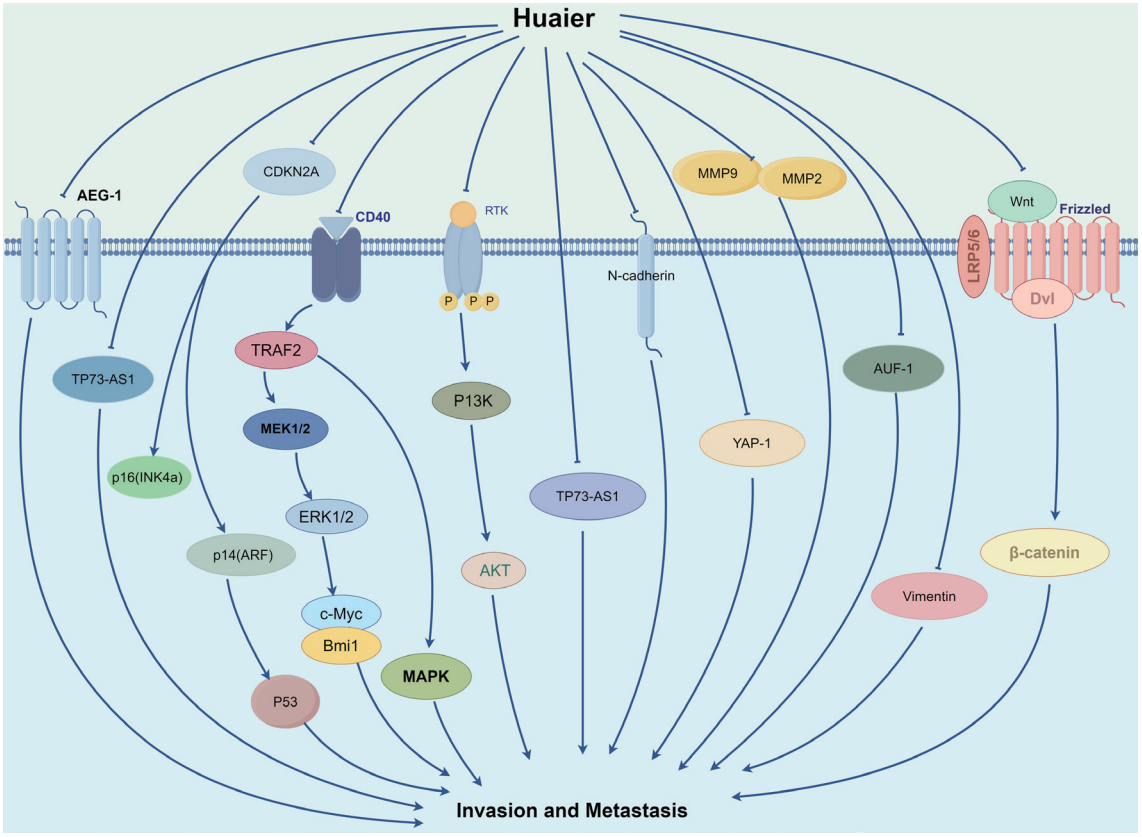

Fig. 5. Mechanism of Huaier inhibiting cancer cell invasion and metastasis. AKT, protein kinase B; Dvl, disaster victim identification; LRP5/6, lipoprotein receptor-related protein 5/6; MAPK, mitogen-activated protein kinase; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; RTK, receptor Tyrosine Kinases; TRAF2, tumor necrosis factor receptor associated factor 2.

effect of HPs on the stem-like characteristics of triple-negative breast cancer cells, the mammary gland globular formation assay was used to evaluate its inhibitory effect.[72] The results showed that the number and size of mammospheres in Mb436 and SUM159 cells significantly decreased, and their forming ability was also significantly reduced. To further evaluate HP's impact on triple-negative breast cancer cell stemness, researchers observed reduced levels of the stem cell-associated transcription factors Nanog, Oct 4, and Bmi1 following HP treatment. Moreover, HPs effectively suppressed ERα36 expression in Mb436 and SUM159 cells. However, in the absence of ERα36 or under HP treatment, the expression of stem cell-related genes was notably disrupted. This suggests that HPs significantly diminish ERα36 expression, targeting breast cancer stem cells in triple-negative breast cancer, resulting in decreased mammosphere formation and the downregulation of stem cell-associated gene expression (Table 2).

The Hedgehog signaling molecule is a locally secreted protein ligand produced by signaling cells that regulates cell growth, migration, invasion, and stem cell function in tumors.[133] Of note, after being treated with varying concentrations of Huaier extract for 24 h, there was a noticeable reduction in both the quantity and size of breast cancer MCF7 cells. Concurrently, the levels of stem cell markers (OCT-4, NESTIN, and NANOG) decreased, indicating a potential inhibitory effect of Huaier extract on stem cell formation. This effect was observed to weaken under conditions of Hedgehog pathway inactivation, suggesting a role for Huaier in activating the Hedgehog pathway to inhibit stem cell formation.[73]

Inhibition of tumor angiogenesis

Tumor angiogenesis refers to the process of the formation of new blood vessels specifically within a tumor. It is a critical step in tumor growth and metastasis. Unlike normal tissues, which have a regulated and controlled angiogenesis process, tumors exhibit an abnormal and excessive angiogenic response.[134] Tumor cells release various pro-angiogenic factors that stimulate the development of new blood vessels from the existing vasculature. Therefore, inhibiting tumor angiogenesis restricts tumor cells from obtaining essential oxygen and nutrient resources, effectively impeding the continuous proliferation and migration of tumor cells. Moreover, inhibiting tumor angiogenesis enhances the efficacy of other therapeutic modalities, such as chemotherapy, radiation therapy, and immunotherapy. A well-established blood supply facilitates the efficient delivery of targeted therapeutics to tumor cells and augments the immune cells' capacity to mount an effective antitumor attack.

Vascular endothelial growth factor (VEGF), a potent glycoprotein with significant biological activity, was initially identified as a regulator of vascular permeability. It facilitates the proliferation and migration of vascular endothelial cells, playing a pivotal role in angiogenesis, the formation of new blood vessels from pre-existing ones.[135] Furthermore, VEGF serves as a regulatory factor in tumor angiogenesis during tumor progression. Multiple studies have demonstrated a substantial elevation in VEGF secretion as tumor cell clusters transition into solid tumors.[136,137] A study found that Huaier granule exerted an effect on human umbilical

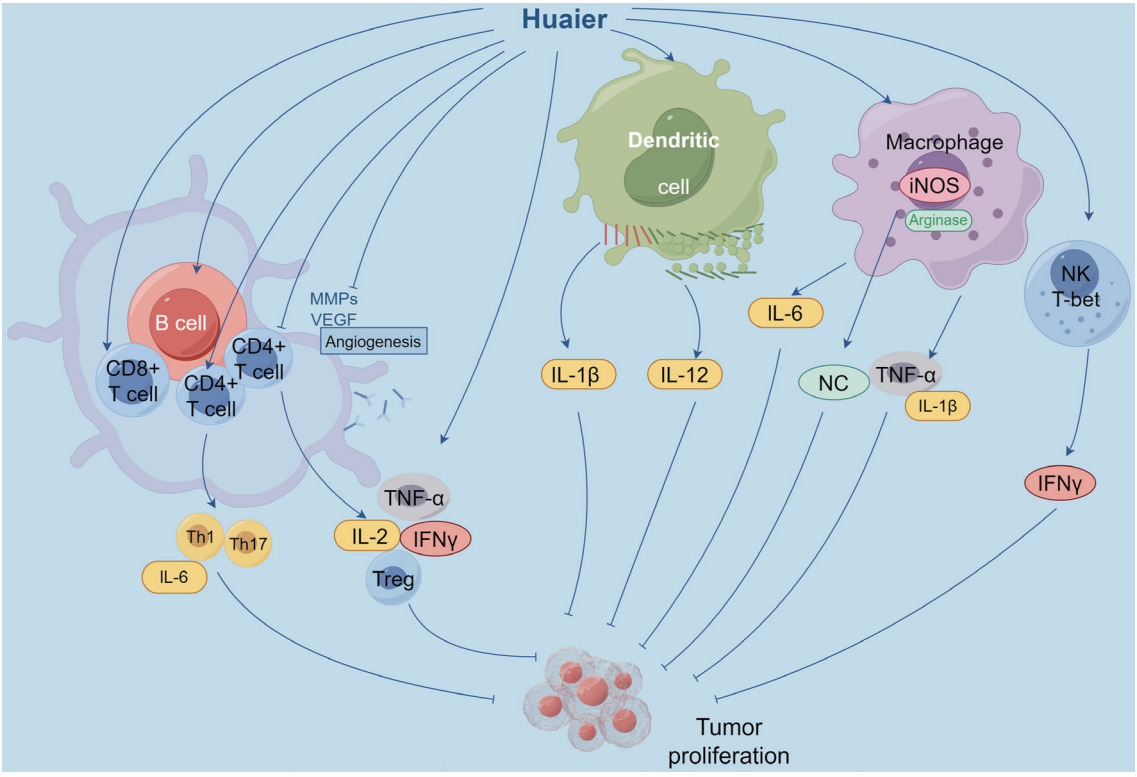

Fig. 6. Mechanism of Huaier enhancing immune function. IFN, Interferon; MMPs, matrix metalloproteinases ; NK, natural Killer cell; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; iNOS, inducible nitric oxide synthase.

vein endothelial cells.[57] It was observed that treatment with Huaier granule led to a significant increase in the G0/G1 phase of the cell cycle, indicating suppression of cell proliferation. Additionally, *in-vitro* experiments demonstrated that Huaier granule inhibited the migration and invasion of human umbilical vein endothelial cells in a dose- and time-dependent manner. Moreover, experiments utilizing tubule formation assays and chicken embryo chorioallantoic membrane models have confirmed the anti-angiogenic properties of Huaier granules. MEK/ERK also has been shown to be the upstream pathway mediating VEGF expression,[138] and the ERK signaling pathway was inhibited after Huaier treatment. Mechanistic studies have shown that this effect downregulates VEGF expression through the ERK pathway, thereby inhibiting tumor angiogenesis.

Hypoxia-inducible factor 1 alpha (HIF1α) is an important transcription factor that is activated in hypoxic environments and promotes the expression of multiple factors, including those associated with tumor growth, drug resistance, and metastasis. Conversely, when HIF1α was inhibited, cell viability and angiogenesis were also significantly inhibited. VEGF is regulated by HIF1α, and the stability and activity of HIF1α increase in a low-oxygen environment, thus promoting the transcription of the *VEGF* gene. VEGF itself can also increase HIF1α expression by activating the HIF1α signaling pathway.[139] They interact with each other during tumor growth and angiogenesis as well as promote tumor angiogenesis and development.[140] It has been found that HPs can significantly inhibit the growth of primary tumors in nude mice, and the number of lung metastases in mice after tail vein transplantation of SMMC-7721 cells showed a dose-dependent decrease with HP treatment.[74] In the tumors of mice under control and HP treatment, a series of protumor mediators related to angiogenesis and tumor development were examined, including HIF1α, VEGF, AU-rich element RNA-binding protein 1, and AEG1. These results indicate that HPs significantly inhibit the expression of HIF1α and VEGF in hepatocellular carcinoma transplant tumors. Therefore, HPs work by downregulating VEGF and HIF1α to inhibit tumor angiogenesis, thereby slowing tumor growth and lung metastasis, demonstrating a significant antitumor effect.

Enhancement of immune function

Natural killer cells are an essential component of the immune system, and they are actively involved in combating tumor development.[141] These cells are capable of recognizing and eliminating cancer cells without prior sensitization or antigen recognition, making them an important component of the innate immune response against tumors. Moreover, natural killer cells can regulate and modulate the activity of other immune cells involved in the antitumor response.[142] They can enhance the function of dendritic cells, T cells, and macrophages, promoting a coordinated and robust immune response against tumor cells. The anticancer properties of Huaier are not only reflected in the direct effect on tumor cells, but it also has a broad spectrum of regulatory effects on many components of the immune system, including immune cells and immune molecules, thereby indirectly killing tumor cells. Huaier has a role in influencing the immune microenvironment and regulating the antitumor immune response by influencing interactions and signaling (Fig. 6).

Lymphocyte proliferation is an indicator of immune enhancement and can be used as a method to evaluate the activity of T

or B lymphocytes. Research has demonstrated that Huaier water extract profoundly suppresses the human cholangiocarcinoma cell lines QBC 939, Sk-ChA-1, and MZ-ChA-1, while also inducing activation in various subsets of lymphocytes to some degree.[143] Additionally, Huaier can effectively enhance the phagocytic capacity of macrophages and the production of nitric oxide. In a study conducted by Sun and colleagues, the relationship between this response and the upregulation of inducible nitric oxide synthase (iNOS) activity was evaluated. The results showed that after treatment with Huaier, the iNOS activity of macrophages was significantly enhanced. Therefore, Huaier significantly stimulates the production of nitric oxide by macrophages through upregulating iNOS activity, demonstrating its *in-vitro* immunomodulatory and antitumor activity.

Interleukin 6 (IL6) is a cytokine belonging to the chemokine family of cell factors. IL6 is a cytokine produced by monocytes, T cells, and other cells as well as cells of the immune system such as B lymphocytes and macrophages.[144] The classical IL6 signaling pathway plays a crucial role in protective innate immune responses and tissue repair processes.[145] Remarkably, Huaier treatment significantly inhibited tumor formation in human liver cancer HepG2 cells and exhibited good antitumor activity in H22 tumor-bearing mice, without apparent toxic side effects. Further studies revealed that Huaier upregulates iNOS mRNA and protein levels in RAW 264.7 cells, stimulating the production of nitric oxide in the cells.[146] Tumor necrosis factor alpha is produced by macrophages, exhibits cytotoxicity to various tumor cells, and is an important mediator of the immune response against bacterial infections.[147] To further investigate the immunomodulatory activity of Huaier, it was observed that the mRNA levels of tumor necrosis factor alpha were significantly upregulated after Huaier treatment, while the expression and secretion of IL6 were significantly enhanced. Moreover, there was a notable elevation in the expression of cyclooxygenase-2, an immune factor released by macrophages, in RAW 264.7 cells.[148] Examination of RNA-sequencing data revealed that the administration of Huaier substantially augmented the phosphorylation of p38, ERK, and JNK within RAW 264.7 cells, indicating that TPG-1 enhances the immune capabilities of RAW 264.7 cells through activation of the nuclear factor kappa B and MAPK signaling pathways (Table 2).

Conclusion

Huaier, as a TCM, has low toxicity and obvious antitumor activity in many types of cancer. *In-vitro* studies have demonstrated that Huaier can inhibit tumor cell growth and proliferation through classical pathways such as p53-MAPK. It can increase the ratio of Bax/Bcl2 and promote the expression of cleaved caspase 3, thereby inducing tumor cell apoptosis, inhibiting angiogenesis, and suppressing tumor cell invasion and metastasis. Huaier also reduces the number of $CD44^{+}/CD24^{-}$ cells and decreases the expression of stem cell markers, thereby enhancing the body's immune response. However, research into effective anticancer treatment strategies for Huaier is still in the experimental stage, and these results indicate the potential applications of Huaier in the development of anticancer drugs as well as in cancer treatment and prevention. *In-vivo* studies in animal models have shown that Huaier has a high tumor suppression rate and low toxicity, laying a solid foundation for its clinical use in humans and confirming its clinical value. In addition to the mentioned benefits, the multifaceted nature of Huaier presents both challenges and opportunities for further research. Although it is reassuring to see the effectiveness of Huaier as a novel anticancer agent mentioned in this review, there are still significant limitations in the current situation. In most of the available studies, the observed biological effects of Huaier have been observed using Huaier water extract, which may be due to the combined actions of various components present in Huaier compounds. Therefore, further research is needed to investigate the specific active anticancer ingredients unique to Huaier. We hope that this review can help more relevant researchers understand the potential of Huaier in cancer treatment and open up possibilities for innovative treatment strategies.

Future research directions

Given the results discussed above, a large number of basic experimental studies have demonstrated that Huaier has significant antitumor effects, and it can be speculated that it has great potential in cancer treatment. However, there are only a few studies in clinical trials on Huaier adjuvant therapy for postoperative liver cancer. In addition, no studies have shown that Huaier can be fully absorbed in the human body, and the distribution and metabolism of the drug are unknown. Therefore, in-depth clinical studies, either alone or in combination with existing therapies, are needed to elucidate the untapped potential of chemoprophylaxis and treatment. Second, it is necessary to improve the extraction rate and product quality control of Huaier, identify the components, and analyze the specific active compounds. Despite these challenges, the complex composition and broad pharmacological actions of Huaier provide promising avenues for further exploration. The objective, qualitative, and quantitative research on its antitumor effect and mechanism from multiple levels, multiple angles, and multiple targets still needs to be explored. It is believed that with the deep integration of TCM theory and modern medical technology, further development and research of Huaier's anticancer effects will make a breakthrough.

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Conflict of interest

JJQ has been an associate editor of *Oncology Advances* since 2023. The authors have no other conflicts of interest related to this publication.

Author contributions

Manuscript writing (XNZ) and manuscript editing (SQT, JJQ); critical revision, critical funding, and administration (XQG). All authors have made a significant contribution to this study and have approved the final manuscript.

Data sharing statement

The data used in support of the findings of the study are included within the article.

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