



## Pharmacology, toxicity and pharmacokinetics of acetylshikonin: a review

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### ABSTRACT

**Context:** Acetylshikonin, a naphthoquinone derivative, is mainly extracted from some species of the family Boraginaceae, such as *Lithospermum erythrorhizon* Sieb. et Zucc., *Arnebia euchroma* (Royle) Johnst., and *Arnebia guttata* Bunge. As a bioactive compound, acetylshikonin has attracted much attention because of its broad pharmacological properties.

**Objective:** This review provides a comprehensive summary of the pharmacology, toxicity, and pharmacokinetics of acetylshikonin focussing on its mechanisms on the basis of currently available literature.

**Methods:** The information of acetylshikonin from 1977 to 2020 was collected using major databases including Elsevier, Scholar, PubMed, Springer, Web of Science, and CNKI. Acetylshikonin, pharmacology, toxicity, pharmacokinetics, and naphthoquinone derivative were used as key words.

**Results:** According to emerging evidence, acetylshikonin exerts a wide spectrum of pharmacological effects such as anticancer, anti-inflammatory, lipid-regulatory, antidiabetic, antibacterial, antifungal, antioxidative, neuroprotective, and antiviral properties. However, only a few studies have reported the adverse effects of acetylshikonin, with respect to reproductive toxicity and genotoxicity. Pharmacokinetic studies demonstrate that acetylshikonin is associated with a wide distribution and poor absorption.

**Conclusions:** Although experimental data supports the beneficial effects of this compound, acetylshikonin cannot be considered as a therapy drug without further investigations, especially, on the toxicity and pharmacokinetics.

### ARTICLE HISTORY

Received 24 April 2020  
Revised 6 August 2020  
Accepted 28 August 2020

### KEYWORDS

Naphthoquinone derivative;  
bioactive compound;  
pharmacological  
effects; mechanism

### Introduction

Zicao, a Chinese Materia Medica, is the root of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), *Arnebia euchroma* (Royle) Johnst. (Boraginaceae), and *Arnebia guttata* Bunge (Boraginaceae) (Chen et al. 2002; Zhang et al. 2019). Furthermore, there are other plants of the genus *Onosma* (Boraginaceae) also named Zicao in some areas of China (Table 1) (Hu et al. 2006). Zicao has been widely used for the treatment of burns, carbuncles, measles, and macular eruptions in traditional Chinese medicine (Chen et al. 2002; Guo et al. 2019). For example, Shiunko, a clinical ointment made of Zicao and *Angelica sinensis* (Oliv.) Diels (Apiaceae), has been used in treating wounded skin caused by cuts, burns, and abrasions (Sakaguchi et al. 2001; Huang et al. 2004; Lu et al. 2008).

Acetylshikonin (Figure 1) is a naphthoquinone derivative mainly isolated from Zicao. However, there are obvious differences in the content of acetylshikonin in different species (Hayashi 1977; Fu et al. 1984; Yazdinezhad et al. 2009). As shown in Table 1, the content of acetylshikonin is  $19.7 \pm 0.66$  mg/g and  $0.123 \pm 0.002$  mg/g in *Arnebia euchroma* and *Onosma confertum* W.W. Smith (Boraginaceae), respectively (Hu et al. 2006). Apart from zicao, there are other plants containing acetylshikonin, such as *Alkanna frigida* Boiss. (Boraginaceae), *Echium italicum* L. (Boraginaceae), or *Onosma visianii* Clem (Boraginaceae) (Albrecht et al. 2009; Yazdinezhad et al. 2009; Vukic et al. 2017).

Currently, acetylshikonin has attracted much attention for its broad pharmacological effects such as anticancer, anti-inflammatory, antioxidative, antibacterial, antifungal, lipid-regulatory, antidiabetic, neuroprotective, and antiviral properties (Sasaki et al. 2000; Cheng et al. 2008; Kim et al. 2009; Gwon et al. 2012; Li et al. 2012; Wang et al. 2013; Xue and Li 2017; Huang et al. 2019; Liao et al. 2019). However, previous studies have reported the toxicity of acetylshikonin with respect to genotoxicity and reproductive toxicity (Li et al. 2015; He et al. 2016). In addition, acetylshikonin exhibits low water solubility and poor absorption rate after oral administration, which may affect its efficacy (Sun et al. 2008a; Peng et al. 2014). Overall, this review discusses the pharmacology, toxicity, and pharmacokinetics of acetylshikonin with an emphasis on its mechanisms, and provides future research opportunities.

### Pharmacology

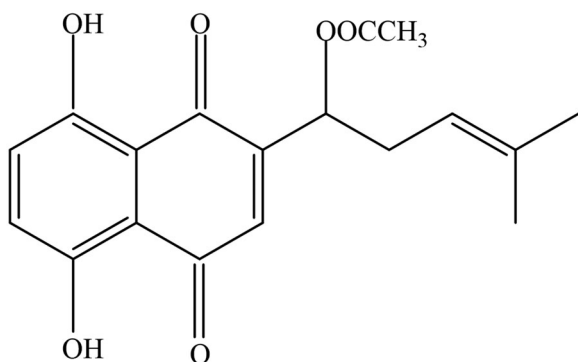
#### Anticancer effect of acetylshikonin

##### Colorectal cancer

Vukic et al. (2017) reported that treatment with acetylshikonin arrested the cell cycle at the G2/M phase, and induced apoptosis and necrosis in HCT116 cells. Furthermore, acetylshikonin suppressed the invasion of HCT116 cells via inhibition of epithelial-mesenchymal transition (EMT) process. Upregulation of the

**Table 1.** Quantification of acetylshikonin in nine species of *Zicao*.

Plant ( family Boraginaceae)	Content (mg/g)	Source (provinces of China)	Reference
<i>Arnebia euchroma</i> (Royle) Johnst.	19.7 ± 0.66	Xinjiang	Hu et al. 2006
<i>Arnebia guttata</i> Bunge	1.36 ± 0.04	Xinjiang	Hu et al. 2006
<i>Lithospermum erythrorhizon</i> Sieb. et Zucc.	7.49 ± 0.11	Jilin	Hu et al. 2006
<i>Onosma paniculatum</i> Bur. et Franch.	2.86 ± 0.03	Yunnan	Hu et al. 2006
<i>Onosma waltonii</i> Duthic	2.60 ± 0.13	Tibet	Hu et al. 2006
<i>Onosma hookerii</i> Clarke	0.747 ± 0.01	Tibet	Hu et al. 2006
<i>Onosma exsertum</i> Hemsl.	0.485 ± 0.01	Yunnan	Hu et al. 2006
<i>Onosma hookerii</i> Clarke var. <i>longiflorum</i> Duthie	0.248 ± 0.01	Tibet	Hu et al. 2006
<i>Onosma confertum</i> W.W. Smith	0.123 ± 0.002	Yunnan	Hu et al. 2006

**Figure 1.** Chemical structure of acetylshikonin [(+)-acetic acid 1-(5,8dihydroxy-1,4-dioxo-1,4-dihydro-naphthalen-2-yl)-4-methyl-pent-3-enyl ester].

expression of E-cadherin mRNA and downregulation of N-cadherin, vimentin mRNA, and fibronectin were stated to be involved (Xue and Li 2017). Acetylshikonin/ $\beta$ -cyclodextrin inclusion complex decreased Bcl-2/Bax ratio and activated caspase-3, thereby inducing apoptosis in HCT116 cells. Moreover, this inclusion complex exerted long-term cytotoxicity via suppression of autophagy and excess accumulation of intracellular reactive oxygen species (ROS) (Vukic et al. 2020). Acetylshikonin suppressed the proliferation of colon cancers (HT29, DLD-1, and Caco-2 cells) in a dose-dependent manner. The inhibitory effects were related to induction of cell cycle arrest at G0/G1 phase and early apoptosis via inhibition of PI3K/Akt/mTOR pathway (Zhu et al. 2018). Besides, acetylshikonin downregulated the expressions of matrix metalloproteinase (MMP)-2 and MMP-9 to inhibit the invasion of HT29 cells, and suppressed the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway to inhibit its proliferation (Zhao and Yang 2019).

### Liver cancer

Zhao et al. (2008) reported that acetylshikonin injection showed inhibitory effect on H22 hepatocarcinoma cells, with little adverse effects on immune organs such as thymus and spleen. Co-treatment with acetylshikonin and  $\beta,\beta$ -dimethylacrylshikonin concentration- and time-dependently suppressed the growth of SMMC-7721 cells through induction of apoptosis and cell cycle arrest at G2/M phase (Wu et al. 2011). In another study, acetylshikonin was shown to suppress the proliferation of hepatitis B virus X protein (HBX)-expressing Hep3B cells by inducing apoptosis. The apoptotic mechanisms were associated with upregulation of orphan nuclear receptor 77 (Nur77), activation of Jun-N-terminal kinase (JNK) and endoplasmic reticulum stress (Moon et al. 2014). Park et al. (2017) indicated that acetylshikonin-induced apoptotic cell death of HepG2 cells was mediated via downregulation of Bcl-2 and upregulation of p53, Bax, and caspase-3. In addition, co-treatment with acetylshikonin and tumour necrosis factor-related apoptosis-inducing ligand increased intracellular ROS production, thereby inducing DNA damage and

activating p53/PUMA/Bax signalling. These resulted in activation of caspases and sensitisation of the HepG2 cells to apoptosis (Hong et al. 2019).

### Lung cancer

Previous studies demonstrated that the inhibition of acetylshikonin on LLC cells was involved in induction of apoptosis via release of cytochrome c and activation of caspase-3 (Miao et al. 2007; Xiong et al. 2009). Furthermore, acetylshikonin inhibited the metastasis and angiogenesis of LLC cells via suppression of vascular endothelial growth factor (VEGF) production and downregulation of urokinase-type plasminogen activator (uPA) (Lee et al. 2008). In NIH-H460 cells, acetylshikonin activated Nur77 pathway through regulation of Nur77 expression, nuclear export, and mitochondrial targeting to induce apoptosis (Liu et al. 2008). To enhance the antiproliferative property, acetylshikonin was incorporated into a solid-lipid nanoparticle. This compound showed a higher anticancer potential against A549 cells than intact acetylshikonin in terms of DNA damage and IC<sub>50</sub> (Eskandani and Nazemiyeh 2014).

### Melanoma

Chen et al. (2016) reported that co-treatment with acetylshikonin and pterostilbene showed marked synergistic effect on the proliferation of B16F10 cells. The anticancer activity was attributed to induction of apoptosis and cell cycle arrest via activation of p53 pathway. Jiang et al. (2019) demonstrated that acetylshikonin acted on A375 cells and induced apoptosis. The suppressions of migration and invasion of A375 cells also occurred through reversal of EMT process via PI3K/Akt/mTOR pathway. In addition, cyclopropylacetylshikonin, a structural analogue of acetylshikonin, suppressed the proliferation of melanoma cell lines (WM164 and MUG-Mel2). The mechanisms were associated with inducing caspase-dependent apoptosis, leading to double-stranded DNA breaks, and increasing the protein levels of cleaved Poly ADP-ribose polymerase (Durchschein et al. 2018).

### Leukaemia

Studies confirmed that acetylshikonin produced antiproliferative effect on HL-60 cells by suppressing the extension of telomeres (Lu et al. 2002). Co-treatment with acetylshikonin and  $\beta,\beta$ -dimethylacrylshikonin loaded with jacalin-capped silver nanoparticles resulted in inhibitory effect on K562 cells. The mechanisms were related to excess production of ROS, upregulation of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), loss of mitochondrial membrane potential, and DNA damage (Ahmed et al. 2016). Furthermore, acetylshikonin activated the mitochondria-regulated intrinsic apoptotic pathway through regulation of ROS accumulation, inhibition of the NF- $\kappa$ B pathway and Bcr-Abl fusion protein expression in K562 cells (Hao et al. 2020).

### Oral cancer

It is known that oral squamous cell carcinoma (OSCC) is one of the most common malignant neoplasms in the oral cavity (Greenlee et al. 2001). Acetylshikonin-induced apoptotic cell death of OSCC was mediated through G2 cell cycle arrest and excess ROS via JNK and p38 mitogen-activated protein kinase (MAPK) pathway (Kim et al. 2016). Treatment with acetylshikonin suppressed the invasion of *Porphyromonas gingivalis*-infected YD10B cells by inhibition of interleukin (IL)-8 release and IL-8-dependent MMP release (Cho et al. 2018). In another study, the antiproliferative effect of acetylshikonin against cisplatin-resistant KB-R5 cells could be due to induction of apoptosis and activation of the mTOR/PI3K/Akt pathway (Wang et al. 2019).

### Other cancers

It was shown that acetylshikonin inhibited L-1 sarcoma-induced cutaneous angiogenesis in Balb/c mice (Pietrosiuk et al. 2004a). Treatment with acetylshikonin injection could lead to the inhibition of S180 sarcoma by inducing cell necrosis, while there were no abnormal changes in other organs of the mice (Zeng et al. 2008). Zeng et al. (2009) demonstrated that the suppression of acetylshikonin on SGC-7901 cells was associated with inducing apoptosis through upregulation of Bax and downregulation of Bcl-2. Acetylshikonin suppressed the proliferation of MDA-MB-231 cells through cell cycle arrest at G0/G1 phases, autophagy inhibition, and accumulation of intracellular ROS (Vukic et al. 2020). NF- $\kappa$ B-dependent productions of cytokines and MMP-9 were found to be decreased after acetylshikonin treatment, which led to the inhibition of the proliferation and invasion of PANC-1 cells (Cho and Choi 2015). In another study, acetylshikonin induced cell cycle arrest at S phase and apoptosis by activation of caspase-3 and caspase-8 in Siha cells (Sun et al. 2014). He et al. (2019) reported that acetylshikonin-induced suppression of SKOV3 cells was associated with apoptosis, followed by cell cycle arrest at G0/G1 phase. Acetylshikonin and other shikonin analogues could bypass cancer drug resistance mediated by P-gp, Bcl-2, Bcl-xL, MRP1, and BCRP1, through induction of necroptosis (Xuan and Hu 2009). A study was conducted on acetylshikonin-induced broad blocking of multidrug resistance (MDR) efflux pumps in parental and their drug resistant cell lines (EPG85.257, EPG85.257RDB, MCF7, MCF7MX, A2780, A2780RCIS). The results revealed that acetylshikonin increased drug accumulation inside these cells via reduction of the pump activities of BCRP, MDR1, and MRP2 (Mirzaei et al. 2018).

In general, acetylshikonin has a significant inhibitory effect on a variety of cancers, and regulates genes and proteins involved in cell apoptosis, metastasis, cell invasion, DNA damage, and cell cycle arrest. The synergistic anticancer effect of acetylshikonin in combination with other drugs has attracted attention as a treatment avenue.

### Anti-inflammatory effect of acetylshikonin

It was reported that acetylshikonin possessed inhibitory effects on local vascular hyperpermeability and rat foot edoema, but this had no association with corticotropin-like effect (Hayashi 1977; Lin et al. 1980). Wang et al. (1994) found that acetylshikonin suppressed the biosynthesis of leukotriene B<sub>4</sub> in leukocytes. In addition, acetylshikonin displayed anti-edematous effects via inhibition of preformed chemical mediators release and protection of the vasculature from mediator challenge (Wang et al. 1995). Inositol phosphate formation and phospholipase C (PLC)

activity were suppressed by acetylshikonin in crude cytosolic preparations. Moreover, acetylshikonin blocked Ca<sup>2+</sup> release from internal neutrophils, suggesting that the inhibition of phosphatidylinositol signalling may be involved (Wang and Kuo 1997). Acetylshikonin attenuated the carrageenan-induced acute inflammation, and relieved paw edoema by inhibiting the activation of TNF- $\alpha$  promoter (Kundakovic et al. 2006). Cheng et al. (2008) suggested an association between the anti-inflammatory properties of acetylshikonin and suppression of nitric oxide (NO), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and inducible NO synthase (iNOS) protein via NF- $\kappa$ B pathway in RAW 264.7 cells. Further study showed that acetylshikonin inhibited TNF- $\alpha$  and IL-1 in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells (Wei et al. 2011). In rat neutrophils, acetylshikonin inhibited the production of thromboxane B<sub>2</sub> and leukotriene B<sub>4</sub> through reduction in [Ca<sup>2+</sup>]<sub>i</sub>, which in turn attenuated membrane-associated cytosolic phospholipase A<sub>2</sub>, and blocked cyclooxygenase and 5-lipoxygenase activity (Hsu et al. 2009).

Acetylshikonin produced a strong chondro-protective effect in OA rats, probably through mechanisms related to anti-inflammation and suppression of MMPs production (Kim et al. 2013). Acetylshikonin also downregulated the expression of NO and PGE<sub>2</sub> by inhibiting ROS/PI3K/Akt-dependent NF- $\kappa$ B activation, and mediating haem oxygenase-1 (HO-1) expression in LPS-stimulated BV2 microglial cells (Jayasooriya et al. 2015). Macrophages play an important part in inflammatory responses. Acetylshikonin showed cytotoxic effect on LPS-stimulated macrophages through caspase-dependent apoptotic cell death pathway (Koike et al. 2016). Zorman et al. (2016) demonstrated that acetylshikonin suppressed IL-1 $\beta$  release and the second step of Nod-like receptor protein 3 (NLRP3) inflammasome activation in C57BL/6 mice. In another study, acetylshikonin suppressed atherogenesis, vascular inflammation, and blood lipid levels via suppression of NF- $\kappa$ B pathway in apolipoprotein E-deficient mice (Cui et al. 2018). Acetylshikonin had the potential to ameliorate non-alcoholic steatohepatitis by inhibiting hepatic levels of IL-1 $\beta$  and TNF- $\alpha$ , and downregulating the expressions of fibronectin and fibrotic markers ( $\alpha$ -SMA, collagen I, and collagen III). Hepatocyte autophagy was also induced via AMPK/mTOR pathway (Zeng et al. 2018). Acetylshikonin attenuated cigarette smoke-induced pathological changes and lung tissue damage, and downregulated expressions of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and monocyte chemoattractant protein-1 (MCP-1) in mice. These effects were produced via upregulation of nuclear factor-erythroid 2-related factor 2 (Nrf2) and Nur77-mediated dyclooxygenase-2 (COX-2) (Zhang et al. 2019). In addition, Fan et al. (2019) observed that ovalbumin-induced allergic rhinitis was ameliorated by acetylshikonin due to its inhibitory effects on the production of IgE, IgG1, IL-4, IL-5, and IL-13.

In summary, the anti-inflammatory effect of acetylshikonin is associated with upregulation of HO-1 and Nrf2, and downregulation of TNF- $\alpha$ , leukotriene B<sub>4</sub>, NO, PGE<sub>2</sub>, iNOS, IL-1, IL-4, IL-5, IL-6, IL-13, IL-1 $\beta$ , MMPs, and MCP-1. In addition, acetylshikonin is shown to regulate NF- $\kappa$ B and Nur77/COX-2 pathway.

### Lipid-lowering effect of acetylshikonin

Acyl-CoA: cholesterol acyltransferase (ACAT) is responsible for the intracellular esterification of free cholesterol with fatty acyl-CoA to produce cholesterol esters (Chang et al. 2006). An et al. (2007) demonstrated that acetylshikonin was found to weakly suppress human ACAT-1 and ACAT-2, but its analogue propionylshikonin exerted strong inhibitory effect on ACAT, indicating that the inhibitory potency was associated with the length of the acyl group. In 3T3-L1 cells, acetylshikonin suppressed



adipocyte differentiation and the expression of adipogenic transcription factors (PPAR $\gamma$  and C/EBP $\alpha$ ) (Gwon et al. 2012). Furthermore, treatment of high fat diet (HFD)-induced obese rats with acetylshikonin led to reductions in body weight, white adipose tissue content, serum triglycerides, and free fatty acid levels. The lipid-regulatory effect was attributed to triacylglycerol hydrolysis via regulation of protein kinase A (PKA) signalling in 3T3-L1 cells (Su et al. 2016a). Acetylshikonin had an excellent therapeutic effect on obesity and non-alcoholic fatty liver disease in spontaneous obese db/db mice. The mechanisms were involved in marked suppression of alanine aminotransferase, aspartate aminotransferase, and pro-inflammatory cytokines (TNF- $\alpha$ , IL-6 and IL-1 $\beta$ ), as well as downregulation of sterol regulatory element-binding protein-1 (SREBP-1), fatty acid synthetase (FAS), and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) (Su et al. 2016b).

These studies indicate that acetylshikonin may exhibit beneficial effects on obesity diseases, and the mechanisms involved in the suppression of obesity are associated with regulation of related factors including ACAT, PPAR $\gamma$ , C/EBP $\alpha$ , PKA, SREBP-1, FAS, and HMGCR.

### Antidiabetic effect of acetylshikonin

Protein-tyrosine phosphatase 1B (PTP1B) is an effective target for the treatment of type 2 diabetes (Goldstein 2001). Wang et al. (2016) reported that acetylshikonin and other shikonin derivatives showed significant inhibitory effects on PTP1B due to 2-substituted long lipo-chain of naphthoquinone. Acetylshikonin treatment could ameliorate diabetic nephropathy (DN) in mice through attenuation of renal inflammation, decrease in kidney fibrosis, and inhibition of the activation of TGF- $\beta$ 1/Smad pathway (Li et al. 2018). In another study, acetylshikonin enhanced glucose transporter 4 translocation and induced glucose uptake in L6 myotubes via the PLC- $\beta$ 3/PKC $\delta$  pathway to reduce blood glucose (Huang et al. 2019). Free fatty acid receptor (FFA) 4 is a member of the G-protein coupled receptors, which may play a role in diabetes treatment and other diseases (Milligan et al. 2017). Acetylshikonin induced dynamic mass redistribution (DMR) response in CHO-FFA1 and CHO-FFA4 cells. It was noteworthy that the agonist response of acetylshikonin was comparable to that of  $\alpha$ -linolenic acid, indicating that acetylshikonin could be a novel FFA4 agonist (Xu et al. 2019).

Summarising the beneficial effect of acetylshikonin on diabetes, these reports prove the role of acetylshikonin in the amelioration of DN via TGF- $\beta$ 1/Smad pathway and the regulation of blood glucose via PLC- $\beta$ 3/PKC $\delta$  pathways.

### Antioxidant properties of acetylshikonin

Acetylshikonin reduced superoxide anion generation in guinea pig polymorphonuclear leukocytes by suppressing the production of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, but it had no inhibitory effect on already generated NADPH oxidase (Kawakami et al. 1996). Weng et al. (2000) and Jiang et al. (2002) reported that acetylshikonin exerted antioxidant property in lard containing Fe $^{3+}$ , and showed synergistic effects with D,L- $\alpha$ -tocopherol. Another study proved that acetylshikonin suppressed Cu $^{2+}$ -induced oxidation of human low-density lipoprotein (Kim et al. 2009). Skrzypczak et al. (2015) found that acetylshikonin exhibited DPPH radical-scavenging property by using electron paramagnetic resonance spectroscopy.

In general, acetylshikonin plays a potential role in antioxidation by inhibiting the production of NADPH oxidase complex and DPPH radical. Further research is still needed to investigate other antioxidative properties of acetylshikonin and clarify its mechanisms.

### Antibacterial and antifungal effects of acetylshikonin

Studies demonstrated that acetylshikonin exhibited a wide antifungal spectrum against *Cladosporium herbarum*, *Candida albicans*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Trichosporon cutaneum*, and *Aspergillus fumigatus* (Li et al. 1998; Sasaki et al. 2000, 2002). Acetylshikonin not only showed inhibitory effects on some Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus megaterium*, *Microbacterium arborescens*, and *Micrococcus luteus*, but also suppressed the proliferation of some Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Hafnia alvei*, *Yersinia intermedia*, and *Citrobacter koseri* (Damianakos et al. 2012; Vukic et al. 2017). Moreover, acetylshikonin produced antibacterial effects against different species of oral bacteria such as *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Streptococcus mutans*, and the minimum inhibitory concentration (MIC) values were 0.08, 0.04, and 0.08 mg/mL, respectively (Li et al. 2012). Further investigations revealed that acetylshikonin suppressed the activities of trypsin-like protease and glycosidase enzyme of *Porphyromonas gingivalis* (Li et al. 2014).

Although acetylshikonin exhibits extensive spectrum of antibacterial and antifungal effects, there are currently very few studies on the mechanisms underlying these effects. Therefore, the mechanisms involved in the antibacterial and antifungal effects of acetylshikonin require further investigation.

### Neuroprotective effect of acetylshikonin

The flavin-containing enzymes monoamine oxidase (MAO)-A and MAO-B are involved in the treatment of Alzheimer's disease (Wu et al. 2007; Schedin-Weiss et al. 2017). Choi et al. (2005) demonstrated that acetylshikonin inhibited mouse brain MAO-A and MAO-B activity in a competitive manner assayed by a modification of Kraml's fluorometric method. Acetylshikonin could be used as a novel acetylcholinesterase inhibitor to suppress the loss of mitochondria membrane potential and the generation of ROS, thereby possessing anti-apoptotic effect on neuronal SH-SY5Y and PC12 cells. Besides, acetylshikonin-induced increase in HO-1 also played an important role in mediating anti-apoptosis in SH-SY5Y cells under oxidative stress (Wang et al. 2013). Acetylshikonin attenuated D-galactose-induced cognitive impairment and hippocampal senescence in mice, through reduction in oxidative stress and neuroinflammation, and inhibition of the activation of SIRT1/p53/p21 pathway (Li et al. 2018).

Acetylshikonin possesses powerful anti-apoptotic properties in the neuronal cells and attenuates brain aging in mice. Its neuroprotective effect was due to upregulation of HO-1, attenuation of oxidative stress and regulation of SIRT1/p53/p21 pathway.

### Other properties of acetylshikonin

Chang et al. (1993) demonstrated that acetylshikonin suppressed platelet aggregation induced by platelet-activating factor, collagen, arachidonic acid, or thrombin in washed rabbit platelets. Another study proved that acetylshikonin inhibited the generation of [ $^3$ H]inositol monophosphate and blocked increase in intracellular [ $Ca^{2+}$ ] via suppression of phosphoinositide

breakdown, thereby inhibiting platelet activation (Ko et al. 1995). Acetylshikonin attenuated protein tyrosine phosphorylation, and influenced the assembly of a functional NADPH oxidase complex via suppression of respiratory burst in neutrophils (Wang et al. 1997). Pietrosiuk et al. (2004b) reported that splenocytes treated with acetylshikonin had high proliferative potential *in vivo* and active migratory potential *in vitro*. In addition, acetylshikonin promoted the proliferation of pellet-induced granulation tissue, and enhanced re-epithelialization and angiogenesis in wounded skin (Ozaki et al. 1994; Lu et al. 2008). Acetylshikonin suppressed acetylcholine and histamine-induced relaxation in PE pre-contracted aorta, suggesting that acetylshikonin might be an inhibitor of NO synthesis in endothelium (Hu et al. 2004). Liao et al. (2019) demonstrated that acetylshikonin exerted anti-replicative effect on HCV virus through autophagy pathway by down-regulation of autophagy marker LC3. Acetylshikonin protected cells from coxsackievirus A16 (CVA16) via inactivation of viral particles and blockage of cellular entry. It also reduced CVA16-induced diseases in neonatal mouse model, decreased viral loads in internal organs, and alleviated inflammation via downregulation of interferon- $\gamma$  and IL-6 (Liu et al. 2019). Shon et al. (2017) reported that acetylshikonin could act as a novel non-selective cytochrome P450 inhibitor to produce significant inhibitory effects on nine cytochrome P450 isoforms (i.e., CYP 1A2, CYP 2A6, CYP 2B6, CYP 2C8, CYP 2C9, CYP 2C19, CYP 2D6, CYP 2E1, and CYP 3A). In another study, the proliferation and migration of angiotensin II-induced cerebrovascular smooth muscle cells were inhibited by acetylshikonin through suppression of Wnt/ $\beta$ -catenin pathway (Li et al. 2018).

In general, acetylshikonin plays a potential role in anticoagulation through inhibition of phosphoinositide breakdown, and shows antiviral activity via autophagy pathway and inactivation of viral particles. Acetylshikonin also possesses the properties of wound healing and immunoregulation, but further studies are needed to make clear of its mechanisms of action.

### Toxicity of acetylshikonin

Acetylshikonin exerted toxic effects on DNA by binding to the minor groove of DNA and slightly intercalating into the DNA base pairs by using the fluorescence spectrometry with two kinds of probes and resonance light scattering spectroscopy (Li et al. 2015). In another study, acetylshikonin increased methanesulfonate-induced genotoxicity, and had cytotoxic effect on a normal cell line (V79 cells;  $EC_{50} = 0.49$  mg/L), based on LDH assay (Figat et al. 2018). In acetylshikonin-treated Sprague-Dawley rats, reproductive toxicity effect was observed due to decrease in the populations of developing and mature follicles. The mechanisms involved in the reproductive toxicity effect were related to down-regulation of follicle stimulating hormone and luteinizing hormone through inhibition of the secretion of gonadotropic hormone (GTH) (He et al. 2016).

Based on these data, acetylshikonin exhibits genotoxicity by binding to the minor groove of DNA, and induces reproductive toxicity through affecting the secretion of GTH. However, since the risk of human exposure to acetylshikonin remains unclear, more investigations are needed to evaluate its toxicity.

### Pharmacokinetics of acetylshikonin

Lin et al. (1981) reported that radioactivity could be detected in the mice plasma after oral administration of [ $^3$ H]-acetylshikonin for 15 min, and the radioactive intensity reached a peak after 1 h.

Furthermore, [ $^3$ H]-acetylshikonin was mainly distributed in the stomach and intestine, secondarily in the gallbladder, liver, kidneys, lungs, and least in the brain and spinal cord. The cumulative excretion rate of faeces and urine was nearly 80% within 48 h, mainly through faecal excretion. These results indicated that acetylshikonin had a wide distribution and poor absorption in mice. The binding rate of acetylshikonin to human plasma proteins was high, and no prototype of it could be detected in mice blood, which blocked its further content determination *in vivo* (Sun et al. 2008a). To solve this problem, an LC-ESI-MS/MS method after precolumn derivatization with 2-mercaptoethanol was developed for determining the pharmacokinetics of acetylshikonin after oral administration to macaque monkeys at the dose of 0.40 mg/kg. The pharmacokinetic parameters obtained from the non-compartmental analysis showed that the terminal elimination half-life ( $t_{1/2}$ ), the mean residual time (MRT), and the area under the concentration-time curve to the last measurable concentration ( $AUC_{0-t}$ ) were  $12.3 \pm 1.6$  h,  $10.2 \pm 0.7$  h, and  $615.4 \pm 206.5$  ng/mL/h, respectively (Sun et al. 2008b).

In general, acetylshikonin has a wide distribution, poor absorption, and high binding rate to human plasma proteins. More attention should be paid to possible competition for binding to plasma proteins between acetylshikonin and any other conjunctive chemical agent. There is need to study the mechanisms involved in these interactions. In fact, previous researches on the pharmacokinetics of acetylshikonin are far from enough to make clear of its metabolic mechanisms. Thus, further studies are needed to clarify its metabolic pathway and investigate acetylshikonin metabolites in different kinds of experimental models.

### Conclusion and future perspectives

Acetylshikonin is a naphthoquinone derivative with broad pharmacological benefits, including anticancer, anti-inflammatory, lipid-regulatory, antidiabetic, antibacterial, antifungal, antioxidant, neuroprotective, and antiviral properties (Table 2). The various pharmacological effects of acetylshikonin are strongly associated with regulation of the expression of proteins and genes such as upregulation of Bax, Bak, Nur77, caspase-3, caspase-8, p53, p21, HO-1, as well as COX-2; and downregulation of Bcl-2, IL-10, IL-6, IL-1 $\beta$ , MCP-1, IgG1, uPA, MMP, and interferon- $\gamma$ . Moreover, acetylshikonin was shown to regulate the PI3K/Akt/mTOR, NF- $\kappa$ B, MAPK, AMPK/mTOR, PLC- $\beta$ 3/PKC $\delta$ , Wnt/ $\beta$ -catenin, and SIRT1/p53/p21 pathways (Figure 2).

Available data indicates that combination treatment with acetylshikonin and other chemotherapeutic agents has attracted attention because of its good pharmacological effect in cancer treatment. However, there is need to determine the effect of acetylshikonin on the metabolism of other drugs. This is important for the safe use of drugs. Besides, some analogues of acetylshikonin also show effective, or even more effective inhibitory effects against a variety of cancers, than acetylshikonin. This provides new opportunities for future research.

However, the toxicity of acetylshikonin should not be neglected. Previous studies have reported the adverse effects of acetylshikonin, with respect to reproductive toxicity and genotoxicity. In particular, the reproductive toxicity of acetylshikonin should be paid more attention to, especially when it is used in the treatment of diseases in women of childbearing age and pregnant women. Overall, studies on the toxicity of acetylshikonin are far from sufficient. Thus, further studies are needed before acetylshikonin can be safely used for therapeutic purposes.

**Table 2.** Pharmacology of acetylshikonin.

Pharmacological effect	Cell lines/model	Activity/Mechanism(s) of action	Application	Reference
Anticancer activity	HCT116 cells	Inhibition of EMT and autophagy; Upregulation of ROS; Induction of apoptosis and necrosis	<i>In vitro</i>	Xue & Li 2017; Vukic et al. 2017; Vukic et al. 2020
	HT29 cells	Suppression of invasion; Regulation of NF- $\kappa$ B and PI3K/Akt/mTOR pathway	<i>In vitro</i>	Zhu et al. 2018; Zhao and Yang 2019
	SMMC-7721 cells	Induction of apoptosis; Cell cycle arrested at G2/M phase	<i>In vitro</i>	Wu et al. 2011
	Hep3B cells	Upregulation and export of Nur77; Regulation of JNK pathway; Induction of ER stress	<i>In vitro</i>	Moon et al. 2014
	HepG2 cells	Induction of apoptosis; Regulation of p53/PUMA/Bax pathway; Upregulation of ROS	<i>In vitro</i>	Park et al. 2017; Hong et al. 2019
	LLC cells	Downregulation of VEGF and uPA; Induction of apoptosis	<i>In vivo</i> and <i>in vitro</i>	Lee et al. 2008; Xiong et al. 2009
	NIH-H460 cells	Regulation of Nur77 pathway	<i>In vitro</i>	Liu et al. 2008
	A549 cells	DNA fragmentation	<i>In vitro</i>	Eskandani and Nazemiyeh 2014
	B16F10 cells	Regulation of p53 pathway	<i>In vitro</i>	Chen et al. 2016
	A375 cells	Inhibition of EMT process; Regulation of PI3K/Akt/mTOR pathway	<i>In vitro</i>	Jiang et al. 2019
	HL-60 cells	Inhibition of the extension of telomeres	<i>In vitro</i>	Lu et al. 2002
	K562 cells	Induction of apoptosis; Upregulation of ROS and TNF- $\alpha$ ; DAN damage; Regulation of NF- $\kappa$ B pathway	<i>In vitro</i>	Ahmed et al. 2016; Hao et al. 2020
	OSC cells	Regulation of JNK and p38 MAPK pathway	<i>in vitro</i>	Kim et al. 2016
	YD10B cells	Downregulation of IL-8 and MMP	<i>in vitro</i>	Cho et al. 2018
	KB-R5 cells	Regulation of m-TOR/PI3K/Akt pathway	<i>In vitro</i>	Wang et al. 2019
	L-1 sarcoma cells	Inhibition of cutaneous angiogenesis	<i>In vivo</i>	Pietrosiuk et al. 2004a
	S180 sarcoma	Induction of necrosis	<i>In vivo</i>	Zeng et al. 2008
	SGC-7901 cells	Induction of apoptosis	<i>In vitro</i>	Zeng et al. 2009
	MDA-MB-231 cells	Cell cycle arrest at G0/G1; Inhibition of autophagy; Upregulation of ROS	<i>In vitro</i>	Vukic et al. 2020
	Anti-inflammatory activity	PANC-1 cells	Regulation of NF- $\kappa$ B pathway	<i>In vitro</i>
Siha cells		Activation of caspase-3 and caspase-8	<i>In vitro</i>	Sun et al. 2014
SKOV3 cells		Induction of apoptosis	<i>In vitro</i>	He et al. 2019
EPG85.257RDB, MCF7MX, A2780RCIS cells		Reduction of BCRP, MDR1, and MRP2 pump activity	<i>In vitro</i>	Mirzaei et al. 2018
Leukocyte cells		Inhibition of leukotriene B <sub>4</sub>	<i>In vitro</i>	Wang et al. 1994
Carrageenan-induced paw edoema		Inhibition of TNF- $\alpha$ activation	<i>In vivo</i>	Kundakovic et al. 2006
RAW 264.7 cells		Downregulation of NF- $\kappa$ B activation; Inhibition of TNF- $\alpha$ , IL-1, and NO	<i>In vitro</i>	Cheng et al. 2008; Wei et al. 2011
Rat neutrophils		Suppression of phosphatidylinositol signalling; Inhibition of cyclooxygenase and 5-lipoxygenase activity	<i>In vitro</i>	Wang and Kuo 1997; Hsu et al. 2009
BV2 microglial cells		Regulation of NF- $\kappa$ B pathway; Upregulation of HO-1	<i>In vitro</i>	Jayasooriya et al. 2015
Macrophages		Induction of apoptosis	<i>In vitro</i>	Koike et al. 2016
C57BL/6 mice		Suppression of IL-1 $\beta$ release and NLRP3 activation	<i>In vitro</i>	Zorman et al. 2016
Apolipoprotein E deficient mice		Regulation of NF- $\kappa$ B pathway	<i>In vivo</i>	Cui et al. 2018
MCD diet-induced mice		Regulation of AMPK/mTOR pathway	<i>In vivo</i>	Zeng et al. 2018
Smoke-induced mice	Downregulation of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and MCP-1; Upregulation of COX-2 and Nrf2	<i>In vivo</i>	Zhang et al. 2019	
TH2 cells	Downregulation of IgE, IgG1, IL-4, IL-5, and IL-13	<i>In vivo</i>	Fan et al. 2019	
Lipid-regulatory activity	AC-29 cells	Inhibition of ACAT-1 and ACAT-2	<i>In vitro</i>	An et al. 2007
	3T3-L1 cells	Inhibition of adipocyte differentiation and adipogenic transcription factor; Regulation of PKA signalling	<i>In vitro</i>	Gwon et al. 2012; Su et al. 2016a
	Spontaneous obese db/db mice	regulation of lipid metabolism	<i>In vivo</i>	Su et al. 2016b
Antidiabetic activity	Diabetic mice	Regulation of TGF- $\beta$ 1/Smad pathway	<i>In vivo</i>	Li et al. 2018
	Alloxan-induced mice	Regulation of PLC- $\beta$ 3/PKC $\delta$ pathway	<i>In vivo</i>	Huang et al. 2019
	CHO cells	Induction of DMR response	<i>In vitro</i>	Xu et al. 2019
Antioxidative activity	leukocytes	Reduction of NADPH oxidase	<i>In vitro</i>	Kawakami et al. 1996
Neuroprotective effect	SH-SY5Y and PC12 cells	Suppression of loss of mitochondria membrane potential and the generation of ROS	<i>In vitro</i>	Wang et al. 2013
	D-galactose-induced mice	Regulation of SIRT1/p53/p21 pathway	<i>In vivo</i>	Li et al. 2018

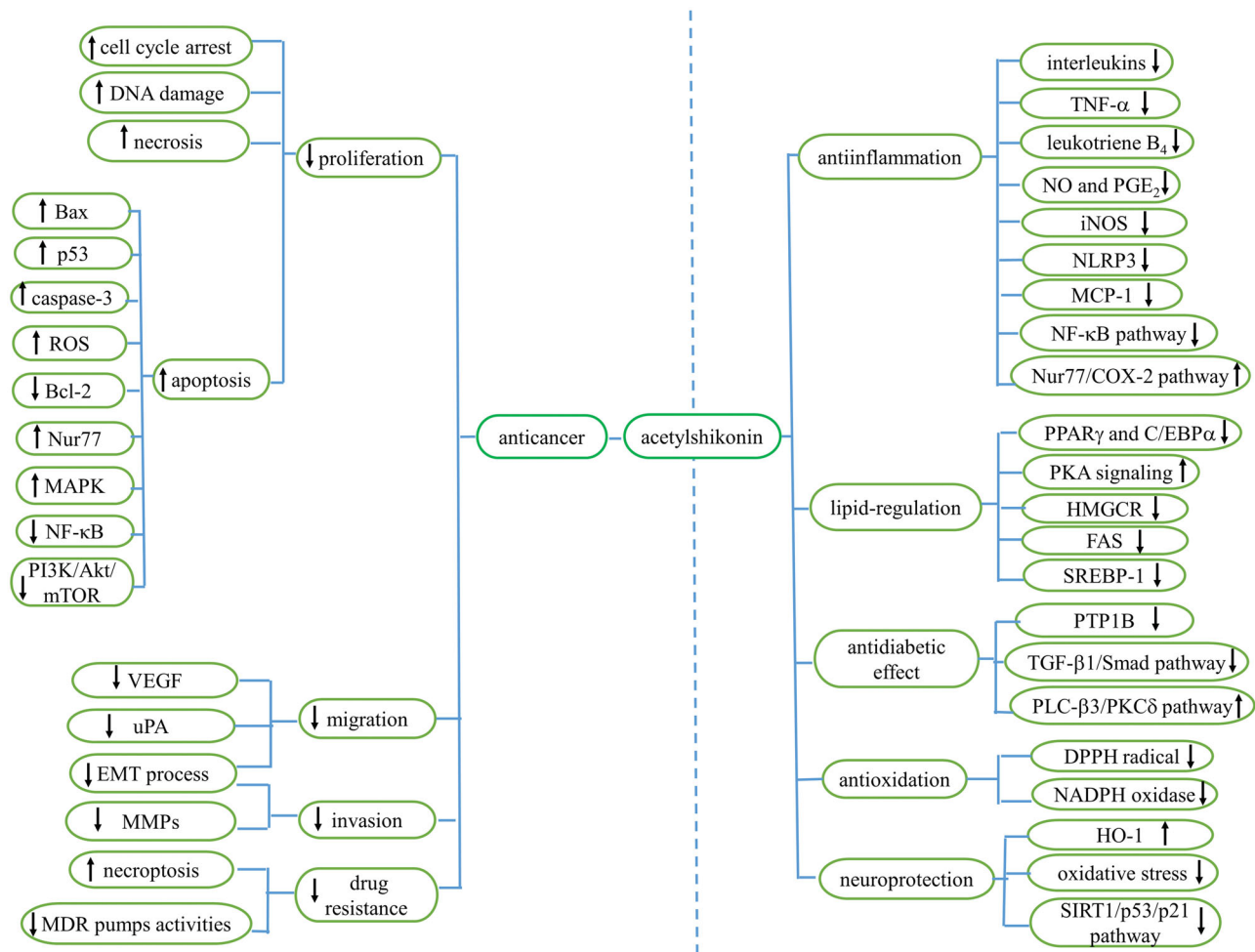


Figure 2. Pharmacological activities and the mechanisms of acetylshikonin.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

This work was financially supported by the Beijing Natural Science Foundation [7194289], the National Natural Science Foundation of China [81703715].

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