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Review

Advances in extraction, purification, and pharmacological mechanisms of calycosin: a comprehensive review

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ABSTRACT

Calycosin, a pivotal isoflavonoid active constituent derived from *Astragalus membranaceus*, is designated as a key marker compound for the quality assessment of Astragalus and its products in the *Pharmacopoeia of the People's Republic of China* (2020 Edition). It exhibits a broad spectrum of pharmacological activities and holds significant potential for clinical application. This article systematically reviews the research progress on calycosin. In terms of extraction, isolation, and purification, techniques such as flash extraction and hydrolytic extraction enable efficient enrichment of the compound, whereas methods such as macroporous adsorption resin and high-speed counter-current chromatography allow for high-purity preparation. The biosynthetic pathways of calycosin encompass the phenylpropanoid pathway in planta, the chemical "one-pot" method *in vitro*, and synthesis *via* microbial cell factories, offering diverse strategies for large-scale production. Structural derivatization, particularly through modification of the 7- and 3'-hydroxyl groups, significantly enhances its solubility and antitumor activity. With respect to pharmacological mechanisms, calycosin exerts multi-pathway and low-toxicity effects in diseases such as inflammation, cancer, and neural injury by modulating multiple signaling pathways, including NF-κB, PI3K/AKT, and MAPK. Furthermore, pharmacokinetic studies indicate that its absorption depends on deglycosylation, that it undergoes substantial hepatic first-pass metabolism, and that its tissue distribution is organ-specific. Safety evaluations suggest low toxicity at therapeutic concentrations. This review aims to clarify the core issues concerning the translation of basic research on calycosin into clinical practice, thereby providing a theoretical foundation for subsequent development.

1. Introduction

Astragalus membranaceus (Huangqi), renowned as the "supreme herb for replenishing Qi" in traditional Chinese medicine (TCM), was first documented in the *Shennong Bencao Jing* (*Divine Farmer's Materia Medica*) and classified as a superior-grade herb (Figs. 1A–1B). Its dried root, used medicinally for over a thousand years, is clinically applied for conditions such as Qi deficiency and fatigue, chronic diarrhea and rectal prolapse, and non-healing carbuncles and abscesses [1]. Modern pharmacological studies have further confirmed its diverse pharmacological activities, including immunomodulation, antioxidation, and cardiocerebrovascular protection [2]. Calycosin, a key isoflavonoid active compound isolated and purified from the roots and rhizomes of *Astragalus*, is characterized by functional groups such as phenolic hydroxyl groups at the C7 and C3' positions (Fig. 1C). It is not only designated as a core marker for the quality assessment of Astragalus and its products in the *Pharmacopoeia of the People's Republic of China* (2020 Edition), which requires the content of calycosin-7-O-β-*D*-glucoside in the dried material to be no less than 0.02%, but has also become a hotspot molecule in recent pharmacological research on natural medicines [3, 4]. This compound appears as white needle-shaped crystals, is readily soluble in organic solvents such as methanol, ethanol, and dimethyl sulfoxide, and its content in *Astragalus membranaceus* var. mongholicus is significantly higher than in *Astragalus membranaceus*. Furthermore, its accumulation is greater in the fine roots (lateral roots) of cultivated varieties compared with the coarse roots (taproots) of wild varieties. This distribution characteristic provides a crucial basis for its targeted extraction and industrial application [5, 6].

In recent years, with the deep integration of molecular biology and pharmacological research methods, the spectrum of pharmacological activities of calycosin has continued to expand. Studies have shown that this compound exhibits significant biological effects in multiple areas, including anti-inflammatory, anti-

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Fig. 1 *Astragalus membranaceus* plants, decoction pieces, and their monomers. (A): *Astragalus membranaceus* plants; (B): *Astragalus membranaceus* slices; (C): Calycosin.

oxidant, antitumor, and neuroprotective activities, as well as in the repair of heart and kidney damage. Particularly in complex disease models such as chronic inflammation-related diseases, neurodegenerative diseases, and malignant tumors, calycosin demonstrates the unique advantages of being "multi-pathway and low-toxicity", indicating promising clinical application prospects [7-9]. However, its specific targets, signaling network pathways, and synergistic mechanisms have not yet been fully elucidated. Accordingly, this review systematically summarizes key research progress on the isolation and purification, biosynthesis, structural derivatization, pharmacological signaling pathways, pharmacokinetics, and safety evaluation of calycosin. It aims to clarify the core scientific issues in translating basic research into clinical application and to provide a theoretical basis and research directions for subsequent precise target discovery, development of new formulations, and rational clinical use.

2. Isolation and purification of calycosin

Calycosin, an isoflavonoid with significant pharmacological activity found in leguminous plants, is based on an isoflavone core structure featuring a methoxy group at the 4' position and a hydroxyl group at the 3' position. This structural feature confers strong biological activity and target-binding capacity. Owing to the complexity of plant extracts, the content of calycosin is typically low (0.1–1.0 mg·g^{-1}), and it often coexists with structurally similar isoflavonoids such as formononetin and daidzein, posing challenges for its isolation and purification [10]. Therefore, establishing an efficient and stable isolation and purification process is a core step in realizing its medicinal value. This section systematically reviews the extraction techniques and separation/purification methods for calycosin, provides a detailed analysis of the optimized parameters, separation mechanisms, and application outcomes of each method, compares the advantages and limitations of different technologies, and offers a theoretical basis for the efficient preparation and industrial application of calycosin (Fig. 2).

2.1. Extraction techniques

Extraction is the prerequisite for isolation and purification, aiming to maximize the enrichment of calycosin while minimizing impurity interference. Well-established extraction methods currently include flash extraction, hydrolytic extraction, and ultrasound-assisted enzymatic hydrolysis.

2.1.1. Flash extraction

Flash extraction, based on the principles of mechanical shearing and cell disruption, accelerates the dissolution of calycosin through high-intensity agitation. Studies showed that, using Astragalus as the raw material with an extraction temperature of 70 °C, 5% ethanol concentration, and a system pH of 10, and employing a JHBE-50S flash extractor for two extraction cycles (1 min each with a 1-min interval), calycosin and its glycosides were effectively extracted, with the extraction yield increased by 30%–40% compared with traditional decoction [11]. The advantages of this method include short extraction time (total time < 5 min), reduced degradation of thermosensitive components, and

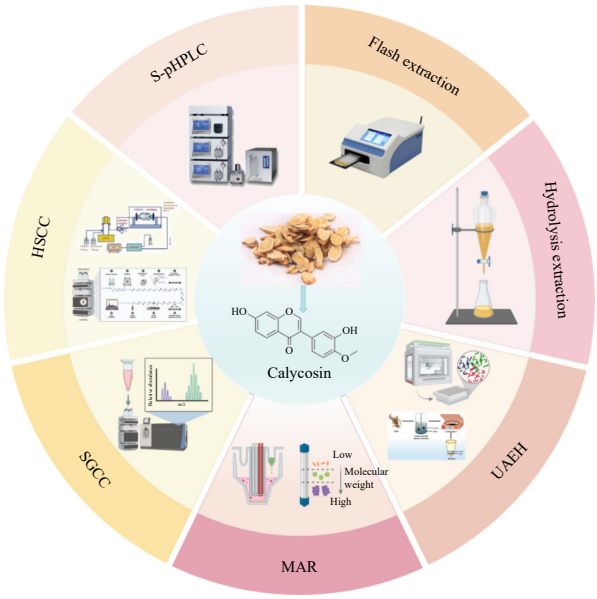

Fig. 2 Total separation and purification of calycosin

suitability for large-scale production. However, the extract contains relatively high levels of impurities (e.g., polysaccharides and proteins), necessitating subsequent purification steps.

2.1.2. Hydrolytic extraction

Calycosin predominantly exists in plants in glycosidic forms (e.g., calycosin-7-O-β-*D*-glucoside) and requires hydrolysis to its free aglycone form to improve extraction efficiency. One study reported that, using 100% ethanol as the extraction solvent, 2.5 mol·L^{-1} hydrochloric acid as the hydrolyzing agent, a solid-to-liquid ratio of 1 : 40, and extraction in a 37 °C water bath for 2 h, a glycoside conversion rate of over 90% was achieved, with the extraction amount of free calycosin reaching 1.116 mg·g^{-1} [12]. Acid hydrolysis releases the aglycone by cleaving the glycosidic bond, but it requires strict control of hydrochloric acid concentration and reaction time to avoid excessive hydrolysis that could damage the isoflavone core structure.

2.1.3. Ultrasound-assisted enzymatic hydrolysis (UAEH)

For extraction of calycosin from chickpeas, cellulase-assisted ultrasound extraction significantly improves efficiency. The optimal process parameters determined by response surface methodology combined with a genetic algorithm were as follows: enzymatic hydrolysis time, 100 min; enzymatic hydrolysis temperature, 40 °C; enzyme dosage, 15 mg·g^{-1} ; ultrasound time, 1.73 h; ethanol concentration, 78.64%; and liquid-to-material ratio, 24.9 mL·g^{-1}. Under these conditions, the extraction yield reached 0.76 mg·g^{-1} [13]. Enzymatic hydrolysis disrupted the cell wall structure, whereas ultrasound cavitation enhanced component dissolution; their synergistic effect increased the extraction yield by 25% compared with ultrasound alone.

2.2. Separation and purification methods

2.2.1. Enrichment by macroporous adsorption resin (MAR)

Macroporous adsorption resins are widely used for the enrichment and purification of calycosin owing to their porous structure and selective adsorption capacity. Among them, HPD500 macroporous adsorption resin has been confirmed to exhibit good enrichment performance for calycosin. The optimized process conditions were as follows: calycosin sample concentration, 0.69 mg·mL^{-1}; sample solution pH, 5; sample volume, 12 BV (108

mL); sample flow rate, 0.1 mL·min^{-1}; 60% ethanol solution as the desorption solvent; and desorption volume, 15 BV (135 mL). Under these conditions, the content of calycosin increased from 1.75% to 7.72%, with an enrichment factor of 4.41 and a recovery rate of 85.6%. This process achieves preliminary purification by specifically adsorbing calycosin onto the resin, thereby removing water-soluble impurities such as sugars and amino acids. Kinetic studies indicate that the adsorption of calycosin onto HPD-500 resin follows a pseudo-second-order kinetic model, and the adsorption isotherm can be described by both Langmuir and Freundlich models, suggesting that the adsorption process is predominantly chemisorption with heterogeneous adsorption sites [14, 15].

2.2.2. Purification by silica gel column chromatography (SGCC)

Silica gel column chromatography, which separates compounds based on polarity differences, is a key step in the purification of calycosin. Samples enriched by macroporous adsorption resin can be further purified using silica gel column chromatography. Using a petroleum ether-acetone system for gradient elution, crude calycosin can be obtained from the fraction eluted with petroleum ether-acetone (100 ∶ 3.5). Combined with low-temperature crystallization and recrystallization, the final calycosin crystals reached a purity of 98.5% with an overall yield of 32.6%. Silica gel exhibits strong adsorption for the relatively polar calycosin; by gradually increasing the proportion of acetone in the eluent, separation from other flavonoid components can be achieved. In studies on *Astragalus membranaceus* var. mongholicus, the combined use of silica gel column chromatography and polyamide column chromatography with a cyclohexane-acetone (25 ∶ 100–0 ∶ 100) elution system successfully isolated high-purity calycosin monomer from a 70% ethanol extract [14].

2.2.3. Refinement by high-speed counter-current chromatography (HSCC)

High-speed counter-current chromatography (HSCCC), a liquid-liquid partition chromatography technique without a solid support, effectively avoids irreversible sample adsorption and is suitable for high-purity preparation of calycosin. Using a two-phase solvent system of n-hexane-ethyl acetate-ethanol-water (3 ∶ 5 ∶ 3 ∶ 5, *V/V*), with the upper phase as the stationary phase and the lower phase as the mobile phase, at a flow rate of 2 mL·min^{-1}, a rotor speed of 850 r·min^{-1}, and a column temperature of 20 °C, 1.3 mg of calycosin with a purity of 95.8% and a recovery rate of 85.9% was isolated from 264.9 mg of crude extract [16, 17]. This method achieves efficient separation from impurities by optimizing the two-phase solvent ratio to adjust the partition coefficient (*K* value) of the target compound. Compared with traditional column chromatography, HSCCC offers advantages such as high separation efficiency, high sample recovery, and operational simplicity, making it suitable for preparation of high-purity calycosin standards [16, 17].

2.2.4. Semi-preparative high-performance liquid chromatography (S-pHPLC)

Semi-preparative HPLC is a core technique for obtaining high-purity monomers. Using a Waters SunFire™ C_{18} column (100 mm × 19 mm, 5 μm) as the stationary phase and water-acetonitrile as the mobile phase for gradient elution (5% acetonitrile → 65% acetonitrile, 5–60 min), at a flow rate of 5 mL·min^{-1} and detection wavelength of 254 nm, calycosin isolated from chickpea extract achieved a purity of 97.45% [11]. This method offers high separation efficiency and good reproducibility but has a small preparation scale, making it suitable for laboratory-scale sample preparation.

In practice, a strategy combining multiple methods is often adopted to improve the purity and yield of calycosin. For example, Astragalus waste residue subjected to flash extraction with 70% methanol, followed by sequential enrichment with HPD500 macroporous adsorption resin, separation by silica gel column chromatography, and low-temperature crystallization and recrystallization, ultimately yielded calycosin with a purity exceeding 98% [14]. This combined process integrates the high efficiency of flash extraction, the enrichment capability of macroporous adsorption resin, and the fine separation capability of silica gel chromatography, enabling efficient preparation of high-purity calycosin from complex matrices.

Furthermore, a 70% ethanol extract of *Astragalus membranaceus* var. mongholicus, after preliminary separation with HPD-100 macroporous adsorption resin and purification by silica gel column chromatography and Sephadex LH-20 gel column chromatography, can yield relatively high-purity calycosin and its derivatives [18].

Several mature methods currently exist for the isolation and purification of calycosin. Flash extraction combined with macroporous adsorption resin enrichment is a common process for industrial production, whereas the combination of silica gel column chromatography and HSCCC is suitable for preparation of high-purity samples. These methods have their respective advantages, but some shortcomings remain, such as the need for further purity improvement after macroporous resin enrichment and the complexity of solvent system selection for HSCCC. Future research should focus on optimizing process parameters to improve separation efficiency and product purity; developing new adsorption materials and separation technologies, such as molecularly imprinted polymers and supercritical fluid chromatography, to reduce organic solvent use and lower costs; and strengthening the integrated application of various separation technologies to achieve efficient and green preparation of calycosin, providing the material foundation for its pharmacological research and industrial application.

3. Synthesis pathways of calycosin

Calycosin shows broad application prospects in medicine and health foods. This section reviews the synthesis pathways of calycosin, encompassing its biosynthesis in planta, chemical synthesis *in vitro*, and microbial synthesis, aiming to provide a comprehensive overview of the current research status on calycosin synthesis pathways and to offer a theoretical basis for its further development and utilization.

3.1. Biosynthesis pathway

The biosynthesis of calycosin initiates from the phenylpropanoid pathway. Within this pathway, *L*-phenylalanine is converted to trans-cinnamic acid by phenylalanine ammonia-lyase (PAL). Subsequently, cinnamate 4-hydroxylase (C4H) hydroxylates trans-cinnamic acid to p-coumaric acid, and 4-coumaroyl-CoA ligase (4CL) further converts p-coumaric acid to p-coumaroyl-CoA (Fig. 3). p-Coumaroyl-CoA serves as a key precursor participating in subsequent reactions, laying the foundation for the synthesis of calycosin [19]. This series of reactions constitutes crucial initial steps for the synthesis of numerous secondary metabolites in plants and is of great significance for maintaining physiological functions and defense mechanisms.

Chalcone synthase (CHS) catalyzes the condensation of p-coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin chalcone, which is then converted to naringenin by chalcone isomerase (CHI). Alternatively, for the synthesis of liquiritigenin, a direct precursor for certain isoflavonoids including those in calycosin biosynthesis, CHS acts in concert with chalcone reductase (CHR) to produce isoliquiritigenin, which is subsequently isomerized to liquiritigenin by CHI (Fig. 3). In *Astragalus* species, this process is a key step in calycosin synthesis,

and the activity and expression levels of the relevant enzymes directly affect the yield of calycosin [19, 20].

After formation of the basic isoflavone skeleton, it undergoes a series of post-modification processes, including methylation, hydroxylation, and glycosylation. O-Methyltransferases (OMTs) catalyze the transfer of a methyl group from S-adenosyl-*L*-methionine (SAM) to the hydroxyl moiety of isoflavones, participating in the synthesis of calycosin. Studies have found that AmOMT2 from *Astragalus membranaceus* exhibits catalytic activity toward various flavonoids, with relatively strong specificity for isoflavones, and can perform 4'- or 3'-O-methylation on different flavonoid substrates. Isoflavone 3'-hydroxylase (I3'H) catalyzes the hydroxylation at the 3' position of the B ring of formononetin, a key step in the synthesis of calycosin. Glycosyltransferases (UGTs) transfer sugar molecules from sugar donors such as UDP-glucose to acceptor molecules, participating in the synthesis of calycosin glycosides (Fig. 3). In *Astragalus*, AmUGT88E29 and AmUGT88E30 show high *in vitro* catalytic activity toward isoflavones. Moreover, overexpression of AmUGT88E29 in Astragalus hairy roots significantly increased the content of calycosin-7-O-β-*D*-glucoside [21].

Furthermore, the synthesis of calycosin in planta is regulated by various factors. Research indicates that chitosan, acting as a biological elicitor, can induce the accumulation of both calycosin and formononetin in Astragalus hairy root cultures. Following chitosan treatment, an oxidative burst is triggered, which induces the expression of genes associated with the mitogen-activated protein kinase (MAPK) signaling cascade (specifically, MPK3 and MPK6). This, in turn, promotes the transcriptional activation of pathogenesis-related (PR) genes (such as those encoding β-1,3-glucanase, chitinase, and PR-1), as well as eight biosynthetic genes within the calycosin and formononetin synthesis pathways, ultimately enhancing calycosin synthesis [22].

3.2. Chemical synthesis methods

Fu et al. [23] synthesized calycosin using a "one-pot" method with resorcinol and 3-methoxy-4-hydroxyphenylacetic acid as starting materials. The specific process involves the condensation of resorcinol and 3-methoxy-4-hydroxyphenylacetic acid under specific conditions to yield calycosin (Fig. 4). This method provides a novel chemical synthesis pathway for calycosin. Compared with traditional methods, the "one-pot" approach offers advantages such as operational simplicity and streamlined reaction steps, providing a valuable reference for further research on the chemical synthesis of calycosin.

3.3. Microbial synthesis methods

The use of microorganisms for calycosin synthesis has been a research hotspot in recent years. Construction of suitable microbial cell factories enables highly efficient production of calycosin. Regarding the choice of microbial host, both *Escherichia coli* and yeast systems offer distinct advantages. *E. coli*, prized for its simple genetics and rapid growth, is frequently employed to construct pathways for hydroxylated flavonoid precursors such as those of calycosin. Conversely, yeast (e.g., *Saccharomyces cerevisiae*), owing to its superior eukaryotic protein processing machinery and functional expression of plant-derived enzymes (e.g., P450s), demonstrates unique potential for reconstituting complete flavonoid pathways.

In terms of specific synthesis strategies, Hu et al. [24] isolated a UDP-dependent glycosyltransferase (UGT), UGT88E18, from soybeans and expressed it in *Escherichia coli*. The recombinant UGT88E18 was capable of selectively and efficiently synthesizing calycosin and further glycosylating the C7 hydroxyl group of calycosin to produce calycosin-7-O-β-*D*-glucoside. The authors also developed a one-pot reaction coupling UGT88E18 with soybean sucrose synthase (SuSy). The UGT88E18-SuSy cascade reaction facilitates the recycling of expensive uridine diphosphate glucose (UDP-Glc) by utilizing inexpensive sucrose and a catalytic amount of uridine diphosphate (UDP). Under further optimized reaction conditions, through stepwise addition of calycosin, a final production of 10.5 $g \cdot L^{-1}$ calycosin-7-O-β-*D*-glucoside was achieved. The molar conversion rate of calycosin reached 97.5%, the space-time yield was 747 $mg \cdot L^{-1} \cdot h^{-1}$, and UDP-Glc was recycled 78 times. Therefore, by introducing glycosyltransferases and optimizing UDP-glucose supply, the production of calycosin and its

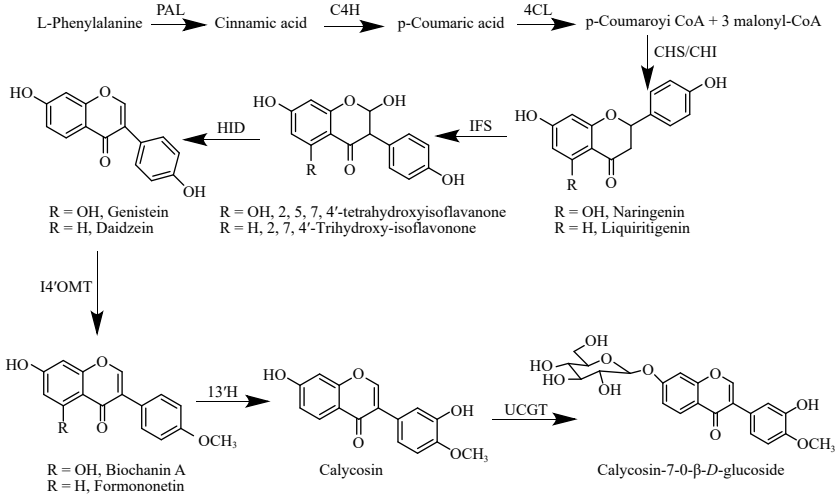

Fig. 3 Calycosin biosynthesis pathway.

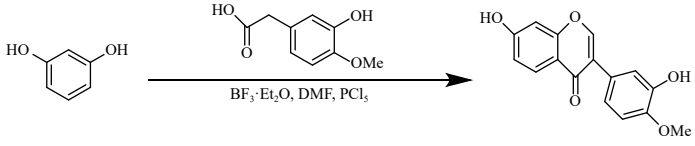

Fig. 4 Chemical synthesis pathway of calycosin.

glycoside derivatives holds significant promise. This microbial synthesis approach offers advantages such as being environmentally friendly and cost-effective, providing a new direction for large-scale production of calycosin.

To achieve higher yields and economic feasibility, a series of metabolic engineering optimization strategies are being applied in microbial synthesis, including: ① Directed evolution of key enzymes: engineering enzymes such as UGTs through methods like error-prone PCR to enhance their catalytic efficiency, stability, and substrate specificity. ② Precursor supply regulation: enhancing the shikimate and malonyl-CoA pathways to increase the intracellular availability of key precursors (e.g., p-coumaroyl-CoA) for the flavonoid skeleton. ③ Cofactor engineering: optimizing the regeneration cycles of crucial cofactors such as NADPH and UDP-glucose, which is critical for alleviating synthesis bottlenecks. ④ Module co-regulation: modularizing and fine-tuning the synthetic pathway to balance metabolic flux and reduce the cytotoxic accumulation of intermediates.

Therefore, by introducing glycosyltransferases and optimizing UDP-glucose supply, the production of calycosin and its glycoside derivatives holds significant promise. This microbial synthesis method offers advantages such as being environmentally friendly and cost-effective, providing a new direction for the large-scale production of calycosin.

However, several bottlenecks must be overcome for successful industrial-scale application: ① Host selection and compatibility: different hosts vary in their capacity for heterologous expression of plant enzymes and in their native metabolic backgrounds, necessitating rational selection and adaptive engineering based on the target product. ② Pathway efficiency and toxicity: heterologous pathways can suffer from low efficiency, and certain intermediate metabolites may exert toxicity on host cells, limiting high-tier production. ③ Scale-up fermentation process: scaling from shake flasks to bioreactors introduces significant engineering challenges related to mass transfer, oxygen supply, and substrate inhibition. ④ Downstream separation and purification costs: the efficient and cost-effective isolation of high-purity calycosin from complex fermentation broths is crucial for economic viability.

4. Calycosin derivatives and their pharmacological activities

Inherent drawbacks of calycosin, such as poor solubility and low bioavailability, limit its clinical application. Structural modification of calycosin to synthesize derivatives with improved physicochemical properties and pharmacological activities has become a research hotspot in recent years. This section reviews the synthesis and pharmacological activities of calycosin derivatives.

Yang et al. [25] used calycosin as the starting material, acetone as the solvent, triethylamine to provide a basic environment, and 4-dimethylaminopyridine (DMAP) as a catalyst to conduct condensation reactions with various acyl chlorides. This approach modified the hydroxyl groups at the 7- and 3'-positions of calycosin, yielding ten calycosin derivatives (A1–A10). Further research found that derivative A10 could concentration-dependently inhibit ovarian cancer cell (A2780 and SKOV3) clone formation, induce cell apoptosis, and reduce cell migration and invasion capabilities. Its IC_{50} values were 2.64 μmol·L^{-1} and 5.23 μmol·L^{-1}, respectively, superior to both the parent compound calycosin and the positive control tamoxifen. Proteomic analysis indicated that its mechanism of action might involve regulation of signaling pathways such as the cell cycle and ferroptosis, downregulating the expression of cyclins CCNB1, CDC20, CDK1, and CCKN1B, and the ferroptosis protein GPX4 (Fig. 5). Furthermore, Fu et al. [23] synthesized nine 7-alkoxy calycosin derivatives *via* substitution reactions between calycosin and bromoalkanes (Fig. 6) and evaluated the inhibitory activity of these derivatives against breast cancer cells (MCF-7), colon cancer cells (SW480), and pancreatic cancer cells (HepG2) using the MTT assay. The results showed that 3'-hydroxy-4'-methoxy-7-cyclopentyloxyisoflavone (B5) exhibited the best antitumor activity, with inhibition rates of 89.20%, 77.33%, and 44.32% against MCF-7, SW480, and HepG2, respectively. Yin et al. [26] used MCF-7 and MDA-MB-231 human breast cancer cell lines as research models and treated them with different concentrations of the calycosin derivative CA028 for 48 h. The results demonstrated that the calycosin derivative inhibited cell clone formation, induced cell apoptosis, upregulated the expression of the pro-apoptotic factor Bax, and downregulated the expression of the anti-apoptotic factor Bcl-2, with the inhibitory effects strengthening as the concentration increased. Further transcriptomic analysis revealed that CA028 exerted anti-breast cancer effects by regulating genes such as AP-1, EGR1, and CBL, as well as signaling pathways related to immune cell function and inflammatory response. Additionally, Wei et al. [27] found, during intervention on ER-positive breast cancer cells MCF-7 and T47D with calycosin derivative A10, that A10 concentration-dependently arrested the cell cycle at the G_2/M phase, inhibited cell migration and invasion, and downregulated the expression of EMT-related proteins N-cadherin, Vimentin, β-catenin, Snail, and MMP9. Its mechanism of action was potentially mediated through regulation of the PI3K/AKT signaling pathway by ER-α36.

Through structural modification, calycosin derivatives show significant improvements over the parent compound in solubility, bioavailability, and pharmacological activity, demonstrating particularly promising application prospects in antitumor therapy. Current research has clarified the anti-breast and anti-ovarian cancer activities of some derivatives and their related mechan-

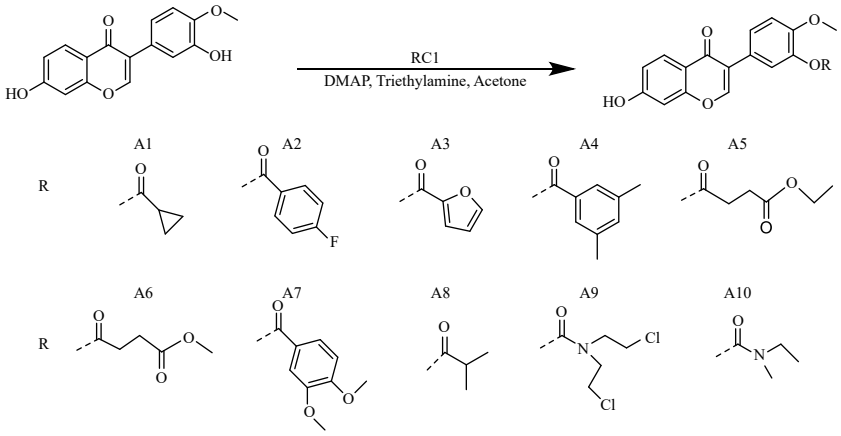

Fig. 5 Synthesis of calycosin derivatives (1)[25].

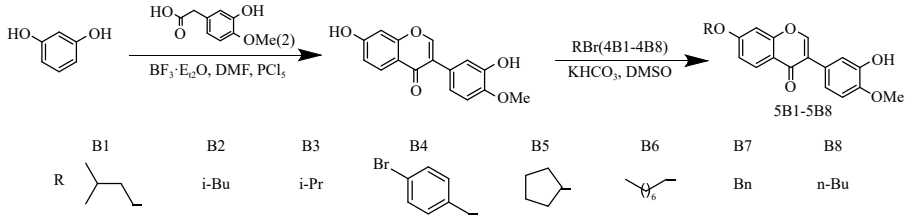

Fig. 6 Synthesis of calycosin derivatives (2)[23].

isms, but some shortcomings remain, such as relatively few in vivo efficacy studies and the need for deeper exploration of the mechanisms of action. Beyond its antitumor applications, the core anti-inflammatory and antioxidant properties of calycosin suggest that its derivatives also hold potential for treating cardiovascular and neurodegenerative diseases. For example, these derivatives may offer therapeutic benefits in pathological processes driven by chronic inflammation and oxidative stress, such as atherosclerosis and Alzheimer's disease. In the future, further *in vivo* experimental research on calycosin derivatives should be conducted to thoroughly investigate their molecular mechanisms of action, providing a more solid theoretical basis for their clinical application. Synthesis methods should also continue to be optimized to develop more efficient and low-toxicity calycosin derivatives, expanding their application in the treatment of other diseases.

5. Biological effects of calycosin *via* multiple pathways

5.1. Biological effects of calycosin via modulation of the NF-κB signaling pathway

Nuclear Factor-kappa B (NF-κB) is a family of transcription factors widely present in eukaryotic cells, primarily consisting of protein complexes formed by homo- or heterodimers of Rel family proteins. Common members include p65 (RelA), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2), with the p65/p50 heterodimer being the predominant active form [28]. Typically, NF-κB is bound to its inhibitory protein, IκB (such as IκBα), and retained in the cytoplasm in an inactive state. When cells are stimulated (e.g., by inflammatory factors, pathogens, or oxidative stress), IκB is phosphorylated and degraded, leading to the release and nuclear translocation of NF-κB. NF-κB then binds to promoter or enhancer regions of target genes to initiate gene transcription [29, 30]. Therefore, it is also an important target for current drug development (e.g., anti-inflammatory and anti-tumor drugs) [31]. Calycosin, a major active component of Astragalus, has been demonstrated in various disease models in recent years to exert broad biological activities by modulating the NF-κB signaling pathway, with its mechanisms of action showing disease specificity and diversity in pathway regulation.

In inflammatory disease models, the inhibitory effect of calycosin on the NF-κB pathway may be its core anti-inflammatory mechanism. In a dextran sulfate sodium (DSS)-induced mouse colitis model, calycosin significantly inhibited the phosphorylation of IκBα and p65, reduced the expression of pro-inflammatory factors such as IL-1β and IL-6 in colon tissue, and ameliorated intestinal mucosal damage and inflammatory cell infiltration, suggesting that it alleviates intestinal inflammation by blocking the NF-κB-mediated inflammatory cascade [32]. Similarly, Dong et al. [33] confirmed in LPS-stimulated RAW 264.7 macrophages that calycosin decreased the transcriptional activity of p65, iNOS, and COX-2, and reduced the secretion of NO, PGE2, and pro-inflammatory cytokines, further validating its inhibitory effect on the NF-κB pathway. In allergic inflammation, calycosin targeted the TLR4-MyD88-TAK1 axis, inhibited NF-κB activation, reduced the release of TSLP and IL-33, and simultaneously upregulated the expression of tight junction proteins occludin and ZO-1, indicating that calycosin has dual roles in maintaining epithelial barrier integrity and exerting anti-inflammatory effects [34]. The pathological core of acute pancreatitis is an excessive inflammatory response triggered by pancreatic acinar cell injury, in which activation of the NF-κB pathway is a key driver of the massive release of pro-inflammatory factors (e.g., TNF-α and IL-6) [35]. Studies showed that calycosin inhibited the phosphorylation of p38 MAPK, blocking its activation of NF-κB, thereby downregulating the expression of pro-inflammatory factors such as IL-1β and IL-6, reducing neutrophil infiltration, and alleviating pancreatic edema and acinar cell necrosis [36]. The onset of gouty arthritis is closely related to the inflammatory cascade induced by urate crystal deposition, and the synergistic effect of AIM2 inflammasome activation and the NF-κB pathway is a key link in amplifying the inflammatory response [37]. Tian et al. [38] found that calycosin upregulated the expression of p62, promoted its binding to Keap1, and thereby relieved Keap1's inhibition of Nrf2. It also competitively inhibited the interaction between the AIM2 inflammasome and NF-κB, reducing Caspase-1-mediated IL-1β release. The central inflammatory response in parasitic meningitis is mainly caused by persistent activation of the NF-κB pathway due to pathogen stimulation, leading to the release of excessive pro-inflammatory factors (e.g., IL-1β and TNF-α) and resulting in meningeal edema and neural damage [39]. Research by Lu et al. [40] confirmed that calycosin induced the expression of heme oxygenase-1 (HO-1), indirectly inhibiting the nuclear translocation of NF-κB p65 and reducing the secretion of pro-inflammatory factors. This mechanism indicates that calycosin achieves dual suppression of central inflammation in parasitic meningitis through the antioxidant–anti-inflammatory cross-regulation of HO-1/NF-κB. The aforementioned studies demonstrate that calycosin exerts therapeutic effects in different inflammatory diseases by directly inhibiting the NF-κB pathway.

In metabolic disease and organ injury models, the regulation of the NF-κB signaling pathway by calycosin demonstrates tissue-protective properties. Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes and a leading cause of end-stage renal disease (ESRD). Its high incidence and poor prognosis pose a major challenge to global public health [41]. Zhang et al. [42], using a high glucose-induced db/db mouse model, found that calycosin significantly inhibited IκBα and NF-κB p65, reduced the expression of TNF-α and IL-1β in the kidneys, and alleviated glomerulosclerosis and renal interstitial fibrosis, suggesting that calycosin improves renal inflammatory injury by inhibiting the NF-κB pathway. In severe acute pancreatitis-associated acute lung injury (SAP-ALI), studies confirmed that calycosin directly bound to HMGB1, blocked its interaction with TLR4, subsequently downregulated NF-κB p65 expression, and reduced neutrophil infiltration and pulmonary inflammatory cytokine levels, thereby mitigating lung injury [43]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key signaling molecule regulating intracellular antioxidant stress. It can alleviate excessive inflammation by inhibiting the nuclear translocation of NF-κB or reducing the expression of its downstream pro-inflammatory factors [44]. In an intracerebral hemorrhage model, it was found

that calycosin could synergistically activate Nrf2 to inhibit NF-κB, thereby reducing cerebral oxidative stress and inflammatory response, reflecting the characteristic of multi-pathway synergistic regulation [45]. These studies suggest that calycosin's regulation of the NF-κB pathway is not limited to direct inhibition but can also achieve tissue protection by targeting upstream molecules (e.g., HMGB1 and TLR4) or synergizing with other pathways (e.g., Nrf2).

Furthermore, in cancer research, calycosin exhibits bidirectional regulation of the NF-κB pathway. In HepG2 liver cancer cells, Liu et al. [46] found that calycosin induced ROS generation to activate the NF-κB pathway, upregulated the expression of Bax and caspase-3, and induced mitochondria-dependent apoptosis. In osteosarcoma studies, Qiu et al. [47] observed that calycosin downregulated miR-223, inhibited the expression of NF-κB p65, decreased Bcl-2, and increased caspase-3 activation, thereby inhibiting cell proliferation. Subsequent research further confirmed that calycosin inhibited the NF-κB-mediated IκBα/ECT2 pathway, reduced MMP2 expression and cell migration ability, and exerted anti-metastatic effects [48]. This bidirectional regulation may be related to tumor type, cellular microenvironment, and drug concentration, indicating the need for precise dose control in potential tumor therapy.

In summary, calycosin regulates the NF-κB signaling pathway in a disease-specific manner, with three core regulatory patterns. In inflammatory diseases, it primarily directly inhibits NF-κB core molecules (IκBα and p65) or upstream activation axes (TLR4-MyD88 and p38 MAPK), supplemented by crosstalk with pathways such as Nrf2 and HO-1, to block NF-κB-mediated pro-inflammatory responses. In metabolic diseases and organ injury, it focuses on tissue protection, either by directly inhibiting NF-κB activation in target organs or by targeting upstream molecules such as HMGB1 and synergizing with the Nrf2 pathway to alleviate tissue damage caused by inflammation and oxidative stress. In cancer, it exhibits context-dependent bidirectional regulation: activating NF-κB to induce tumor cell apoptosis in liver cancer (HepG2 cells), and inhibiting NF-κB to block cell proliferation and metastasis in osteosarcoma—both serving antitumor effects.

However, its tissue specificity, dose dependency, and clinical translation potential still require in-depth exploration. On the one hand, it is necessary to identify the key molecular differences in NF-κB pathway regulation across different diseases to provide a basis for precise targeting. On the other hand, the connection between preclinical research and clinical trials should be strengthened to verify its efficacy and safety in humans. Future research could focus on the detailed interactions between calycosin and key proteins of the NF-κB pathway, as well as synergistic regulation strategies combined with other drugs, to lay the foundation for clinical application (Fig. 7).

5.2. Biological effects of calycosin via modulation of the PI3K/AKT signaling pathway

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway is a crucial intracellular pathway regulating survival and metabolism. Its aberrant activation is closely associated with the pathological processes of various diseases, including tumorigenesis, fibrosis, inflammation, and neural damage [49]. Phosphatidylinositol 3-kinase (PI3K), a key regulator of this pathway and numerous cellular processes, is a heterodimeric lipid kinase composed of a p85 regulatory subunit and a p110 catalytic subunit, possessing dual activities of phosphatidylinositol kinase and serine/threonine protein kinase [50,51]. Protein kinase B (Akt, also known as PKB) is an evolutionarily highly conserved serine/threonine kinase. As a downstream effector of PI3K, it is primarily involved in cell metabolism, growth, survival, and proliferation [52]. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 acts as a second messenger by binding to the PH domain of AKT, recruiting it to the plasma membrane. Upon recruitment, AKT is phosphorylated and activated by phosphoinositide-dependent kinase 1 (PDK1) and the mammalian target of rapamycin complex 2 (mTORC2) (key phosphorylation sites are Thr308 and Ser473) [53]. Activated AKT exerts its biological functions by phosphorylating downstream target molecules.

Calycosin can exert therapeutic effects in various disease models, such as fibrosis and neural damage, by modulating the PI3K/AKT signaling pathway. In oncology, the inhibitory effect of calycosin on the PI3K/AKT pathway is a core mechanism of its antitumor activity. In colorectal cancer research, calycosin inhibited PI3K/AKT pathway activation and reduced the expression of the anti-apoptotic protein Bcl-2. This ultimately inhibited the proliferation and induced apoptosis of HCT116 and SW480 cells, and significantly suppressed tumor growth in a nude mouse xenograft model [54]. Similarly, in ER-positive MG-63 osteosarcoma cells, Sun et al. [55] found that calycosin inhibited the PI3K/AKT/

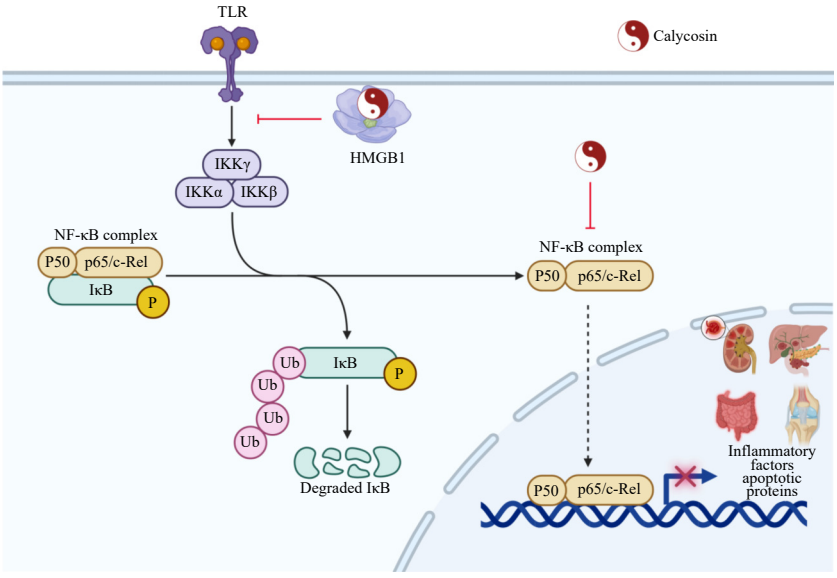

Fig. 7 Calycosin regulates NF-κB signaling pathway. NF-κB: Nuclear Factor Kappa-B; IκB: Inhibitor of NF-κB; TLR: Toll Like Receptors; HMGB1: High mobility group box-1 protein.

mTOR pathway and induced cell apoptosis. In glioblastoma, calycosin indirectly inhibited the PI3K/AKT pathway and downstream MMP9 expression by downregulating the c-Met receptor, thereby inhibiting the invasion and proliferation of U251 and U87 cells [56, 57]. These studies indicate that calycosin's inhibition of the PI3K/AKT pathway is tumor-type specific and often associated with targeting upstream molecules (e.g., c-Met) or downregulating downstream effectors.

In fibrotic diseases, calycosin alleviates tissue fibrosis by inhibiting the PI3K/AKT pathway. In a bleomycin-induced pulmonary fibrosis model, calycosin significantly reduced the phosphorylation levels of AKT and GSK3β, thereby blocking the epithelial-mesenchymal transition (EMT) process and reducing the expression of mesenchymal markers such as α-SMA and vimentin. This effect could be reversed by the AKT activator SC79 [58]. This suggests that calycosin inhibits fibrosis via the PI3K/AKT/GSK3β/β-catenin axis.

Furthermore, in neuroprotection and inflammation-related diseases, the regulation of the PI3K/AKT pathway by calycosin exhibits "bidirectional" or "synergistic" characteristics. In an H_2O_2-induced astrocyte injury model, calycosin was found to activate the PI3K/AKT pathway, promote the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), and upregulate the expression of heme oxygenase-1 (HO-1) and superoxide dismutase (SOD), thereby mitigating oxidative stress damage. This protective effect could be reversed by the PI3K inhibitor LY294002 [59]. In spinal cord injury research, Li et al. [60] found that rehabilitation training enhanced the activating effect of calycosin on the PI3K/AKT pathway, further inhibiting ASK1/p38-mediated oxidative stress and apoptosis, and promoting neurological functional recovery. Additionally, in an osteoarthritis model, calycosin inhibited the PI3K/AKT/FoxO1 pathway, reducing IL-1β-induced chondrocyte apoptosis, the release of inflammatory factors (TNF-α and IL-6), and extracellular matrix degradation. The PI3K activator 740Y-P attenuated this protective effect [61].

In summary, the regulation of the PI3K/AKT signaling pathway by calycosin is disease-dependent: in cancer, it primarily inhibits pathway activity, exerting antitumor effects by downregulating molecules related to proliferation and invasion; in fibrotic and inflammatory diseases, it either inhibits excessive pathway activation to alleviate tissue damage or activates the pathway to enhance antioxidant and repair capacities. Its core mechanisms involve directly regulating PI3K/AKT phosphorylation, targeting upstream receptors (e.g., ERβ and c-Met), and synergizing with downstream transcription factors (e.g., Nrf2 and FoxO1). Future research should further clarify its dose-effect relationship and tissue-specific molecular targets in different diseases to provide an experimental basis for clinical translation (Fig. 8).

5.3. Biological effects of calycosin via modulation of the MAPK signaling pathway

The mitogen-activated protein kinase (MAPK) signaling pathway belongs to the serine/threonine protein kinase family and is a core transduction network through which cells respond to external stimuli. When the cell membrane is stimulated, the upstream MAPK kinase kinase (MAPKKK) and MAPK kinase (MAPKK) undergo sequential phosphorylation [62, 63]. MAPKK performs dual phosphorylation on the C-terminal TXY motif, thereby activating downstream MAPK, which subsequently participates in regulating numerous physiological processes such as cell growth, apoptosis, proliferation, and inflammatory responses. To date, 12 MAPKs have been identified in mammals, primarily including subtypes such as extracellular signal-regulated kinase (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) [64].

p38 MAPK, a core target in signal transduction research, plays an indispensable pivotal role in mediating cellular events such as inflammatory and stress responses. Its regulatory mechanism exhibits dual dimensions: on one hand, p38 MAPK activates transcription factors such as ATF-2 and ELK1 through phosphorylation, driving the transcription of downstream target genes (e.g., pro-inflammatory factors and apoptosis-related genes) to regulate cell function [65]; on the other hand, p38 MAPK can directly activate kinases such as p38-regulated protein kinase (PRAK), which, *via* a phosphorylation cascade, activates the low-molecular-weight heat shock protein HSP27, inducing actin filament remodeling and mediating adaptive changes in cell morphology and function under stress conditions [66].

ERK1/2 regulates cell proliferation by phosphorylating transcription factors such as cellular oncogene fos (c-Fos) and cellu-

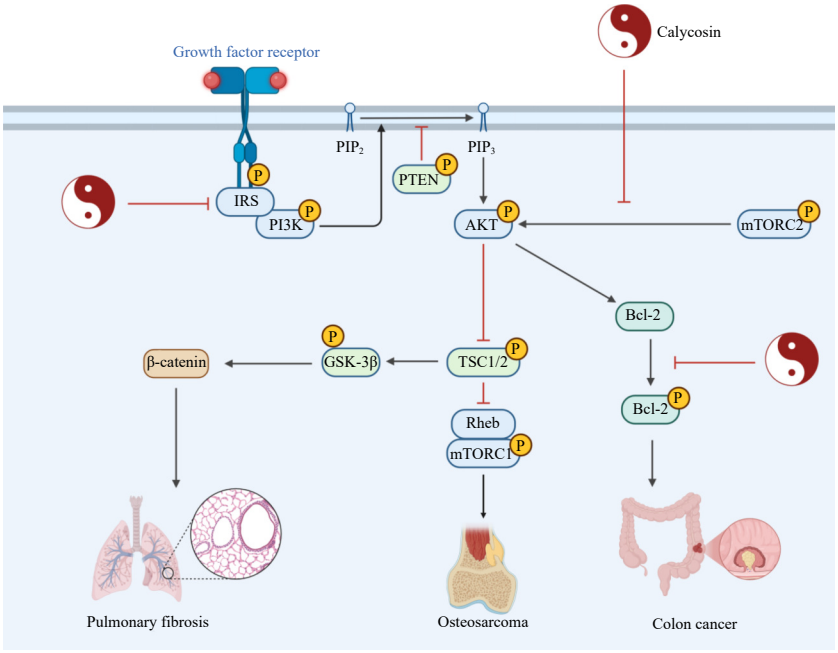

Fig. 8 Calycosin regulates PI3K/AKT signaling pathway. PTEN: phosphatase and tensin homolog deleted on chromosome ten; PI3K: Phosphoinositide 3-Kinase; IRS: insulin receptor substrate; AKT: Protein kinase B; Bcl-2: B-cell lymphoma-2.

lar oncogene Jun (c-Jun), promoting the formation of the activator protein-1 (AP-1) complex and facilitating the transition from the G_1 to S phase of the cell cycle. In addition, ERK1/2 is involved in cellular stress responses and metabolic regulation, indirectly affecting cell survival by acting on oxidative metabolism and energy balance [67, 68].

The JNK-MAPK pathway can be activated by inflammatory factors or growth factors. Upon receptor-ligand binding, intracellular mitogen-activated protein kinase kinase kinase 1 (MEKK1), mitogen-activated protein kinase kinase 3 (MKK3/MAP2K3), and apoptosis signal-regulating kinase 1 (ASK1) are activated. These in turn activate downstream MAPKKK4/7, leading to the activation of JNK1/2 [69]. Ultimately, activated JNK1/2 translocates into the nucleus and binds to target genes such as c-Jun, activating transcription factor-2 (ATF-2), ELK1, and p53, thereby regulating transcription. Furthermore, ASK1 can directly promote apoptosis [70].

Recent studies have found that calycosin exerts significant protective effects against various diseases, including neural injury, cancer, and fibrosis, by modulating the MAPK signaling pathway. You et al. [71] discovered in a cerebral ischemia-reperfusion model that calycosin significantly increased the pERK/ERK ratio and reduced SH-SY5Y cell apoptosis and rat cerebral infarction volume. However, its neuroprotective effects were reversed upon application of the ERK inhibitor (U0126 or pERK-siRNA), indicating that the phosphorylation and activation of ERK1/2 is the core mechanism by which calycosin protects against ischemic brain injury. This finding is consistent with the classical role of ERK1/2 in pro-survival signaling, suggesting that calycosin may maintain neuronal survival by enhancing ERK1/2 activity.

In a colon cancer model, Hu et al. [72] found that calycosin dose-dependently downregulated p-ERK1/2 expression and inhibited the proliferation and migration of Caco2 and HT-29 cells, accompanied by upregulation of the epithelial-mesenchymal transition (EMT) marker E-cadherin and downregulation of N-cadherin and vimentin. Its inhibitory effect was significantly correlated with inactivation of the ERK1/2 pathway. Conversely, in an osteosarcoma 143B cell model, calycosin activated the p38 MAPK pathway, upregulated pro-apoptotic proteins Bax, cleaved caspase-3/9, and PARP, decreased the mitochondrial membrane potential, and induced cell apoptosis [73]. Furthermore, Liu et al., through network pharmacology analysis, identified MAPK3 (ERK1) as one of the core targets of calycosin against nasopharyngeal carcinoma, involved in regulating cell apoptosis and proliferation signals, further supporting the broad-spectrum role of the MAPK pathway in its antitumor effects [74].

The regulation of the MAPK pathway by calycosin may exhibit bidirectionality depending on the tumor microenvironment. Zhang et al. [75] stablished an orthotopic pancreatic cancer xenograft mouse model and found that calycosin, on one hand, promoted EMT and migration of MIA PaCa-2 cells by activating the Raf/MEK/ERK pathway; on the other hand, it induced cell cycle arrest and apoptosis. This paradox was associated with TGF-β1 upregulation. TGF-β1 inhibits tumor growth via the ERK pathway in early stages but promotes metastasis through the same pathway in later stages. This bidirectionality suggests that calycosin's regulation of the MAPK pathway may depend on the stage of tumor development. In a gastric cancer model, calycosin induced ROS generation, activated the p38 and JNK pathways while inhibiting ERK phosphorylation, synergistically regulated the MAPK/STAT3/NF-κB axis, and induced apoptosis and G_0/G_1 phase arrest in AGS cells, demonstrating its coordinated regulatory effect on different MAPK subtypes.

In fibrotic diseases, calycosin primarily alleviates the pathological process by inhibiting the MAPK pathway. Ding et al. [76] found in an Ang II-induced glomerular mesangial cell model that calycosin downregulated p-ERK1/2 expression and reduced cell proliferation and the expression of autophagy markers LC3 and Beclin-1, thereby mitigating renal interstitial fibrosis.

In summary, calycosin exerts effects in neuroprotection, tumor suppression, and inflammation reduction by selectively regulating subtypes such as ERK1/2, p38 MAPK, and JNK. Its regulatory mode exhibits disease type and pathway subtype specificity. Future research should further clarify the details of its interaction with key molecules of the MAPK pathway and its dose-effect relationship to provide a precise basis for clinical application (Fig. 9).

5.4. Biological effects of calycosin via modulation of the STAT3 signaling pathway

The signal transducer and activator of transcription (STAT) family plays a key role in cell proliferation, apoptosis, and inflammatory responses. Its aberrant activation is closely associated with various diseases, including neural injury, myocardial ischemia, fibrosis, and cancer [77]. STAT3 is a member of the STAT family, primarily composed of an N-terminal domain (NTD), a coiled-coil domain (CCD), a DNA-binding domain (DBD), a linker domain (LD), a Src homology 2 (SH2) domain, and a trans-activation domain (TAD) [78, 79]. Under pathological conditions, cytokines such as IL-6, interferon-alpha (IFN-α), epidermal growth factor (EGF), and fibroblast growth factor bind to cell surface receptors, causing dimerization of the receptor extracellular domains, which in turn activates the Janus tyrosine kinases (JAKs) associated with the receptor intracellular regions [80]. Activated JAKs phosphorylate specific tyrosine residues on the receptor cytoplasmic tails and recruit STAT3 to the receptor complex via interaction with its SH2 domain. Subsequently, JAKs phosphorylate the key tyrosine residue at Tyr705 of STAT3. The phosphorylated STAT3 (p-STAT3) forms homo- or heterodimers through SH2 domain interactions. After dissociation, the p-STAT3 monomer binds to importin β1 (IMPB1) and is transported into the nucleus, where it regulates the transcription of downstream target genes [81].

Ischemic stroke (IS), a common cerebrovascular disease in the elderly, is characterized by high disability and mortality rates. Excessive autophagy triggered by IS is closely related to neuronal death, making the inhibition of excessive autophagy a potential new direction for IS treatment. Xu et al. [82] established a rat middle cerebral artery occlusion/reperfusion (MCAO/R) model using the suture method and simulated cerebral ischemic injury *in vitro* using oxygen-glucose deprivation/reoxygenation (OGD/R). They found that calycosin significantly downregulated the phosphorylation levels of STAT3 and FOXO3a, decreased the LC3II/LC3I ratio, and upregulated the expression of the autophagy substrate SQSTM1. By inhibiting excessive autophagy, calycosin reduced cerebral infarction volume and neurological deficits in rats. The STAT3 inhibitor Stattic reversed this protective effect, indicating that calycosin inhibits excessive autophagy by downregulating the STAT3/FOXO3a pathway. This discovery reveals a novel mechanism by which calycosin exerts neuroprotective effects in ischemic brain injury through negative regulation of the STAT3 pathway. Furthermore, Liu et al. [83] established a myocardial ischemia-reperfusion injury mouse model by ligating the left anterior descending (LAD) coronary artery. They found that calycosin upregulated the expression of IL-10, p-JAK2, and p-STAT3, and this effect could be blocked by an IL-10 receptor antagonist, suggesting that the IL-10/JAK2/STAT3 axis is a key pathway for the cardioprotective effect of calycosin. Thus, calycosin demonstrates clear protective effects in both cerebral and myocardial ischemic injury. Although both mechanisms involve the STAT3 pathway, they are achieved through distinct routes: downregulating STAT3/FOXO3a to inhibit autophagy (cerebral ischemia) and upregulating IL-10/JAK2/STAT3 to exert anti-in-

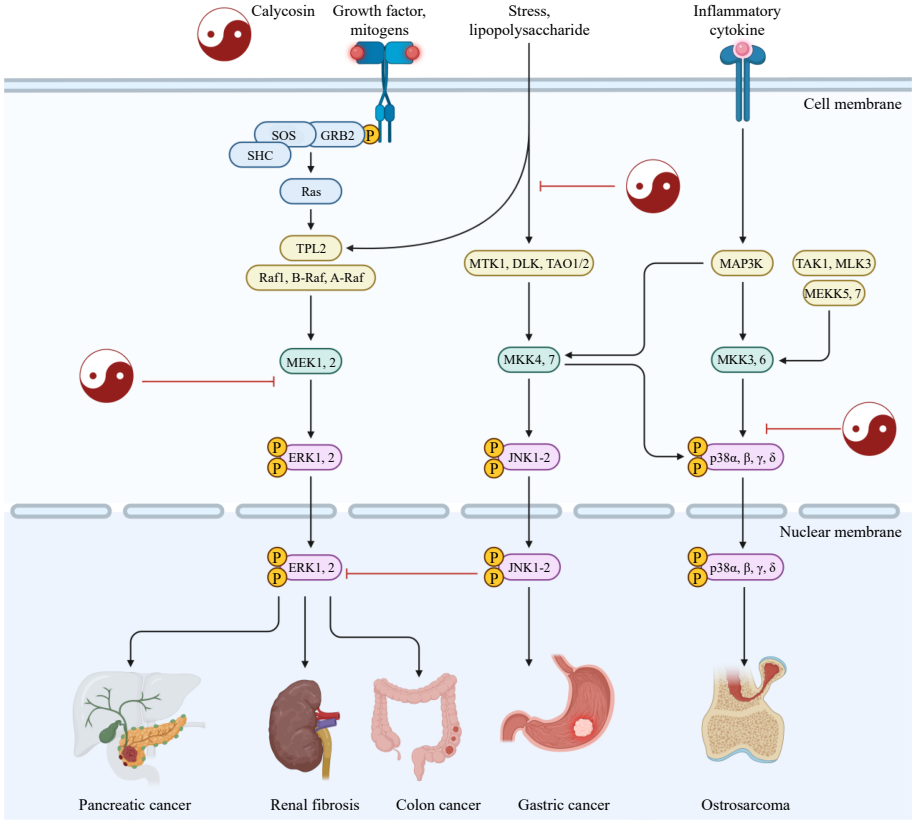

Fig. 9 Calycosin regulates MAPK signaling pathway. GRB2: growth factor receptor-bound protein 2; TPL2: Tumor progression locus 2; MEK1,2: Mitogen-activated protein 1,2ERK1,2: Extracellular regulated protein kinases 1,2; JNK1-3: c-Jun N-terminal kinase 1-3.

flammatory effects (myocardial ischemia). This exemplifies the multi-pathway regulation and tissue adaptability of natural products. This finding not only provides a new candidate drug for the treatment of ischemic diseases but also offers an experimental paradigm for research on precise regulation based on tissue specificity.

In a study on oxidative damage in spinal cord astrocytes, it was found that calycosin could alleviate H_2O_2-induced cell apoptosis by inhibiting the JAK/STAT3 signaling pathway, simultaneously downregulating the level of the pro-inflammatory cytokine IL-6. The STAT3 inhibitor Stattic could reverse its promotive effect on cell proliferation [84]. These findings suggest that calycosin mitigates oxidative stress-mediated astrocyte damage through negative regulation of the JAK2/STAT3 pathway. In a liver fibrosis model, Wang et al. [85] found that that overexpression of estrogen receptor beta (ERβ) enhanced the inhibitory effect of calycosin on STAT3 phosphorylation, synergistically reducing the expression of fibrosis markers such as α-SMA and Col-I, while upregulating MMP-1 to promote collagen degradation. Inhibition of the JAK2/STAT3 signaling pathway may be the primary mechanism by which calycosin exerts its anti-fibrotic effects. Furthermore, in gastric cancer precancerous lesions, calycosin significantly downregulated STAT3 protein expression, inhibited the integrin β1/NF-κB pathway, reduced intestinal metaplasia and dysplasia, and improved gastric mucosal ultrastructural damage [86]. Its mechanism of action likely involves inhibiting STAT3-mediated angiogenesis and inflammatory responses, thereby blocking the progression of precancerous lesions.

In summary, the regulation of the STAT3 signaling pathway by calycosin exhibits significant disease specificity: it primarily negatively regulates STAT3 to inhibit autophagy and oxidative stress in neural and spinal cord injury; it exerts protective effects by activating JAK2/STAT3 in myocardial injury; and it alleviates pathological processes by inhibiting STAT3 phosphorylation in liver fibrosis and gastric precancerous lesions. This diversity is closely related to its selective regulation of different STAT subtypes and its crosstalk with signals such as ERβ and cytokines (Fig.10).

5.5. Biological effects of calycosin via modulation of the AMPK signaling pathway

Adenosine monophosphate-activated protein kinase (AMPK), a key regulatory element in maintaining cellular material and energy metabolic balance, is widely involved in fundamental physiological processes such as cell growth, development, and programmed cell death [87]. It is primarily composed of an α catalytic subunit and β, γ regulatory subunits. The α subunit has two isoforms: α1 (PRKAA1) and α2 (PRKAA2); the β subunit includes β1 (PRKAB1) and β2 (PRKAB2); and the γ subunit includes γ1 (PRKAG1), γ2 (PRKAG2), and γ3 (PRKAG3) [88]. When intracellular energy levels are low, ATP bound to the regulatory γ subunit of AMPK is replaced by AMP, promoting the phosphorylation of the AMPK α subunit at threonine-172, thereby activating AMPK. Subsequently, AMPK blocks anabolic pathways of ATP synthesis and initiates catabolic pathways of ATP breakdown, inhibiting energy consumption and maintaining energy homeostasis [89,90]. This function in maintaining energy dynamics allows AMPK to play a significant role in various pathological processes such as vascular calcification, airway injury, abnormal osteogenesis, skeletal muscle atrophy, and adipose tissue dysfunction. Recent studies have found that calycosin exerts unique therapeutic effects in different disease models by targeting AMPK and its downstream molecular network.

Vascular calcification (VC) is a common pathological phenomenon in patients with chronic diseases such as atherosclerosis, chronic kidney disease, and diabetes. In a calcifying environment, vascular smooth muscle cells (SMCs) transform into osteoblast-like cells, leading to loss of smooth muscle contraction markers such as smooth muscle α-actin and smooth muscle 22α

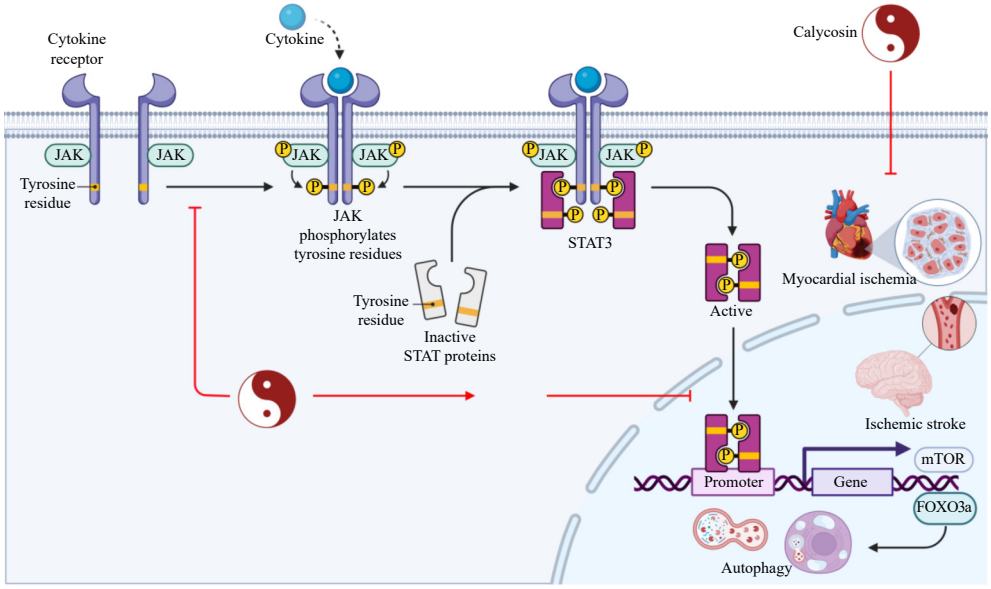

Fig. 10 Calycosin regulates JAK2/STAT3 signaling pathway. JAK: Janus Kinase; STAT3: signal transducer and activator of transcription 3; FOXO3a: Forkhead Box O3A; mTOR: mammalian target of rapamycin.

protein (SM22α), and an increase in the expression levels of osteogenic markers such as osteopontin (OPN), RUNX2 transcription factor, and bone morphogenetic protein 2 (BMP2) [91]. Zhou et al. [92] established a vascular calcification model using rat thoracic aortic smooth muscle cells (A7r5) in vitro and found that calycosin activated the AMPK/mTOR signaling pathway. On one hand, it initiated autophagy onset by phosphorylating ULK1 (Unc-51 like autophagy activating kinase 1); on the other hand, it promoted the formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (STX17-SNAP29-VAMP8), restoring autophagosome-lysosome fusion. This significantly reduced the expression of RUNX2, BMP2, and OPN, as well as calcium deposition, ultimately inhibiting the vascular calcification process. In a human bronchial epithelial cell injury model constructed by PM2.5 exposure, calycosin dose-dependently activated the AMPK signaling pathway and inhibited the nuclear translocation of NF-κB p65, thereby reducing inflammatory cell infiltration and inhibiting the release of pro-inflammatory factors such as IL-6 and IL-8. Further studies showed that the AMPK inhibitor Compound C reversed the protective effect of calycosin, suggesting that AMPK is a key target for its inhibition of airway inflammation [93]. In osteoblast differentiation, Gong et al. [94] sed omics and a chemical knockout method (constructing Danggui Buxue Tang extract DBT without calycosin) and found that calycosin, as a key active component of Danggui Buxue Tang, promoted osteoblast differentiation by regulating the AMPK-related pathway (synergizing with MAPK/ERK and Wnt/β-catenin pathways), upregulating the expression of osteogenic markers such as ALP, Runx2, and secreted protein acidic and cysteine rich (Sparc, osteonectin). Its effect was significantly weakened in DBT, indicating that calycosin coordinates the osteogenic effect of the full prescription through AMPK signaling and is the core component maintaining osteogenic activity. Skeletal muscle atrophy is a common and serious complication of chronic kidney disease (CKD). Oxidative stress and autophagy are the main molecular mechanisms leading to muscle atrophy. Hu et al. [95] used a 5/6 nephrectomy (5/6 Nx) CKD rat model and a TNF-α-induced C2C12 myotube model and found that calycosin downregulated AMPK expression, inhibited the nuclear translocation of FOXO3a, thereby reducing the expression of autophagy markers (LC3A/B, ATG7); simultaneously, it upregulated SKP2 to decrease CARM1 and H3R17me2a levels, thus inhibiting excessive autophagy and oxidative stress and alleviating muscle fiber atrophy. In an obese mouse model of perivascular adipose tissue (PVAT) dysfunction, calycosin upregulated adiponectin secretion, activated the AMPK/eNOS signaling pathway, promoted eNOS phosphorylation and NO generation, and restored the anti-contractile activity of PVAT. Concurrently, it reduced superoxide generation and TNF-α levels in PVAT, improving vascular endothelial function. These effects were reversed by an AMPK inhibitor or an adiponectin receptor blocking peptide [96].

In summary, through precise modulation of the AMPK signaling pathway, calycosin exerts diverse protective effects in vascular calcification, airway injury, osteogenic differentiation, skeletal muscle atrophy, and PVAT dysfunction: it primarily activates AMPK in vascular calcification, airway injury, osteogenic differentiation, and PVAT dysfunction, whereas it mainly inhibits AMPK activity in skeletal muscle atrophy. This difference is closely related to its selective regulation of downstream AMPK targets (such as mTOR, NF-κB, SKP2, and eNOS). These findings provide an important experimental basis for understanding the pharmacological effects of calycosin and expanding its clinical applications (Fig. 11).

5.6. Biological effects of calycosin via modulation of the TGF-β1/Smad signaling pathway

Members of the transforming growth factor-beta (TGF-β) family include TGF-β subtypes (TGF-β1, TGF-β2, and TGF-β3), growth differentiation factors (GDFs), glial-derived neurotrophic factors (GDNFs), and 33 other homodimeric superfamily ligands [97]. Among them, TGF-β1 is the most abundant subtype, primarily expressed in smooth muscle, fibroblasts, and epithelial cells, and is considered a key mediator in the progression of diseases such as cancer and fibrosis [98]. Smad family proteins are the core transcription factors of the TGF-β pathway, capable of transducing the signal from ligand-receptor interaction from the cytoplasm into the nucleus. Based on their function, they can be divided into three categories: receptor-activated Smads (R-Smads), including Smad1, Smad2, Smad3, Smad5, and Smad8; the common-mediator Smad4 (Co-Smad); and inhibitory Smads (I-Smads), including Smad6 and Smad7 [99]. TGF-β1 typically exists in an inactive form on the cell surface and within the extracellular matrix (ECM). It is activated by inflammation, oxidative stress, or the action of enzymes such as plasmin and cathepsins. Activated TGF-β1 binds to cell surface TβR-II, which then recruits and phosphorylates TβR-I. The activated TβR-I phosphorylates

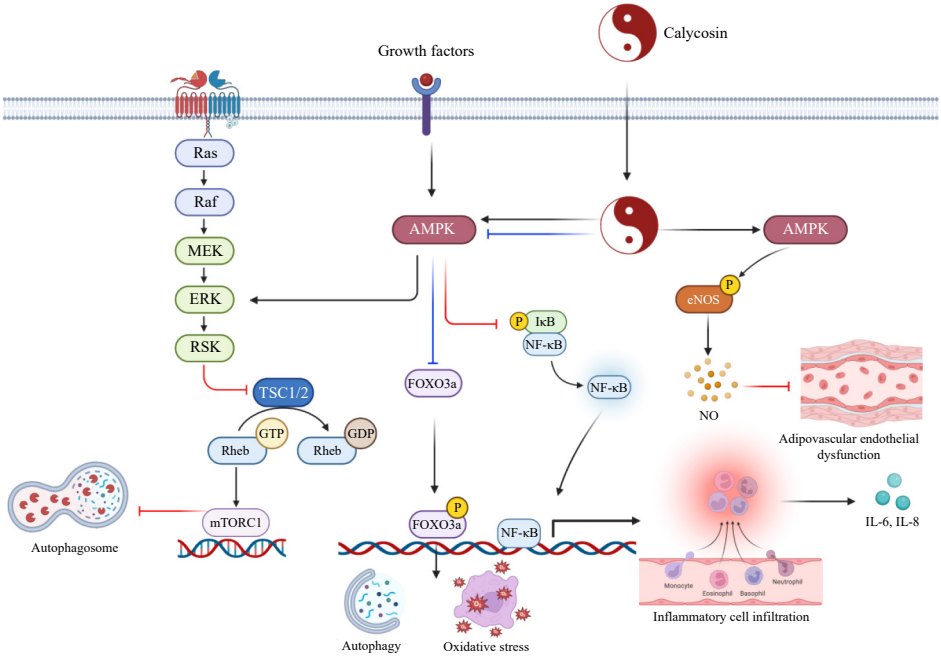

Fig. 11 Calycosin regulates the AMPK signaling pathway. AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase; Rheb: Ras homolog, mTORC1 binding; NF-κB: Nuclear Factor Kappa-B; IκB: Inhibitor of NF-κB; FOXO3a: Forkhead Box O3A

Smad2 and Smad3 at C-terminal serine residues. Phosphorylated Smad2/3 then forms a complex with Smad4, which translocates into the nucleus to mediate the biological effects of the TGF-β1/Smad signaling pathway [100].

Multiple studies have found that calycosin can exert interventional effects in diseases such as breast cancer, intestinal fibrosis, myocardial fibrosis, and renal fibrosis by modulating the TGF-β1/Smad signaling pathway. The basic leucine zipper ATF-like transcription factor (BATF) can directly bind to the P1 region of the TGF-β1 promoter to promote its expression. Zhang et al. [101], using T47D and MCF-7 breast cancer cells as research models, found that calycosin dose-dependently downregulated BATF expression and inhibited the TGF-β1/Smad signaling pathway-mediated epithelial-mesenchymal transition (EMT) process (upregulating the epithelial marker E-cadherin and downregulating the mesenchymal markers N-cadherin, vimentin, MMP-2, and MMP-9), thereby inhibiting the migration and invasion of breast cancer cells. In a TGF-β1-induced human intestinal fibroblast (CCD-18Co) model, calycosin dose-dependently reduced TGF-β1 levels and downregulated the expression of phosphorylated Smad2, Smad3, and Smad4, while upregulating Smad7 (an endogenous inhibitor of the TGF-β1 pathway). This consequently reduced the synthesis and deposition of the fibrosis markers α-smooth muscle actin (α-SMA) and collagen I, alleviating pathological intestinal fibrosis [102]. Chen et al. [103], using a left anterior descending coronary artery ligation-induced myocardial infarction (MI) mouse model and primary cardiac fibroblast (CF) experiments, revealed that calycosin directly targets and binds to TGF-β receptor 1 (TGFBR1), inhibits TGF-β1 activation, and subsequently blocks the phosphorylation and nuclear translocation of downstream Smad2/3. This reduced the expression of collagen I and III in myocardial tissue, decreased collagen deposition in the infarct area, and improved left ventricular ejection fraction (EF) and fractional shortening (FS), ultimately alleviating myocardial fibrosis and improving cardiac function. Obstructive nephropathy, often caused by urinary tract obstruction, leads to renal dysfunction and renal parenchymal destruction. Hu et al. [104], using a unilateral ureteral obstruction (UUO)-induced mouse renal fibrosis model, found that calycosin downregulated the expression of TGF-β1 and Smad2/Smad3, while simultaneously reducing the levels of TNF-α and its receptor 1 (TNFR1), thereby synergistically inhibiting renal tubular epithelial cell injury and renal interstitial collagen deposition. This effect might be related to the enhanced dual inhibition of the TGF-β1/Smad2/Smad3 and TNF-α pathways by calycosin-primed MSCs.

In summary, calycosin modulates the TGF-β1 signaling pathway through network pharmacology effects: in cancer, it targets upstream transcription factors (e.g., BATF) to inhibit TGF-β1 expression; in fibrotic diseases, it acts directly on the receptor (TGFBR1) or downstream Smad molecules, or synergizes with other pathways (e.g., TNF-α/TNFR1). This specific regulatory pattern provides an experimental basis for its clinical application in diseases related to tumor metastasis and tissue fibrosis (Fig. 12).

5.7. Biological effects of calycosin via modulation of the NLRP3 signaling pathway

The NOD-like receptor protein 3 (NLRP3) inflammasome, a core molecule in innate immune responses, is a multiprotein complex composed of NLRP3 protein, apoptosis-associated speck-like protein containing a CARD (ASC), and pro-cysteine aspartase-1 (pro-caspase-1) [105]. The activation of the NLRP3 inflammasome primarily involves a two-signal cascade. The first signal is the "priming signal", whose core biological function is to upregulate the expression levels of inflammation-related factors. Toll-like receptors (TLRs) recognize their corresponding ligands, or cytokines like TNF-α mediate signal transduction, thereby triggering the activation of the NF-κB signaling pathway, which ultimately promotes the expression of NLRP3 and the inflammasome-dependent cytokine pro-IL-1β [106, 107]. The second signal is the "activation signal", whose core role is to trigger the assembly process of the NLRP3 inflammasome. Exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) can be specifically recognized by pattern recognition receptors (PRRs) on the surface of immune cells, subsequently inducing downstream endogenous signals (such as ion transmembrane flux and changes in organelle structure and function), and ultimately initiating the assembly and activation of the NLRP3 inflammasome complex [108, 109].

Yosri et al. [110], in a streptozotocin (STZ)-induced diabetic rat model *via* intraperitoneal injection, found that calycosin dose-de-

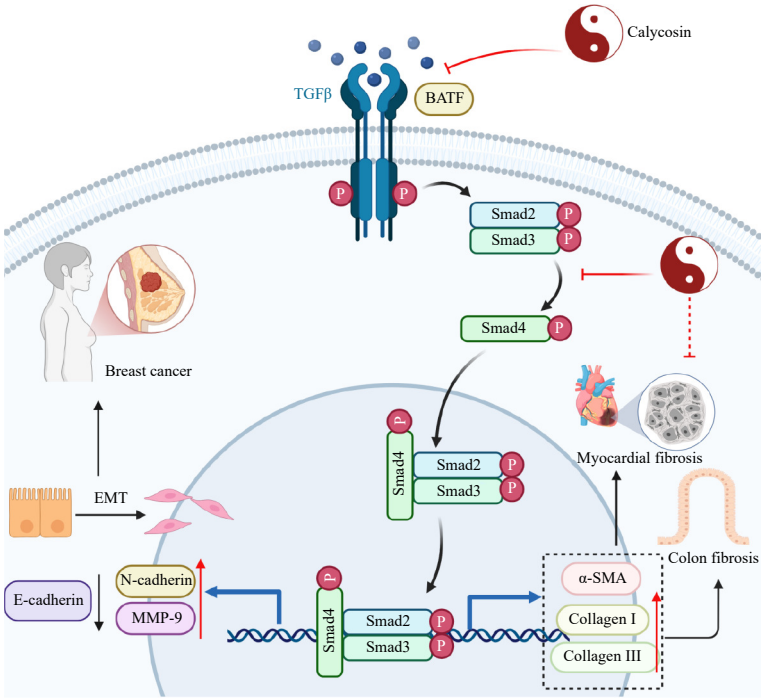

Fig. 12 Calycosin regulates the TGF-β/Smad signaling pathway. TGFβ: transforming growth factor-β; BATF: Basic leucine zipper ATF-like transcription factor; EMT: Epithelial-Mesenchymal Transition; MMP-9: Matrix metallopeptidase 9

pendently downregulated the expression of NLRP3 in kidney tissue, reduced IL-1β release, decreased the activation level of Caspase-1, and thereby improved renal function and pathological kidney damage. Its mechanism of action might be related to inhibition of the binding of thioredoxin-interacting protein (TXNIP) to NLRP3, thereby blocking inflammasome assembly. In a doxorubicin (DOX)-induced cardiotoxicity mouse model, calycosin alleviated myocardial injury by inhibiting NLRP3 inflammasome activation. Further *in vitro* studies showed that calycosin reduced DOX-induced mitochondrial reactive oxygen species (ROS) generation and decreased the interaction between NLRP3 and ASC or pro-caspase-1, thus inhibiting the cleavage and activation of Caspase-1 and the release of IL-1β [111]. This research reveals the molecular basis for calycosin's mitigation of chemotherapy drug cardiotoxicity through a dual mechanism of "antioxidant-inhibition of NLRP3". Xia et al. [112], using lipopolysaccharide (LPS) combined with cecal ligation and puncture (CLP) to construct a sepsis-induced acute lung injury (ALI) mouse model, found that calycosin reduced the expression of NLRP3 and ASC, the activity of Caspase-1, and the release of IL-1β and IL-18 in lung tissue. It also decreased the lung wet/dry weight ratio and myeloperoxidase (MPO) activity, improving lung histopathology. Further in vitro experiments confirmed that its inhibitory effect on NLRP3 depended on the scavenging of mitochondrial ROS, and the mitochondrial-targeted antioxidant MitoTEMPO synergistically enhanced this effect, indicating that mitochondrial ROS is a key upstream signal for calycosin's regulation of NLRP3 [113]. Additionally, Yuan et al. [114], through network pharmacology and animal experiments, found that calycosin, as a core component of the Astragalus-Safflower herb pair, could inhibit the assembly of the NLRP3 inflammasome by binding to the active sites of NLRP3 and caspase-1. Further studies in an ApoE$^{-/-}$ mouse model revealed that calycosin reduced the expression of NLRP3 in aortic plaques, decreased IL-1β release, and simultaneously inhibited the pyroptosis of vascular smooth muscle cells, suggesting its anti-inflammatory and plaque-stabilizing effects in atherosclerosis.

In summary, the regulation of the NLRP3 signaling pathway by calycosin exhibits "multi-step" characteristics. In metabolic diseases (e.g., diabetes), it primarily acts by inhibiting the TXNIP/NLRP3 axis; in drug toxicity and sepsis, its core action is clearing mitochondrial ROS and blocking inflammasome assembly; while in coronary heart disease, it may directly bind to the NLRP3 protein to inhibit its activity. This specificity is closely related to its selective intervention on ROS levels and upstream regulatory molecules (e.g., TXNIP) in the pathological microenvironment (Fig. 13).

5.8. Biological effects of calycosin via modulation of the EGFR signaling pathway

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase and a key member of the erythroblastosis oncogene B (ErbB) family (including EGFR, ErbB2, ErbB3, and ErbB4). It is expressed in many tissues and is primarily involved in cell proliferation, division, and cancer development [115, 116]. In a pathological injury environment, binding of specific ligands to the extracellular domain of EGFR promotes homodimerization and heterodimerization, thereby activating intracellular protein tyrosine kinase activity. This leads to autophosphorylation of tyrosine residues in the C-terminal domain, providing docking sites for signaling molecules containing Src homology 2 (SH2) domains and phosphotyrosine-binding (PTB) domains. Consequently, it initiates downstream pathways such as PI3K/AKT and MAPK, which participate in physiological and pathological processes including cell proliferation, differentiation, and damage repair [117, 118]. Recent studies have shown that aberrant activation or dysfunction of the EGFR signaling pathway is closely associated with diseases such as osteoarthritis and impaired diabetic wound healing. Calycosin can exert disease-intervening effects by targeting and modulating the EGFR signaling pathway. For instance, Su et al. [119], using network pharmacology and molecular docking, demonstrated that calycosin directly binds to EGFR (at binding sites including SER14, SER201, and LYS207). Further validation in a mouse osteoarthritis (OA) model established by anterior cruciate ligament transection confirmed that calycosin significantly inhibited activation of the EGFR signaling pathway and the expression of its downstream inflammation-related molecules COX-2 and MMP-9, while upregulating the expression of cartilage synthesis

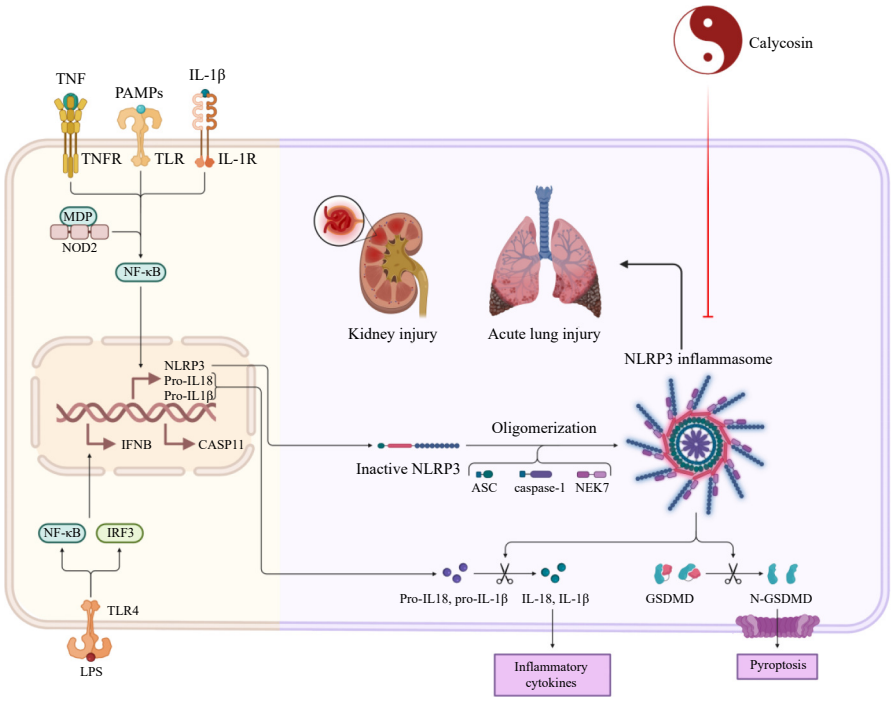

Fig. 13 Calycosin regulates the NLRP3 signaling pathway. TNFR: Tumor Necrosis Factor Receptor; TLR: toll-like receptor; IL-1R: interleukin-1 receptor; MDP: Muramyl Dipeptide; NOD2: Nucleotide-binding oligomerization domain 2.

markers type II collagen (Col-2) and Sox-9. This improved the imbalance between chondrocyte anabolism and catabolism and alleviated OA cartilage damage. Diabetic ulcers are recognized as one of the most common complications in diabetic patients, potentially leading to disability or even death in severe cases. Liu et al. [120] observed, in a db/db diabetic mouse wound model, that calycosin, as one of the main absorbed components of Huiyang Shengji Tang, upregulated the expression of EGFR in wound tissue, promoted epidermal cell proliferation, accelerated granulation tissue formation and epithelialization, and significantly increased the wound healing rate. In vitro experiments further confirmed that calycosin promoted the proliferation of human keratinocytes (HaCaT) by activating the EGFR/PI3K/AKT pathway, and this effect could be partially reversed by an EGFR inhibitor.

In summary, the regulation of the EGFR signaling pathway by calycosin exhibits a bidirectional characteristic: in OA, it primarily inhibits EGFR activity to exert anti-inflammatory and anti-catabolic effects, whereas in diabetic wound healing, it upregulates EGFR expression and its downstream pathway to promote tissue repair. This discrepancy may be related to the basal activity state of EGFR in the specific disease microenvironment and the specific activation of downstream target molecules. These studies provide an experimental basis for the clinical application of calycosin in inflammation-related diseases, cancer, and tissue repair. However, its selectivity for EGFR subtypes and its dose-effect relationship still require in-depth exploration (Fig. 14).

5.9. Biological effects of calycosin via modulation of the HIF signaling pathway

Hypoxia-inducible factor (HIF) is a core transcription factor regulating oxygen homeostasis in the body. It is a heterodimer composed of HIF-1α and HIF-1β subunits and plays a highly specific regulatory role in oxygen homeostasis [121]. Under normoxic conditions, HIF-1α is ubiquitinated by prolyl hydroxylases (PHDs) and rapidly degraded. In a hypoxic environment, HIF-1α translocates to the nucleus, binds to the HIF-1β subunit to form an active HIF-1 heterodimer, and binds to hypoxia response elements (HREs), promoting the upregulation of genes such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), inducible nitric oxide synthase (iNOS), and heme oxygenase-1 (HO-1). This subsequently regulates a series of physiological processes including cell proliferation, differentiation, apoptosis, angiogenesis, and energy metabolism [122, 123]. Calycosin exerts therapeutic effects in various physiological and pathological processes, such as inflammatory responses, tumor progression, hematopoietic function, and epithelial barrier maintenance, by mediating the HIF signaling pathway.

In a cobalt chloride ($CoCl_2$)-induced hypoxia model of retinal pigment epithelial (RPE) cells, it was found that calycosin significantly reduced the release of pro-inflammatory cytokines IL-6 and IL-8 and decreased the levels of apoptosis-related proteins (Cleaved caspase-3, Cleaved PARP), thereby ameliorating the

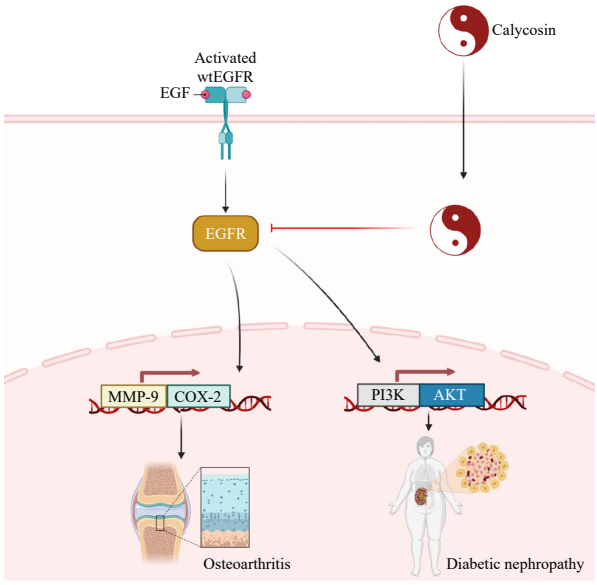

Fig. 14 Calycosin regulates the EGFR signaling pathway. EGF: EpidermalGrowthFactor; MMP-9: Matrix metallopeptidase 9; COX-2: Cyclooxygenase-2; PI3K: Phosphoinositide 3-Kinase; AKT: Protein kinase B.

hypoxia-induced decrease in RPE cell viability. This process likely occurs by blocking the synergistic activation of the HIF-1α/NF-κB axis, alleviating cellular inflammatory damage and apoptosis. This suggests that calycosin can exert a protective effect in hypoxia-related eye diseases, such as retinopathy of prematurity, by inhibiting HIF-1α [124]. Research by Zhang et al. [125] on radioresistance in lung cancer cells showed that calycosin, by targeting and binding to HIF-1α (key binding sites include TYR-131 and GLN-133), directly inhibited the overactivation of the HIF-1α/VEGF pathway. This subsequently reduced the clonogenic ability of lung cancer cells (NCI-A549 and NCI-H1299) and promoted the expression of apoptosis-related proteins (Bax and cleaved caspase-3). *In vivo* experiments further confirmed that calycosin reduced transplanted tumor volume, decreased the levels of HIF-1α and VEGF in tumor tissue, and showed no significant toxicity to normal liver tissue. This mechanism indicates that calycosin enhances the sensitivity of lung cancer cells to radiotherapy by inhibiting HIF-1α, providing a new strategy to address radioresistance. Regarding the regulation of hematopoietic function, Zheng et al. [126] found, using human embryonic kidney fibroblast (HEK293T) cells as a model, that calycosin promotes HIF-1α accumulation through a dual mechanism. On one hand, it upregulates the mRNA transcription level of HIF-1α; on the other hand, it inhibits its ubiquitin-mediated degradation (similar to the effect of the proteasome inhibitor MG132). The accumulated HIF-1α binds to the HRE of the erythropoietin (EPO) gene, activating its transcription and expression. Subsequent studies in embryonic stem cells (NT2/D1 and PC12) further verified that calycosin-induced EPO expression depends on HIF-1α accumulation, revealing the molecular basis for its regulation of hematopoietic function through HIF-1α activation and providing theoretical support for the treatment of diseases such as anemia [127]. Jia et al. [128], in a FITC-induced allergic contact dermatitis mouse model, found that calycosin significantly reduced the release of HIF-1α-mediated inflammatory initiators (TSLP and IL-33), while upregulating the expression of tight junction proteins (occludin, CLDN1, and ZO-1), alleviating skin barrier damage and Th2-type inflammatory responses. Furthermore, HIF-1α knockout experiments confirmed that the barrier repair and anti-inflammatory effects of calycosin depend on the downregulation of HIF-1α, suggesting that calycosin maintains epithelial barrier integrity in allergic skin diseases by inhibiting HIF-1α.

In summary, the regulation of the HIF signaling pathway by calycosin exhibits significant pathological adaptability. In retinal injury, lung cancer radioresistance, and allergic dermatitis, it exerts anti-inflammatory, pro-apoptotic, or barrier repair effects by inhibiting HIF-1α and its downstream pathways. In contrast, in the regulation of hematopoietic function, it promotes HIF-1α accumulation to enhance EPO expression. This specific regulation is closely related to its selective effects on HIF-1α transcription, degradation, and downstream target molecules (Fig. 15).

5.10. Biological effects of calycosin via modulation of other signaling pathways

The Keap1/Nrf2/HO-1 signaling pathway is one of the core defense pathways for cells to counteract oxidative stress, inflammation, and other stimuli. Under normal conditions, Nrf2 binds to the DGR domain of its negative regulator, Kelch-like ECH-associated protein 1 (Keap1), forming a hinge-latch-like complex that is retained in the cytoplasm. Nrf2 is maintained at low activity levels through Keap1-mediated polyubiquitination and degradation by E3 ubiquitin ligase [129]. Under oxidative stress, conformational changes in Keap1 or inhibition of its binding to Nrf2 lead to Nrf2 dissociation and translocation into the nucleus. There, it activates the transcription of antioxidant response element (ARE)-dependent genes, including antioxidant genes such as HO-1, NAD(P)H quinone reductase 1 (NQO1), and superoxide dismutase (SOD), thereby synergistically alleviating oxidative damage and cellular stress [130, 131]. In a bleomycin (BLM)-induced pulmonary fibrosis mouse model, calycosin upregulated the expression of Nrf2 and HO-1, reduced MDA levels, restored SOD and total antioxidant capacity (TAC) activity, and alleviated oxidative stress damage. Simultaneously, calycosin increased the levels of Beclin1 and PINK1, promoted the conversion of LC3-I to LC3-II, reduced p62 accumulation, upregulated the expression of LAMP1 and TFEB, and decreased legumain levels, thereby improving autophagic-lysosomal function [132]. Therefore, calycosin alleviates BLM-induced pulmonary fibrosis by activating the Nrf2/HO-1 pathway to exert antioxidant effects and by regulating the autophagic-lysosomal pathway to clear damaged material (Fig. 16).

Aberrant activation of the Wnt/β-catenin signaling pathway plays a key role in tumorigenesis and development. Upon pathway activation, Wnt3a binds to the Frizzled/LRP5/6 receptors, leading to the accumulation of β-catenin in the cytoplasm and activation of downstream target genes. Glycogen synthase kinase-3β (GSK-3β) maintains pathway homeostasis by promoting β-catenin degradation [133]. In glioblastoma multiforme (U87 cells), calycosin inhibited the expression of phosphorylated GSK3β (pGSK3βser9, the inactive form of GSK3β), reduced the intracel-

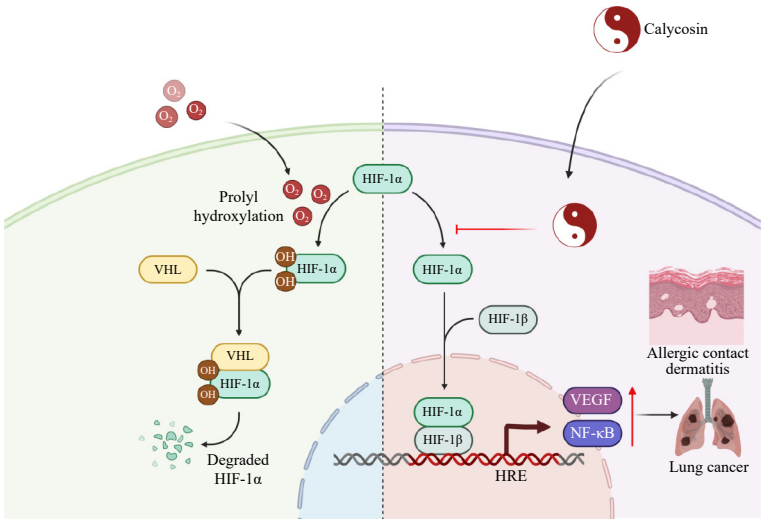

Fig. 15 Calycosin regulates HIF-1α signaling pathway. VHL: Von Hippel-Lindau; HIF-1α: Hypoxia-inducible factor 1α; HIF-1β: Hypoxia-inducible factor 1β; VEGF:Vascular endothelial growth factor; NF-κB: Nuclear Factor Kappa-B.

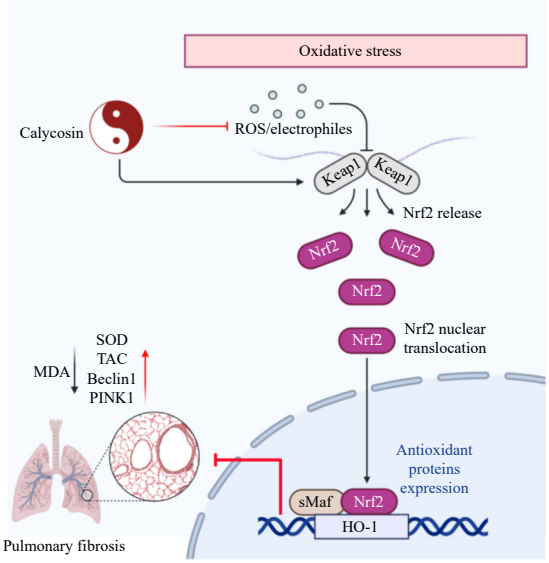

Fig. 16 Calycosin regulates the Keap1/Nrf2/HO-1 signaling pathway. MDA: Malondialdehyde; SOD: Super Oxide Dismutase; PINK1: PTEN induced kinase 1; Nrf2: Nuclearfactor erythroidderived 2-like 2.

lular accumulation of β-catenin, and consequently suppressed the expression of the downstream oncogene c-Myc [134]. Furthermore, calycosin, by mediating the Wnt/GSK3β/β-catenin signaling pathway, exerted antioxidant and pro-apoptotic effects, selectively inhibiting U87 cell proliferation without significant toxicity to normal BV2 microglial cells, providing a potential direction for the treatment of glioblastoma (Fig. 17).

IL-33, a member of the IL-1 family, participates in anti-inflammatory responses to regulate immunity but can also promote inflammatory reactions, exhibiting a dual role. Upon tissue damage, IL-33 is rapidly released into the extracellular space as a damage-associated molecular pattern (DAMP) and binds to its receptor ST2, ultimately driving the expression of specific cytokines and chemokines [135]. In a high-fat diet combined with streptozotocin (HFD/STZ)-induced type 2 diabetes mellitus (T2DM) rat model, calycosin inhibited the activation of the IL-33/ST2 signaling pathway, thereby inhibiting NF-κB p65 nuclear translocation, reducing the levels of TGF-β, TNF-α, IL-1β, and MDA, and alleviating renal fibrosis [136] (Fig. 18).

5.11. Cross-talk network of signaling pathways

As the core active component of *Astragalus membranaceus*, calycosin exerts its biological effects through cross-talk among multiple signaling pathways rather than regulation of a single pathway, forming a synergistic network tailored to different diseases (Table 1). Mechanistically, the crosstalk among core pathways is mainly reflected in three categories. First, the antioxidant–anti-inflammatory synergy between NF-κB and Nrf2. For example, in parasitic meningitis or diabetic nephropathy, calycosin induces HO-1 to scavenge ROS, while Nrf2 directly binds to NF-κB p65 to inhibit its nuclear translocation, thereby dually alleviating inflammatory and oxidative damage. Second, the proliferation–apoptosis balance between PI3K/AKT and MAPK. For instance, in colorectal cancer, inhibition of PI3K/AKT reduces ERK1/2 phosphorylation, and in osteosarcoma, inhibition of PI3K/AKT enhances p38-mediated apoptosis, both jointly achieving antitumor effects. Third, the co-inhibition of fibrosis and inflammation by TGF-β/Smad and NF-κB. For example, in renal fibrosis or myocardial fibrosis, calycosin simultaneously inhibits TGF-β1/Smad-mediated collagen deposition and NF-κB-mediated inflammation, relieving tissue damage. This crosstalk exhibits disease adaptability: in inflammatory diseases, a multi-pathway antagonistic network centered on NF-κB is formed (e.g., dual antagonism of NF-κB by AMPK and ROS-NLRP3 in sepsis-induced ALI); in tumors, a dynamic balance network is formed through bidirectional regulation of PI3K/AKT and MAPK (e.g., synergistic pro-apoptosis by NF-κB and ERK1/2 in liver cancer, and balance of ERK1/2-induced metastasis risk by PI3K/AKT in pancreatic cancer); in fibrotic diseases, a multi-pathway blocking network centered on

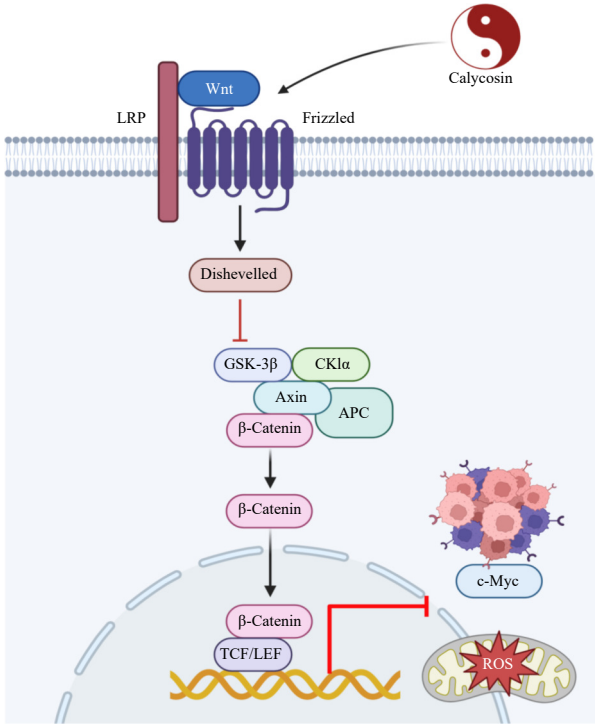

Fig. 17 Calycosin regulates the Wnt/β-catenin signaling pathway. APC: Antigen-presenting cell; CKIα: Cyclin dependent kinase inhibitor α; GSK-3β: Glycogen synthase kinase-3β; TCF/LEF: T cell factor/lymphoid enhancer factor family; c-Myc: Myelocytomatosis viral oncogene homolog.

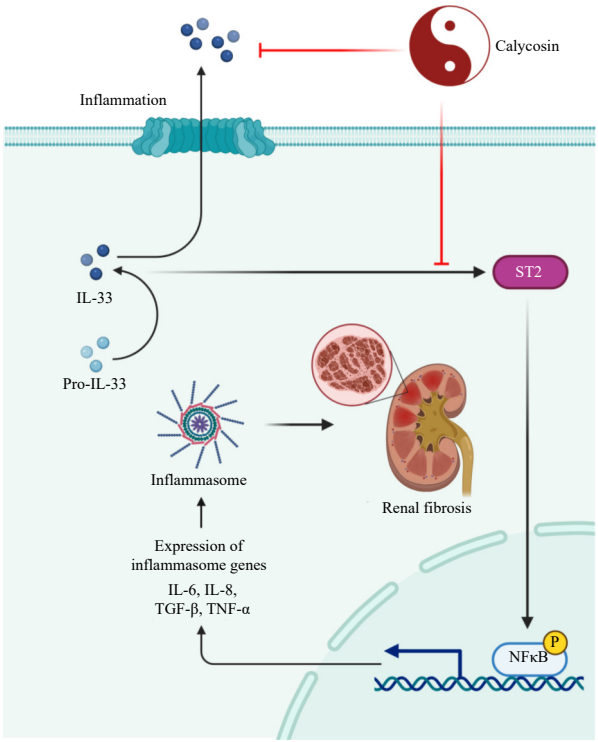

Fig. 18 Calycosin regulates the IL-33/ST2 signaling pathway. IL-33: Interleukin-33; IL-6: interleukin- 6; IL-8: interleukin- 8; TGF-β: Transforming growth factor-β; TNF-α: Tumour necrosis factor α; NF-κB: Nuclear Factor Kappa-B.

Table 1 Biological effects of Calycosin *via* multiple pathways.

Signaling pathway	Subjects	Dosage	Administration method	Mechanism of action	References
NF-κB signaling pathway	Mice	25, 50 mg·kg^{-1}	i.g.	IκBα, p65↓; IL-1β, IL-6↓	32
	RAW 264.7 Macrophages	5, 12.5, 25, 50 μmol·L^{-1}	Calycosin was pretreated for 2 h, then LPS (1 μg·mL^{-1}) was added for co-culture	IκBα, p65↓iNOS, COX-2↓; NO, PGE2↓	33
	Mice; HaCaT cells	0.4, 2, 10 mg·kg^{-1}; 1, 10 μmol·L^{-1}	I.g.; Pretreated with Calycosin for 6 h, and then co-cultured with LPS (10 μg·mL^{-1})	TSLP, IL-33↓; occludin; ZO-1↑	34
	Mice; A549 cells	25, 50 mg·kg^{-1}; 1, 5, 10, 20 μmol·L^{-1}	i.p.; The cells were pretreated with Calycosin for 1 h, and then co-cultured with LPS (1 μg·mL^{-1})	TNF-α, IL-6↓; HMGB1, p-p65↓	35
	Mice	25, 50 mg·kg^{-1}	i.p.	IL-1β, IL-6↓; p65, IκBα↓; p38 MAPK↓	36
	Mice; PBMCs/THP-1 macrophages	50 mg·kg^{-1}; 10 μmol·L^{-1}	i.p.; The cells were pretreated with Calycosin for 1 h, and then co-cultured with MSU (0.2 mg·mL^{-1})	TNF-α, IL-6↓; AIM2, caspase-1↓; p-p65, p-IκBα↓; p62-Keap1↑	38
	Mice	30 mg·kg^{-1}	i.p.	p65, IL-1β, TNF-α↓	40
	Mice; mTEC cells	10 mg·kg^{-1}; 0.01-10 μmol·L^{-1}	i.p.; Pretreated with Calycosin for 2 h, and then added AGEs (50 μg·mL^{-1}) for co-culture	IκBα, p65↓; TNF-α, IL-1β↓	42
	Mice; A549 cells	25, 50 mg·kg^{-1}; 1, 5, 10, 20 μmol·L^{-1}	i.p.; The cells were pretreated with Calycosin for 1h and then co-cultured with LPS (1 μg·mL^{-1})	NF-κB , p65↓; TNF-α, IL-6, HMGB1↓	43
	Mice	10, 25, 50, 75 mg·kg^{-1}	i.p.	TNF-α, IL-6↓; Nrf2/SOD1↑	45
	HepG2, Hep3B, and Huh7 cells	1, 3, 10, 30, 100 μmol·L^{-1}	Add Calycosin for cultivation	Bax, caspase-3↑; TGF-β1/SMAD2/3/SLUG/Vimentin↓; p21/p27↑	46
	Nude mice; 143B cells	0, 30, 60, 120 mg·kg^{-1}; 60, 120, 180 mmol·L^{-1}	i.p.; Add Calycosin for cultivation	Bcl-2, PARP↓; caspase-3↑; miR-223, NF-κBp65, IκBα↓	47
	Nude mice; 143B cells	30, 60, 120 mg·kg^{-1}; 60, 120，180 μmol·L^{-1};	i.p.; Add Calycosin for cultivation	MMP2, PCNA, IκBα, ECT2↓; MMP2, IκBα, ECT2, IL-6↓	48
PI3K/AKT signaling pathway	Mice; HCT116, SW480 cells	20, 40, 60 mg·kg^{-1}; 0, 50, 100, 200 μmol·L^{-1};	i.p.; Add Calycosin for cultivation	Erβ, PTEN↑; p-AKT/AKT↓; Bcl-2↓	54
	Nude mice; MG-63, U2-OS cells	2, 4, 8 mg·kg^{-1}; 0, 25, 50, 100 μmol·L^{-1};	i.p.; 0, 25, 50, 100 μmol·L^{-1};	p-PI3K, p-AKT, p-mTOR↓; cleaved caspase-3↑	55
	U251, U87 cells	0, 25, 50, 100, 200, 400, 800 μmol·L^{-1}	Add Calycosin for cultivation	MMP9, p-AKT↓; c-Met, Dtk, Lyn, PYK2↓	56 57
	Mice; MLE-12 cells	7, 14 mg·kg^{-1}; 10, 20, 40, 60, 80 μmol·L^{-1}	i.g.; Add Calycosin for cultivation	AKT, GSK3β, β-catenin↓; E-cadherin↑; vimentin, α-SMA, fibronectin↓	58
	HEK293T, U251, U81 cells	12.5, 25, 50, 100 μmol·L^{-1}	Add Calycosin for cultivation	Nrf2, HO-1, SOD↑; ROS↓	59
	Rat	50 mg·kg^{-1}	i.p.	SOD, GSH↑; MDA, NO, ROS, LDH↓	60
	CP-H096 cells	1, 5, 10, 20 μmol·L^{-1}	The cells were pretreated with Calycosin for 24 h, and then co-cultured with IL-1β (10 ng·mL^{-1})	TNF-α, IL-6↓; PI3K, AKT, FoxO1↓	61
MAPK signaling pathway	Rat	20 mg·kg^{-1}	i.p.	pERK/ERK↑	71
	HT29, Caco2 cells	3.125–200 μmol·L^{-1}	Add Calycosin for cultivation	p-ERK1/2↓; E-cadherin↑; N-cadherin, Vimentin↓; ERK1/2↓	72
	143B cells	0–160 μg·mL^{-1}	Add Calycosin for cultivation	Bax, Cleaved-caspase-3/9 , PARP↑; p38-MAPK↑; Bcl-2↓	73
	Mice; MIA PaCa-2, PANC1 cells; Pan02 cells	15, 30 mg·kg^{-1}; 25, 50, 100, 200 μmol·L^{-1};	i.p.; Add Calycosin for cultivation	MMP-2, MMP-9↑; Snail, CD31, CD206↑; TGF-β1/Raf/MEK/ERK↑	75
	GMCs cells	0.1, 1, 10 μmol·L^{-1}	Add Calycosin and Ang II for co-culture	p-ERK1/2, LC3, Beclin-1↓; p62↑; Bax↑, Bcl-2↓	76
STAT3 signaling pathway	Rat; PC12 cells	7.5, 15, 30 mg·kg^{-1} 1–120 μg·mL^{-1}	i.g.; After 4 h of OGD and 24 h of reperfusion, Calycosin was added at the time of reperfusion	STAT3, FOXO3a, LC3II/LC3I↓; SQSTM1↑; STAT3/FOXO3a↓	82
	Mice; H9C2 cells	30 mg·kg^{-1}; 1 μmol·L^{-1}	i.v.; Calycosin pretreatment was added before OGD/R treatment	IL-10, p-JAK2, p-STAT3↑	83
	Primary rat spinal astrocytes	5, 10, 20 μmol·L^{-1}	Calycosin was pretreated and then H_2O_2 (100 μmol·L^{-1}) was added for co-culture	IL-6, p-JAK2, p-STAT3, p-AKT, GP130↓	84
	LX-2 cells	25–200 μmol·L^{-1}	The cells were pretreated with Calycosin, and then co-cultured with TGF-β1 (10 ng·mL^{-1})	α-SMA, Col-I, TIMP-1↓; MMP-1↑; p-STAT3↓	85
	Rat	40 , 80 mg·kg^{-1}	i.g.	STAT3, integrin β1/NF-κB↓	86

Continued

Signaling pathway	Subjects	Dosage	Administration method	Mechanism of action	References
AMPK signaling pathway	A7r5 cells	0, 5, 10, 20 μmol·L^{-1}	The cells were treated with Calycosin for 24 hours and then cultured in β-GP (10 mmol·L^{-1}) medium	AMPK/mTOR↑; RUNX2, BMP2, OPN↓	92
	Mice; Beas-2B cells	25 mg·kg^{-1}; 0, 1, 10, 50, 100, 500, 1000 μmol·L^{-1};	i.p.; Calycosin pretreatment was followed by PM2.5 co-culture	p65, IL-6, IL-8↓	93
	Primary osteoblasts of rats	693 ng·mg^{-1}	Join Calycosin for co-cultivation	ALP, Runx2, Sparc↑	94
	Rat; C2C12 cells	15 mg·kg^{-1}; 7.5 μg·mL^{-1}	i.p.; The cells were pretreated with Calycosin and then co-cultured with TNF-α (40 ng·mL^{-1})	AMPK, LC3A/B, ATG7↓; SKP2↑; CARM1, H3R17me2a↓	95
	Mice	50 mg·kg^{-1}	i.g.	AMPK, eNOS↑; TNF-α↓	96
TGF-β1/Smad signaling pathway	T47D, MCF-7 cells	0, 25, 50, 100 μmol·L^{-1}	i.p.; Join Calycosin for co-cultivation	BATF↓ E-cadherin↑; N-cadherin, Vimentin, CD147, MMP-2, MMP-9↓	101
	CCD-18Co cells	12.5, 25, 50 μmol·L^{-1}	The cells were pretreated with TGF-β1 (5.0 μg·L^{-1}) and then cultured with Calycosin	α-SMA, Collagen I↓; TGF-β1, p-Smad2, p-Smad3, Smad4↓; Smad7↑	102
	Mice; CFs cells	25, 50 mg·kg^{-1}; 10, 20, 40 μmol·L^{-1}	i.g.; Join Calycosin for co-cultivation	CDK2, CDK4, PCNA↓; TGFBRI↓	103
	Mice; MSCs, PTECs cells	200 μg·mL^{-1}; 200 μg·mL^{-1}	i.v.; Join Calycosin for co-cultivation	TGF-β1, Smad2/Smad3↓; TNF-α, TNFR1↓	104
NLRP3 signaling pathway	Rat	5, 10 mg·kg^{-1}	i.g.	NLRP3, IL-1β, Caspase-1, p65, TXNIP↓	110
	Mice; H9c2 cells	50 mg·kg^{-1}; 5, 10, 20 μg·mL^{-1}	i.g.; Calycosin was pre-treated and then DOX was added for co-culture	IL-1β, IL-18, BNP, LDH, CRP, MCP-1, ROS, MDA↓; NLRP3/caspase-1/GSDMD↓; SOD, GSH-Px, ATP↑	111
	Mice; BMDMs cells	12.5, 25, 50 mg·kg^{-1}; 2.5, 5, 10 μmol·L^{-1}	i.g.; Calycosin was pretreated and then LPS and ATP were added to co-culture	NLRP3, ASC, Caspase-1, IL-1β, IL-18↓	112
EGFR signaling pathway	Mice; ATDC5 chondrocytes	50 mg·kg^{-1}; 0, 16, 32, 64 μmol·L^{-1}	I.g.; Join Calycosin for co-cultivation	COX-2, MMP-9↓; Col-2, Sox-9↑	119
	Mice; Hacat cells	16.25, 32.5, 65, 130 μmol·L^{-1}	I.g.; Join Calycosin for co-cultivation	EGFR/PI3K/Akt↑	120
HIF signaling pathway	ARPE-19 cells	1, 5, 10 μmol·L^{-1}	Join Calycosin for co-cultivation	IL-6, IL-8, Cleaved caspase-3, Cleaved PARP↓; HIF-1α/NF-κB↓	124
	Mice, Nude mice; NCI-A549, NCI-H1299 , Lewis cells	40 mg·kg^{-1}; 25 μmol·L^{-1}	i.p.; Join Calycosin for co-cultivation	HIF-1α/VEGF↓; Bax, Cleaved caspase-3↑	125
	HEK293T, HepG2 cells	0.1–10 μmol·L^{-1}	Join Calycosin for co-cultivation	HIF-1α, EPO↑	126
	NT2/D1, PC12 cells	0.1-30 μmol·L^{-1}	Join Calycosin for co-cultivation	HIF-1α, EPO↑	127
	Mice; HaCaT cells	50 mg·kg^{-1}; 0.1, 1, 10 μmol·L^{-1}	i.p.; Join Calycosin for co-cultivation	TSLP, IL-33↓; Occludin, CLDN1, ZO-1↑	128
Nrf2/HO-1 signaling pathway	Mice	14 mg·kg^{-1}	i.g.	SOD, TAC, Nrf2/HO-1, Bcl-2, HK-2,Beclin1, PINK1, LC3-II, LAMP1, TFEB↑; Bax, p62, legumain↓	132
Wnt/β-catenin signaling pathway	U87 cells	1-100 μg·mL^{-1}	Join Calycosin for co-cultivation	SOD, CAT, GSH, Bax/Bcl-2, Caspase-9, Caspase-3↑; ROS, p-GSK3β(Ser9), β-catenin, c-Myc↓	134

TGF-β/Smad is formed (e.g., co-inhibition of EMT and collagen synthesis by PI3K/AKT, MAPK, and Nrf2 in pulmonary fibrosis). Moreover, the overall regulation presents characteristics of target orientation (e.g., EGFR as a common upstream of PI3K/AKT and MAPK to achieve bidirectional regulation), pathological adaptability (e.g., HIF-1α is inhibited in hypoxic injury but activated in hematopoietic regulation), and effect synergy (e.g., synergy of PI3K/AKT and Nrf2 in neuroprotection during spinal cord injury, and synergy of multiple MAPK subtypes in inducing apoptosis in gastric cancer). These features define its multi-pathway mechanism of action and provide a theoretical foundation for clinical translation. Future studies should further clarify the details of molecular interactions and regulatory thresholds (Table 2).

6. Pharmacokinetics (PK) of calycosin

Calycosin, a major active component of *Astragalus membranaceus*, exhibits a wide range of pharmacological activities. Based on published studies utilizing HPLC-MS/MS, UHPLC-MS/MS, and LC-MS/MS, this section systematically reviews the absorption, distribution, metabolism, and excretion (ADME) characteristics of calycosin. It analyzes its pharmacokinetic differences under various formulations, pathological states, and drug combinations, and discusses the regulatory roles of sulfotransferases and transporters on its *in vivo* processes, providing a reference for its clinical application and formulation optimization.

6.1. Absorption and first-pass effect

The absorption of calycosin is closely related to its precursor form. When administered orally as calycosin-7-O-β-*D*-glucoside, it requires deglycosylation by intestinal glycosidases to calycosin for effective absorption. *In vitro* studies showed that the intestinal permeability of calycosin was significantly higher than that of calycosin-7-O-β-*D*-glucoside, with an apparent permeability coefficient (Papp) of approximately 3.5 × 10−5 cm/s, 20–40 times

that of the glucoside (about 1 × 10^{-6} cm/s). This is attributed to the absence of the glucose moiety and higher lipophilicity of calycosin [137]. Studies using Caco-2 cells to investigate the absorption and transport characteristics of calycosin and its glucoside found that both are primarily absorbed *via* passive diffusion, and the absorption process is not affected by inhibitors of multidrug and toxin extrusion (MATE) proteins such as P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2) [138]. In Huangqi Chifeng Decoction (HQCF), calycosin exhibited a double-peak absorption profile in rat plasma after oral administration. The first peak time (T_{max}) was 1.5 h, likely related to direct intestinal absorption; the second absorption peak might originate from enterohepatic circulation. In contrast, the Tmax of its precursor, calycosin-7-O-β-*D*-glucoside, in Fangji Huangqi Decoction was 0.35 h, suggesting that excipients or other components in different formulations may affect the absorption rate [139]. Compared to normal rats, the area under the curve ($AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$) and half-life ($T_{1/2}$) of calycosin were significantly increased in pulmonary fibrosis (PF) rats after oral administration of Fei Kang Fu mixture (a Chinese herbal formula containing calycosin 124.4 μg·g^{-1}), indicating that the PF state might enhance the absorption of calycosin [140]. Furthermore, using an in situ single-pass intestinal perfusion model in rats, it was found that within the concentration range of 5–20 μg·mL^{-1}, the effective permeability coefficient and absorption rate constant of calycosin gradually decreased with increasing concentration in various intestinal segments, showing self-concentration inhibition. This suggests possible involvement of active transport mechanisms. Its absorption was better in the colon than in the duodenum, jejunum, and ileum, indicating that the colon is one of its primary absorption sites. Combined with Caco-2 cell model studies, the ratio of apparent permeability coefficients for low, medium, and high concentrations of calycosin was 1.38 < 1.5, indicating that its absorption is primarily *via* passive transport, with a contribution from active transport mechanisms [141].

The hepatic first-pass effect is a key factor leading to low systemic exposure of calycosin. After oral administration of calycosin-7-O-β-*D*-glucoside to rats, the peak concentration (C_{max}) of calycosin in portal vein plasma was 2563.0 ± 487.6 ng·mL^{-1}, significantly higher than that in systemic circulation plasma (34.8 ± 2.0 ng·mL^{-1}), with a hepatic extraction ratio (ER) as high as 98.5%. This phenomenon is related to highly expressed metabolic enzymes in the liver, such as sulfotransferases (SULT1A1 and SULT1B1), which rapidly catalyze calycosin into sulfate conjugates, reducing its transfer into the systemic circulation. In contrast, calycosin-7-O-β-*D*-glucoside has a weaker hepatic first-pass effect (ER = 0.3%), as it is less recognized by hepatic metabolic enzymes and enters the systemic circulation more in its prototype form; however, it ultimately still requires conversion to calycosin to exert activity [142].

6.2. Distribution characteristics

Calycosin exhibits specific tissue distribution in vivo. After oral administration of calycosin-7-O-β-*D*-glucoside to rats, calycosin was only detected in enriched amounts in the liver, kidneys, and heart. The highest concentration was in the liver (C_{max} = 8514.7 ± 332.1 ng·g^{-1}), which was 212.1 times the plasma concentration; concentrations in the kidneys and heart were 30.5 times and 4.7 times the plasma concentration, respectively [142]. This distribution pattern is consistent with its pharmacological target organs; for example, its hepatoprotective effect might be directly related to its high accumulation in the liver. After oral administration of Zhenqi Fuzheng Capsules, the order of calycosin (CA) tissue distribution concentration in rats was: spleen > heart > thymus > lung > kidney > liver > stomach > testis [143]. After oral administration of Buzhong Yiqi Decoction concentrated granules, the peak concentration of calycosin in rats appeared in the stomach, liver, and kidneys at 30 minutes, and in the small and large intestines at 3 hours [144]. In a cerebral ischemia model, the distribution of calycosin in brain tissue significantly increased (approximately 4.98 times that in normal rats), potentially related to increased blood-brain barrier permeability and enhanced active transport, providing an anatomical basis for its neuroprotective effects [145].

6.3. Metabolism and excretion

The metabolism of calycosin is dominated by Phase II reactions, primarily sulfation and glucuronidation. Mouse intestinal perfusion models and pharmacokinetic studies demonstrated that calycosin is metabolized to calycosin-3′-sulfate in the intestine and liver, and sulfotransferase 1A1 (SULT1A1) and sulfotransferase 1B1 (SULT1B1) are primarily responsible for the sulfation of calycosin [146]. Similarly, Yun et al. found that the main metabolic reactions of calycosin are glucuronidation and sulfation [147]. Sulfotransferases (SULT1A1 and SULT1B1) are key enzymes for sulfation, with SULT1A1 exhibiting the highest catalytic efficiency (V_{max} = 11.01 ± 3.27 nmol·min^{-1}·mg^{-1}), generating calycosin-3′-sulfate (C-3′-S). This metabolite is the main form in vivo, accounting for over 80% of the total exposure in plasma. Glucuronidation is mediated by UGT1A9 and UGT1A1, generating glucuronic acid conjugates. However, under HQCF combination, the proportion of sulfated metabolites was significantly higher than that of glucuronidated products, suggesting that other components may regulate metabolic enzyme selectivity [139]. Studies using rat liver microsomes showed that calycosin is primarily metabolized in the liver, involving Phase I and Phase II reactions. The Phase I metabolic rate was 9.41%, and the Phase II metabolic rate was 20.58%, indicating Phase II metabolism is dominant. In Phase II metabolism, it serves as a common substrate for UGT1A1, UGT1A3, UGT1A6, UGT1A9, and UGT2B7, generating conjugates through reactions like glucuronidation [141]. Gut microbiota can hydrolyze calycosin glucoside to calycosin, which is further metabolized to daidzein and formononetin; in primary hepatocytes, hydrolysis and glucuronidation primarily occur, generating corresponding conjugates, verifying key steps of in vivo metabolism [148].

Table 2 Example table of cross-talk between key pathways regulated by calycosin.

Pathway combination	Cross-talk mechanism	Involved disease model	Biological effect
NF-κB + Nrf2	Nrf2 eliminates ROS through HO-1 and directly binds to NF-κB p65 to inhibit its nuclear translocation	Parasitic meningitis, diabetic nephropathy	Synergistically inhibit inflammation and oxidative stress to protect tissue damage
PI3K/AKT + MAPK(ERK1/2)	PI3K/AKT inhibition can weaken ERK1/2 phosphorylation and dual inhibit tumor proliferation	Colorectal cancer (HCT116 cell)	Inhibit cell proliferation and EMT, and reduce the risk of tumor metastasis
PI3K/AKT + MAPK(p38)	PI3K/AKT inhibition reduces the expression of Bcl-2 and enhances the apoptotic effect mediated by p38	143B cell	Synergistically induce apoptosis of tumor cells
TGF-β/Smad + NF-κB	Calycosin simultaneously inhibited TGF-β1/Smad-mediated fibrosis and NF-κB mediated inflammation	Renal fibrosis model	Reduce collagen deposition and inflammatory infiltration in the renal interstitium

The excretion of calycosin metabolites is regulated by the breast cancer resistance protein (BCRP). The MDCKII/BCRP cell model showed that BCRP overexpression increased the apical excretion of C-3′-S by 14-fold; whereas the BCRP inhibitor Ko143 reduced excretion by 2-fold. In Bcrp1$^{-/-}$ mice, the $AUC_{0\text{-}t}$ of plasma C-3′-S increased 40-fold compared to wild-type, and intestinal excretion decreased by 82.6%–98.2%, confirming BCRP is a key transporter for intestinal excretion. Biliary excretion is also an important route; the concentration of C-3′-S in rat bile was significantly higher than in plasma, but the cerebral ischemic state had no significant effect on biliary excretion [146]. Other studies have shown that after oral administration of Astragalus extract to rats, the plasma concentration of calycosin was consistently below the lower limit of quantification. This might be related to partial excretion of calycosin from intestinal epithelial cells back into the intestinal lumen *via* transporters, leading to cycling between intestinal epithelial cells and the gastrointestinal tract tract [149].

In summary, calycosin has low oral bioavailability but high exposure in target organs (e.g., heart and kidneys), which may be related to the targeted distribution of its metabolites. Furthermore, calycosin glucoside can act as a prodrug, being converted to calycosin *in vivo* after oral administration, increasing its exposure in plasma and tissues, and providing the material basis for its efficacy. The pharmacokinetic characteristics of calycosin are as follows: absorption depends on the deglycosylation of calycosin-7-O-β-*D*-glucoside, with a significant hepatic first-pass effect; tissue distribution is organ-specific, with significant enrichment in the liver and kidneys; metabolism is dominated by sulfation; excretion is regulated by BCRP; and its *in vivo* processes are significantly influenced by formulation compatibility and pathological state (Table 3).

At present, it has been confirmed that calycosin's sulfated metabolites (such as C-3'-S) are the main form present in vivo (accounting for over 80% of total plasma exposure). However, research on the direct pharmacological activity (such as anti-inflammatory, antioxidant, anti-fibrotic effects) of these metabolites is still in the preliminary stage. There is insufficient experimental data to confirm whether they have biological effects similar to or unique to the parent drug, nor is it clear whether they are inactive terminal metabolites. Subsequent research should focus on the pharmacological activity of calycosin's sulfated and glucuronidated metabolites, clarify their synergistic or independent effects with the parent drug, and provide experimental basis for comprehensively explaining the *in vivo* basis of calycosin's true pharmacologically active substances and optimizing clinical administration regimens.

7. Safety evaluation of calycosin

In recent years, as the application prospects of calycosin in disease treatment and prevention have attracted increasing attention, comprehensive and systematic evaluation of its safety profile has become particularly important. This section reviews the relevant content on the safety of calycosin, aiming to provide a reference for its further research and application.

Multiple studies have investigated the cytotoxicity of calycosin using various cell-based assays. In studies on human bronchial epithelial cells (BEAS-2B), calycosin applied at concentrations of 1, 10, 50, and 100 μmol·L^{-1} for 24–72 h showed no significant effect on cell absorbance (a measure of cell viability), and cell survival rates did not markedly decrease. However, when the concentration reached 500 μmol·L^{-1} and 1000 μmol·L^{-1}, absorbance significantly decreased, indicating that calycosin exerted cytotoxic effects on BEAS-2B cells at these high concentrations 93. In human renal cortical proximal tubule epithelial cells (HK-2), treatment with different concentrations of calycosin (0, 10, 20, 40, 60, and 80 μmol·L^{-1}) for 24 h, assessed by CCK-8 assay and LDH activity measurement, revealed no toxic effects on HK-2 cells [150]. Research has shown that, while exerting pharmacological effects, calycosin has minimal impact on normal cells and tissues. In the normal cervical epithelial cell line Ect1/E6E7, calycosin at concentrations of 10, 20, 30, and 40 μmol·L^{-1} for 48 h showed no significant effects on cell viability or LDH release, with only a slight upregulation of miR-375 expression observed at 50 μmol·L^{-1} [151]. In normal brain tissue of animals, calycosin alleviated neuronal damage induced by advanced glycation end products (AGEs) and showed no significant adverse effects on normal brain tissue. These findings suggest that calycosin, while acting on diseased cells and tissues, causes minimal damage to normal cells and tissues, indicating a certain degree of targeting and safety [152]. Overall, calycosin exhibits no significant toxicity toward various cell types within a certain concentration range (typically 1–100 μmol·L^{-1}). However, cytotoxicity may occur at excessively high concentrations (e.g., 500 μmol·L^{-1} and above), and this toxic effect appears to be cell type-specific.

Based on existing research, calycosin demonstrates a favorable safety profile in various cell types and experimental animals at appropriate concentrations and doses, with no significant toxic reactions and minimal impact on normal cells and tissues. Its safety is closely related to the specificity of its mechanism of action, allowing it to exert pharmacological effects while reducing interference with normal physiological processes. However, current research on the safety of calycosin still has some limitations. For example, there is a relative lack of safety data concerning long-term, high-dose use, differences among various animal species, and insufficient data from human clinical trials. Future studies should further expand the scope of research, conducting long-term toxicity tests, teratogenicity and carcinogenicity studies, and human clinical trials, to evaluate the safety of calycosin more comprehensively and accurately, thereby providing a more solid theoretical basis for its clinical application.

8. Conclusion and prospects

Calycosin, as a core active component of *Astragalus membranaceus*, has been extensively studied across multiple dimensions, ranging from its isolation and purification to its potential for clinical application. In terms of isolation and purification, the combined use of various techniques (such as flash extraction coupled with macroporous adsorption resin enrichment, and the combination of silica gel column chromatography with high-

Table 3 Identified metabolites of calycosin and key characteristics.

Metabolite name	Metabolic reaction	Catalyzing enzyme (s)	Experimental model for identification	Reference
Calycosin (Aglycone)	Hydrolysis	Gut Microbiota / Intestinal Glycosidases	*In vivo* (Rat), *In vitro*	137, 148
Calycosin-3′-Sulfate (C-3′-S)	Sulfation	SULT1A1, SULT1B1	*In vivo* (Rat, Mouse), MDCKII/BCRP cell model, Recombinant enzyme assay	139, 146, 147
Calycosin glucuronide(s)	Glucuronidation	UGT1A1, UGT1A3, UGT1A6, UGT1A9, UGT2B7	Rat liver microsomes, Recombinant enzyme assay	139, 141, 147
Daidzein	Degradation/further metabolism	Gut Microbiota	*In vitro* (Gut microbiota incubation)	148
Formononetin	Degradation/further metabolism	Gut Microbiota	*In vitro* (Gut microbiota incubation)	148

speed counter-current chromatography) has enabled its efficient preparation, providing the material foundation for pharmacological research. The elucidation of its biosynthetic pathways (including the phenylpropanoid pathway in planta, the chemical "one-pot" method *in vitro*, and the construction of microbial cell factories) offers diverse strategies for large-scale production. Structural derivatization, particularly through modifications at the 7- and 3'-hydroxyl groups, has significantly improved its solubility and activities such as antitumor efficacy. Investigations into its pharmacological mechanisms have revealed that calycosin exerts multi-pathway and low-toxicity effects in diseases such as inflammation, cancer, and neural injury by modulating multiple signaling pathways, including NF-κB, PI3K/AKT, and MAPK. Pharmacokinetic studies indicate that its oral bioavailability is regulated by first-pass effects and metabolic enzymes, and its tissue distribution is organ-specific. Safety evaluations have shown low toxicity at therapeutic concentrations, indicating potential advantages for clinical use.

Despite significant progress in calycosin research, its clinical translation faces multiple scientific and technical bottlenecks, specifically:

(1) Insufficient depth of mechanistic understanding. Although current studies confirm its regulation of multiple signaling pathways, the crosstalk mechanisms between these pathways (e.g., the synergy between NF-κB and Nrf2 in anti-inflammation, and the interaction between PI3K/AKT and MAPK in cancer) remain unclear. Furthermore, the cell specificity of certain effects (e.g., AMPK activation in vascular calcification versus its inhibition in skeletal muscle atrophy) lacks molecular-level explanations, hindering the precise design of targeted intervention strategies. Future research should integrate technologies such as single-cell sequencing and protein interactomics to map dynamic "disease-pathway-target" regulatory networks and identify core effector molecules. (2) Pharmacokinetic characteristics limiting clinical application. Its oral bioavailability is low (a hepatic first-pass effect leads to an extraction ratio of up to 98.5%), its metabolism is dominated by sulfation (sulfate metabolites account for over 80% in plasma), and the activity of these metabolites is unknown. Concurrently, the mechanisms underlying the influence of pathological states (e.g., pulmonary fibrosis) on its absorption and distribution have not been elucidated, making it difficult to formulate individualized dosing regimens. There is a need to develop novel delivery systems (e.g., nanocarriers targeting the enterohepatic circulation) and to utilize metabolomics to track active metabolites, establishing PK-PD models that account for the synergistic effects of the "parent compound–metabolites". (3) Disconnect between preclinical research and clinical needs. Existing studies are predominantly based on *in vitro* cell or animal models, and data from human clinical trials are lacking. The synergistic effects of calycosin within Astragalus complex formulations (e.g., regulation of metabolic enzymes by other components) and the safety of long-term, high-dose use (e.g., potential effects on the endocrine system) have not been evaluated. Moreover, the dose-response relationship in different diseases (e.g., the threshold difference between antitumor efficacy and cytotoxicity to normal cells) is unclear, limiting the precision of clinical medication. Future work should involve efficacy assessments using patient-derived organoids and the design of early-stage clinical trials to explore dose-response curves. (4) Industrial technological bottlenecks restricting material supply. Current isolation and purification techniques suffer from high organic solvent consumption and cumbersome processes (e.g., complex solvent system selection for HSCCC). Although microbial synthesis has achieved a glycoside yield of 10.5 $g \cdot L^{-1}$, the efficient synthesis of the calycosin monomer remains unattained. There is a pressing need to develop green separation technologies (e.g., molecularly imprinted polymer adsorption) and high-yield engineered strains (optimizing metabolic pathways through synthetic biology) to reduce production costs and improve product purity.

In conclusion, future research should focus on achieving breakthroughs across the entire chain of "mechanistic elucidation - formulation optimization - clinical validation". Interdisciplinary approaches (integrating chemical biology, pharmaceutical engineering, etc.) are essential to propel calycosin from the laboratory to the clinic, thereby providing a paradigm for the modern development of natural medicines.

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Declaration of Competing Interests

The authors declare no conflicts of interests.

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