Special Section on Drug Metabolism in Liver Injury and Repair—Minireview

Withaferin A in the Treatment of Liver Diseases: Progress and Pharmacokinetic Insights

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ABSTRACT

Withaferin A (WA) is a natural steroidal compound used in Ayurvedic medicine in India and elsewhere. Although WA was used as an anticancer reagent for decades, its role in the treatment of liver diseases has only recently been experimentally explored. Here, the effects of WA in the treatment of liver injury, systematic inflammation, and liver cancer are reviewed, and the toxicity and metabolism of WA as well as pharmacological potentials of other extracts from Withania somnifera (W. somnifera) discussed. The pharmacokinetic behaviors of WA are summarized and pharmacokinetic insights into current progress and future opportunities are highlighted.

SIGNIFICANCE STATEMENT

This review outlines the current experimental progress of Withaferin A (WA) hepatoprotective activities and highlights gaps in the field. This work also discusses the pharmacokinetics of WA that can be used to guide future studies for the possible treatment of liver diseases with this compound.

Traditional herbs have been key sources for the development of hepatoprotective drugs for decades (Harvey et al., 2015; Yan et al., 2016; Yan et al., 2018; Yan et al., 2020). Impressive examples are silymarin for antifibrotic treatment (Gillesen and Schmidt, 2020), licorice-derived glycyrrhizin to treat viral hepatitis (Li et al., 2019), and berberine to treat nonalcoholic fatty liver diseases (NAFLD) (Sun et al., 2017; Sun et al., 2021). Some traditional herbs as drugs in the clinic or as the dietary supplement in the market still lack fully developed experimental evidence. As a result, the concepts of “reverse pharmacology” (Surh, 2011) and “reverse pharmacokinetics” (Hao et al., 2014) were proposed to advance the preclinical studies for traditional herbs. When meeting the increasing pursuit of precision medicine in modern times, globalization of traditional herbs is still facing great challenges, and extensive studies to evaluate the pharmacological effects of traditional herbs in combination with pharmacokinetic studies are attractive and indispensable for drug discovery.

Withania Somnifera (Ashwagandha, W. somnifera), belonging to the family Solanaceae, is a traditional herbal plant used as medicine or dietary supplement for decreasing inflammation; increasing energy; improving cognitive health, anxiety, and depression; as well as improving the homeostasis of glucose and cortisol in India. It has been used from the time of Ayurvedic and Unani systems of medicine at least since the 8th century (Mirtjali et al., 2009). Traditionally, the berries and leaves of W. somnifera were used as a local treatment of ulcers and tumors (Vand Berghe et al., 2012). In addition, several reports have linked the health benefits of W. somnifera to its anti-diabetic, anti-epileptic, anti-inflammatory, antidepressant, and antiarthritic activities (Dutta et al., 2019; Huang et al., 2020). The clinical trials registered at clinicaltrials.gov using W.

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ABBREVIATIONS: APAP, acetaminophen; BCL-2, B-cell lymphoma-2; CCl4, carbon tetrachloride; CYP, cytochrome P450; DR5, death receptor protein-5; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; GaIN, D-galactosamine; HCC, hepatocellular carcinoma; HFD, high-fat diet; HFHC, high-fat and high-cholesterol; iNOS, inhibitor of nuclear factor xB; IL1β, interleukin 1β; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NAPQI, N-acetyl-p-benzoquinone; NASH, nonalcoholic steatohepatitis; NFkb, nuclear factor xB; NLRP3, NLR family, pyrin domain containing 3; NRF2, NF-E2-related factor-2; SIRT3, sirtuin 3; TNFα, tumor necrosis factor α; WA, withaferin A; W. somnifera, Withania somnifera.
somnifera extract, also known as “ashwagandha” or “sensoril” as a dietary supplement or drug, are summarized in Table 1. Most of the pharmacological activities of *W. somnifera* have been attributed to the steroidal lactones and alkaloids called “withanolides,” among which the most important component is withaferin A (WA) (Wu et al., 2018; Huang et al., 2020).

WA was used as an anticancer reagent, and earlier reviews have documented the effects of WA in treating cancer (Vanden Berghe et al., 2012; Hassannia et al., 2020). Beyond its potential anticancer activities, the effects of WA in the treatment of liver diseases have only recently been studied. This review summarizes the main components in *W. somnifera*, the hepatoprotective effects and mechanisms of *W. somnifera* extracts, and WA for the treatment of various liver diseases, including acute liver injury, chronic liver injury, inflammation, and liver cancer.

**Natural Products in *W. Somnifera***

More than 50 chemical constituents are contained in different parts of the root, leaf, fruit, and seed of *W. somnifera* (Kulkarni and Dhir, 2008). These chemical constituents include alkaloids, steroidal lactones, saponins with an additional acyl group, and withanolides that contain a glucose at carbon 27. Alkaloids and steroidal lactones were reported to be the main constituents of *W. somnifera* (Saleem et al., 2020). Although various alkaloids were found to be enriched in the root, withanine is the main constituent, with the other alkaloids, such as somniferine, somnine, somniferinine, and withananine, also detected. The leaves of *W. somnifera* consist primarily of steroidal lactones, which are commonly called “withanolide,” and are believed to account for its extraordinary medicinal properties (Sun et al., 2016). WA and withanolide D were shown to have most of the pharmacological activity of *W. somnifera* extract, also known as “ashwagandha” or “sensoril” as a dietary supplement or drug, are summarized in Table 1. Most of the pharmacological activities of *W. somnifera* have been attributed to the steroidal lactones and alkaloids called “withanolides,” among which the most important component is withaferin A (WA) (Wu et al., 2018; Huang et al., 2020).

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**Table 1.** Clinical trials for Withania somnifera registered in clinicaltrials.gov

<table>
<thead>
<tr>
<th>Drugs/Dietary Supplements</th>
<th>Study Title</th>
<th>Status</th>
<th>Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug: WSE&lt;sup&gt;a&lt;/sup&gt; Drug: placebo oral tablet</td>
<td>Adjunctive Withania somnifera (ashwagandha) for persistent symptoms in people with schizophrenia</td>
<td>Recruiting</td>
<td>NCT03437668</td>
</tr>
<tr>
<td>Drug: ashwagandha&lt;sup&gt;b&lt;/sup&gt; Drug: placebo</td>
<td>Ashwagandha for cognitive dysfunction</td>
<td>Not yet recruiting</td>
<td>NCT04092647</td>
</tr>
<tr>
<td>Drug: Sensoril&lt;sup&gt;c&lt;/sup&gt; Other: placebo</td>
<td>Withania somnifera: an immunomodulator and anti-inflammatory agent for schizophrenia</td>
<td>Completed</td>
<td>NCT01793935</td>
</tr>
<tr>
<td>Drug: ashwagandha Other: placebo</td>
<td>Effect of ashwagandha on salivary antioxidant and serum C reactive protein in chronic generalized periodontitis</td>
<td>Completed</td>
<td>NCT03533972</td>
</tr>
<tr>
<td>Dietary supplement: ashwagandha extract Dietary supplement: placebo</td>
<td>The effects of ashwagandha in endurance exercise performance</td>
<td>Unknown</td>
<td>NCT03596307</td>
</tr>
<tr>
<td>Dietary supplement: curcumin powder Dietary supplement: ashwagandha extract</td>
<td>Pilot study of curcumin formulation and ashwagandha extract in advanced osteosarcoma</td>
<td>Unknown</td>
<td>NCT00689195</td>
</tr>
<tr>
<td>Dietary supplement: ashwagandha</td>
<td>Ashwagandha: effects on stress, inflammation, and immune cell activation</td>
<td>Completed</td>
<td>NCT00817752</td>
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<tr>
<td>Dietary supplement: smart energy system (a nutritional/herbal combination)</td>
<td>Treatment of fibromyalgia and CFS with ribose, ashwagandha, rhodiola, licorice, schisandra, and green tea extract</td>
<td>Recruiting</td>
<td>NCT04598243</td>
</tr>
<tr>
<td>Drug: Sensoril Other: placebo</td>
<td>Sensoril (ashwagandha) for bipolar disorder</td>
<td>Completed</td>
<td>NCT00761761</td>
</tr>
<tr>
<td>Drug: Withania somnifera Drug: placebo</td>
<td>NF-κB inhibition in amyotrophic lateral sclerosis</td>
<td>Not yet recruiting</td>
<td>NCT05031351</td>
</tr>
<tr>
<td>Dietary supplement: andrographis and Withania Dietary supplement: placebo</td>
<td>Effects of an adaptogenic extract on electrical activity of the brain in elderly subjects with cognitive impairment</td>
<td>Completed</td>
<td>NCT03780621</td>
</tr>
</tbody>
</table>

CFS, chronic fatigue syndrome; NF-κB, nuclear factor κB.

<sup>a</sup>WSE, Withania Somnifera extract.

<sup>b</sup>Ashwagandha, Withania somnifera.

<sup>c</sup>Sensoril is a proprietary extract of Withania somnifera. Each Sensoril capsules will contain 250 mg of standardized extract of Withania somnifera.

**Fig. 1.** Representative withanolides contained in *W. somnifera*. Compound 1, withaferin A; compound 2, withanone; compound 3, withanolide A; compounds 4–7, withanolide D–G; and compounds 8–12, withanolide I–M.
**Hepatoprotective Effects of Natural Products in W. Somnifera**

Effects of W. somnifera Extracts in the Treatment of Liver Diseases. Several studies have evaluated the hepatoprotective potential of *W. somnifera* in animal models of various hepatic disorders. Aqueous root extract of *W. somnifera* administered at a dose of 500 mg/kg by gavage 60 minutes after acetaminophen (APAP) dosing was found to significantly reduce the elevated hepatotoxicity biomarkers, decrease lipid peroxidation, and enhance glutathione, catalase, glutathione reductase, and glutathione peroxidase activity in APAP-treated mice (Malik and Pandey, 2013). In another study, the ethanol extract of *W. somnifera* at 100 mg/kg protected against γ radiation-induced hepatotoxicity in rats; decreased serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γ-glutamyl transpeptidase; decreased hepatic levels of malondialdehyde and total nitrate/nitrite; and increased hepatic antioxidant enzymes, including superoxide dismutase and glutathione peroxidase in rats (Hosny Mansour and Farouk Hafez, 2012). The hepatoprotective activity of a methanolic extract of *W. somnifera* roots was examined in APAP-intoxicated rats, and a significant hepatoprotective activity was observed when *w. somnifera* extract was given 2 hours prior to APAP administration through alleviating inflammation and oxidative stress, accompanied by an inhibitory effect on hepatic levels of proinflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-1β (IL1β), cyclooxygenase 2, and inducible nitric oxide (Dekvar et al., 2016). Among the components of *W. somnifera* extracts, WA was the most extensively studied among the natural compounds isolated from *W. somnifera*, whereas other abundant chemical constituents of *W. somnifera* have been studied to a much lesser extent in the treatment of liver diseases. Hence, in the following section of this review, a summary of the literature related to the effects of WA on the treatment of liver diseases is presented.

**Effects of WA in the Treatment of Liver Injury.** WA (Fig. 1, compound 3) was recognized as the most important bioactive component isolated from *W. somnifera*. Beyond the classic antitumor role of WA, a growing list of recent reports have shown that WA has activity against the obesity-associated metabolic syndromes (Lee et al., 2016), diabetes (Teku et al., 2018), and systemic inflammation and liver disease (Gu et al., 2020). Although the detailed mechanisms by which WA can achieve these activities remains elusive, several hypotheses have been proposed, including leptin sensitizer for obesity treatment, immunoregulator for inflammation and fulminant acute liver injury, and angiogenesis activator for liver cancer. In HCC, WA inhibits proliferation, migration, and invasion of HCC cells (Hep3B, HepG2, Huh7, QGY-7703, SK-Hep1, MHCC97H, and MHCC97L cells) by elevating the expression of antiangiogenic factor such as the transcriptional factor-1, DR5, and ERK and activating liver X receptor α to inhibit nuclear factor kB (NF-kB) transcriptional activity. In orthotopic liver tumors, WA inhibits liver tumor invasion and angiogenesis by downregulating the expression of pyruvate kinase (PKY2), the associated coiled-coil containing protein kinase 1 (ROCK1) and vascular endothelial growth factor (VEGF).
activating antioxidant response for treatment of APAP-induced liver injury dependent on the NF-E2-related factor-2 (NRF2) or carbon tetrachloride (CCL₄)-induced liver injury and fibrosis depending on sirtuin 3 (SIRT3). How WA may improve liver diseases are summarized in Fig. 2 for acute liver injury, Fig. 3 for chronic liver injury, and Fig. 4 for liver cancer.

**D-galactosamine/Lipopolysaccharide-Induced Fulminant Hepatitis.** Fulminant hepatitis is a life-threatening clinical syndrome worldwide. WA was recently reported to attenuate D-galactosamine (GaN)/lipopolysaccharide (LPS)-induced hepatotoxicity in mice, associated with attenuating the inflammatory response via targeting macrophage and NLR family, pyrin domain containing 3 (NLRP3), while largely independent of NRF2 signaling, autophagy induction, and hepatic AMPKz1 and hepatocyte inhibitor of nuclear factor kB (IκB) signaling (Xia et al., 2021). In this GaN/LPS-induced acute liver injury model, WA was found to have both potent preventive and therapeutic effects when mice were dosed with a single intraperitoneal injection of WA at 0.5 hour before or 2 hours after GaN dosing. By using clodronate liposome pretreatment to deplete macrophage, the hepatoprotective effect of WA was abolished, but by further using global Nlrp3-null mice as well as NLGRP3-deficient primary macrophages, the WA effects were partially lost, but not totally abolished (Xia et al., 2021). In this same study, by using gene knockout mouse strains, the hepatoprotective effect of WA was found to be independent of the presence of hepatocyte AMPKz1, NRF2, and IκB (Xia et al., 2021). Given that macrophage depletion abolished the hepatoprotective effects of WA in this model and NLRP3 knockout could not totally, but only partially, abolish the hepatoprotective effect of WA, additional targets located in macrophage, such as macrophage-specific IκB, were suspected to mediate the hepatoprotective effects of WA in this model.

The GaN/LPS-induced acute liver injury model is characterized with systematic inflammation and hepatocyte apoptosis, whereas WA was found to have no direct role in TNFz-induced hepatocyte apoptosis (Xia et al., 2021). Decreasing LPS-induced systematic inflammation, also known as “cytokine storms” (Noori et al., 2020), was inferred to play a dominant role in the hepatoprotective effect of WA. Thus, with WA as a chemical probe, the results suggest that a strategy targeting the immune system, such as macrophage, to treat acute liver injury is achievable. This study suggests WA as a potential immunoregulator to normalize the altered immune response for the treatment of acute liver injury. Future studies on the effects of WA as one potent herbal immunoregulators in the treatment of other types of systematic inflammatory disorders are promising.

**APAP-Induced Acute Liver Injury.** WA was reported to protect against APAP-induced liver injury in two reports, indicating that NRF2 activation plays an important role in mediating the hepatoprotective effects of WA (Jadeja et al., 2015; Palliyaguru et al., 2016). In an earlier study, a single dose of 40 mg/kg WA administered via intraperitoneal injection at 1 hour after APAP dosing significantly rescued APAP-induced hepatotoxicity, compared with the control vehicle ethanol (2 μL/g) (Jadeja et al., 2015), which suggests an efficient therapeutic effect. APAP-induced Jun N-terminal kinase (JNK) activation, mitochondrial B-cell lymphoma-2 (BCL-2)-associated X protein translocation, nitrotyrosine production, and hepatic inflammation were reduced by WA, accompanied by upregulation of NRF2 target genes, including Nrf2, Gclc, and Nqo1. In addition, WA alleviated H₂O₂-induced hepatocyte death and oxidative stress in the AML12 cell line, a nontransfected normal hepatocyte-derived cell line, in vitro (Jadeja et al., 2015). To better evaluate the therapeutic effect of one drug in the APAP model, APAP-treated mice are usually administered drugs for 2 hours or longer after APAP dosing to evaluate the effects on late-stage liver injury (Abdullah-Al-Shoeb et al., 2020). Comparing the hepatoprotective activity of a drug candidate to the clinical drug N-acetyl cysteine (Dear et al., 2021) could help to further assess the translational significance. Therefore, whether WA has a superior hepatoprotective effect against APAP-induced acute liver injury compared with N-acetyl cysteine, and whether WA still has a therapeutic effect at the later stages of APAP-induced liver injury warrant future study. In addition, this work only described the NRF2 activation effect of WA in APAP-treated mice (Jadeja et al., 2015); however, whether WA improvement of APAP-induced acute liver injury depends on its effect on NRF2 activation remains unexplored.

In a later study, WA was found to have an NRF2-dependent protective effect toward APAP-induced hepatotoxicity (Palliyaguru et al., 2016). WA exhibited direct NRF2-inducing activity both in mice in vivo and in mouse embryonic fibroblasts generated from fibroblasts isolated from 13.5-day-old mouse embryos in vitro. WA induced NRF2 signaling in a kelch like ECH associated protein 1 (KEAP1)-independent and phosphatase and tensin homolog (PTEN)/phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase 1 (AKT1)-dependent manner in vitro as revealed by using NRF2/kelch like ECH associated protein 1 (KEAP1) double-knockout mouse embryonic fibroblasts (Palliyaguru et al., 2016). More importantly, this study employed a hepatocyte-specific Nrf2-null mouse line to confirm the ameliorating effect of WA on APAP-induced liver injury was dependent on the presence of hepatocyte NRF2 (Palliyaguru et al., 2016), thereby supporting the contribution of WA-induced NRF2 signaling to the hepatoprotective effect of this compound in APAP-dosed mice. However, a major limitation of this study is that 100 μL of DMSO or 7 mg/kg of WA was administrated to mice via oral gavage 22 hours before APAP dosing. Given that WA showed a short retention time in vivo in previous pharmacokinetic studies (Thaiparambil et al., 2011; Vanden Berghe et al., 2012; Devkar et al., 2015; Dai et al., 2019), it is doubtful that WA could still achieve a hepatoprotective effect if only dosed once via gavage 22 hours before APAP, considering the anticipated low plasma concentration of this compound. DMSO is not suitable as a control vehicle for drugs when studying the APAP model, since DMSO has a hepatoprotective effect against APAP-induced liver injury even when used at a very low dose (Yoon et al., 2006). Thus, the dose and route of administration need to be considered when interpreting results. More studies are required to determine the extent by which Nrf2 is involved in mitigating APAP toxicity by WA treatment.

Although the parent drug APAP is not hepatotoxic, APAP is metabolized to a toxic metabolite N-acetyl-p-benzoquinone (NAPQI) in the liver, a reaction catalyzed by cytochrome P450 (CYP) 2E1 (Lee et al., 1996; Cheung et al., 2005) and to a lesser degree by CYP3A11 and CYP1A2 (Zaher et al., 1998; Yan et al., 2016). Any factors that inhibit the generation of NAPQI could contribute to decreasing APAP-induced hepatotoxicity. How W. somnifera and WA affect APAP metabolism has not been examined in the APAP model in vivo and in vitro. However, several studies reported no significant inhibitory effects of W. somnifera extract on the CYPs that are involved in NAPQI generation from APAP (Savai et al., 2013; Varghese et al., 2014; Dey et al., 2015; Savai et al., 2015), indicating that it is less likely that WA exerts hepatoprotective effect toward APAP-induced liver injury by inhibiting CYP-mediated APAP metabolic activation. However, W. somnifera extract was found to exhibit inductive effects on CYP3A4 and CYP1A enzymes (Kumar et al., 2021). Thus, further studies are warranted to determine the extent by which metabolic activation of APAP is involved in reducing APAP-induced hepatotoxicity after W. somnifera and WA treatment.

**Bromobenzene-Induced Hepatotoxicity.** In an earlier study, a protective effect of W. somnifera extract was found in treating bromobenzene-induced nephrotoxicity and mitochondrial oxidative stress in rats through a proposed mechanism of decreasing mitochondrial oxidative stress. Therefore, whether WA has a superior hepatoprotective effect against APAP-induced acute liver injury compared with N-acetyl cysteine, and whether WA still has a therapeutic effect at the later stages of APAP-induced liver injury warrant future study. In addition, this work only described the NRF2 activation effect of WA in APAP-treated mice (Jadeja et al., 2015); however, whether WA improvement of APAP-induced acute liver injury depends on its effect on NRF2 activation remains unexplored.
mice were employed to generate a leptin signaling-depressed cell line in order to induce a lean NASH model, the 40% high-fat and high-cholesterol diet (HFD) model (Patel et al., 2019). Beyond the preventive effects, WA was found to improve methionine-choline-deficient-induced NASH, a lean NASH model, the hepatoprotective effect of WA against NASH was inferred to be at least partially independent of its antiobesity effect. Using ob/ob mice, WA was confirmed to produce an anti-NASH effect independent of leptin signaling (Patel et al., 2019).

WA in Non-NASH-Associated Liver Fibrosis. The effect of WA in non-NASH-associated fibrosis is summarized in Fig. 3B. In a bile duct ligation-induced liver fibrosis model, WA inhibited epithelial mesenchymal transition process by inhibiting the expression of enzymes such as MMP2, TIMP1, and LOXL2, and the transcriptional repressor SNAI1, thus enhancing the expression of CDH1 leading to the reversal of epithelial mesenchymal transition (Sayed et al., 2019). In the liver fibrosis models induced by platelet-derived growth factor BB and CCL4, WA attenuated liver fibrosis by inhibiting oxidative stress in a SIRT3-dependent manner as revealed by using SIRT3 knockout mice and Sirt3 silencing in J51 cells, an immortalized mouse hepatic stellate cell line (Gu et al., 2020). These studies further extend the antifibrosis scope of WA in different types of hepatic fibrosis models. The direct effects and detailed mechanisms of WA in alleviating hepatic fibrogenesis, such as hepatic stellate activation, macrophage activation, and hepatocyte damage, warrant additional studies.

WA in Alcoholic Liver Injury

The hepatoprotective effect of WA in alcoholic liver injury was investigated in an acute- upon-chronic liver injury model (Hamada et al., 2022). In this binge model, WA significantly decreased binge ethanol-induced liver injury and hepatic lipid accumulation both in a preventive and therapeutic manner. Mechanistically, WA inhibits ethanol-induced lipogenesis in vivo as well as in primary hepatocytes in vitro. This study suggests the therapeutic potential of WA in the treatment of alcoholic liver injury (Hamada et al., 2022). The direct target of WA and the role of WA in long-term alcohol-induced alcoholic steatohepatitis and fibrosis warrants further study.

WA in Liver Cancer

The effect of WA on tumor growth and metastasis in liver was investigated in a nude mouse model. WA significantly decreased tumor growth, the incidence of lung metastasis, and macrophage infiltration in liver tumors and vessels by decreasing cell migration and tumor vascular endothelial cell damage, and inducing tumor necrosis through inhibiting the expression of pyruvate kinase PYK2, rafa-associated coiled-coil containing protein kinase 1 and vascular endothelial growth factor (Wang et al., 2015). In another study, WA was found to suppress the proliferation, migration, invasion, and anchorage-independent growth of HCC by inhibiting NFκB signaling through liver X receptor α activation in HCC cells, including Hep3B, HepG2, Huh7, and QGY-7703 cells (Shiraganna et al., 2021). In addition, WA sensitized tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in several human HCC cell lines, including Huh7, SK-Hep1, and Hep3B cells, by death receptor protein-5 (DR5) upregulation and CASP8 and FADD like apoptosis regulator downregulation (Lee et al., 2009). In addition to inducing apoptotic cell death, the WA inhibitory effect on the growth of HCC cells, including Huh7, HepG2, MHCC97H, and MHCC97L, was diminished by the concomitant induction of autophagy, which could be rescued by cotreatment with chloroquine, an autophagy inhibitor (Siddharth et al., 2019). Administration of WA was also reported to decrease the level of serum tumor marker α-fetoprotein and rescue the diethylnitrosamine-induced HCC in rats (Murugan et al., 2015). Oral administration of WA effectively inhibited HepG2-xenograft growth and diethylnitrosamine-induced HCC by concomitantly increasing p90-ribosomal S6 kinase phosphorylation and activating ETS-like transcription factor-1 and DR5 through...
increasing phosphorylation of extracellular signal-regulated kinases (ERK) (Kuppusamy et al., 2017). The roles of WA in the treatment of liver tumor are summarized in Fig. 4.

**WA in Systematic Inflammation**

Although inhibiting inflammation potentially decreases liver injury (Zhang et al., 2019), WA was reported to have potent anti-inflammation activities. WA inhibited the expression of inducible nitric oxide at both the protein and mRNA levels in LPS-stimulated RAW264.7 cells, a macrophage-like, Abelson leukemia virus-transformed cell line derived from BALB/c mice, by blocking the phosphorylation of AKT serine/threonine kinase 1 (9AKT1) and ERK via inhibition of NFκB phosphorylation and subsequent NFκB activation (Oh et al., 2008). WA was found to exert its anti-inflammatory effects by inhibiting NFκB activation through targeting Iκκβ in HEK293T cells, a cell line derived from human embryonic kidney cells (Heyninck et al., 2014). In another study, WA inhibited inflammation by potently inhibiting TNFα-induced NFκB activation via inhibition of Iκκbα kinase activity in vitro in the mouse fibroblasts cell line L929SA and human embryonic kidney 293T cells, and in an acute inflammatory mouse model in vivo (Kaileh et al., 2007). WA dose-dependently inhibited the IL1β secretion as well as the cleavage of IL1β and caspase 1 in LPS-primed macrophage via inhibiting NLRP3 inflammasome activation (Kim et al., 2015). WA also reduced the release of IL1β and IL18 via inhibition of NFκB activity and suppression of NLRP3 inflammasome activation in THP-1 cells (Dubey et al., 2018).

**Hepatoprotective and Anti-Inflammation Potential of Other Extracts**

Both WA and another withanolide constituent from *W. somnifera*, withanone, significantly decreased the production of proinflammatory cytokines TNFα and IL6 at both the mRNA and protein levels in LPS-stimulated bone marrow-derived macrophages (Purushotham et al., 2017). Further mechanistic studies showed that WA markedly inhibited the mitogen-activated protein kinase, including ERK, JNK, and p38 as well as NFκB activation, whereas withanone regulated only ERK and JNK signaling pathways (Purushotham et al., 2017). One withanolide constituent protected liver cells from oxidative stress-induced damage by increasing cellular survival rate and activities of superoxide dismutase, glutathione, and catalase and reducing the accumulation of reactive oxygen species via activating nuclear translocation of NRF2 and increasing expression of the NRF2 target gene heme oxygenase 1 (Wang et al., 2018). Six withanolides showed anti-inflammatory activities through inhibition of nitric oxide production in murine macrophage RAW 264.7 cells stimulated by LPS (Wu et al., 2018). Withanolide A inhibited oxidative and inflammation in the immortalized mouse microglial cell line BV-2 via simultaneously suppressing the NFκB pathways, inhibiting LPS-induced nitric oxide production, and stimulating the NRF2/heme oxygenase 1 pathway, but this antioxidative and anti-inflammatory effect was 10-fold less efficacious than WA (Sun et al., 2016).

**Toxicity of WA**

The toxicity of WA was investigated both in animal models and in humans by using *W. somnifera* extracts standardized for WA or pure WA. An oral LD₅₀ of *W. somnifera* extracts in rats was greater than 2000 mg/kg body weight in acute toxicity studies (Patel et al., 2016). Further, a subacute toxicity study also showed that rats administered *W. somnifera* extracts for 28 days did not exhibit any toxicologically significant changes in the brain, liver, and kidney compared with the control group (Patel et al., 2016). A safety evaluation of *W. somnifera* extracts standardized for WA in a phase I trial demonstrated that the capsule formulation of WA comprised of 72, 108, 144, and 216 mg of WA administered in two or four divided doses per day for at least 30 days was generally well-tolerated with no severe adverse events, but slight adverse effects, such as elevation of liver enzymes (5/11) and skin rash (2/11), were observed (Pires et al., 2020). Toxicity studies with pure WA has not been reported, and only two studies suggested that WA was toxic to mice with an LD₅₀ of 54 mg/kg body weight, which is much higher than the pharmacological doses typically employed that range between 1.25–10 mg/kg (Shohat et al., 1967; Shohat et al., 1970; Lee et al., 2016; Patel et al., 2019; Xia et al., 2021). The toxicity between pure WA and plant extracts of *W. somnifera* could differ because of purity and composition concerns, which is an issue that demands future study.

**Metabolism of WA**

**Metabolism of WA.** Studies on the pharmacokinetics and bioavailability of WA are summarized in Fig. 5 and Table 2. WA was found to be impermeable in an in vitro absorption model using MDCK cells derived from canine kidney (Devkar et al., 2015). WA is readily transported across Caco-2 cell plasma membranes in vitro, whereas WA had an oral bioavailability of 32.4 ± 4.8% in vivo, based on the studies of intravenous (5 mg/kg) and oral (10 mg/kg) dosing in male rats (Dai et al., 2019). Extensive first-pass metabolism of WA was further suggested by rat intestine-liver in situ perfusion, where WA concentration in the media was rapidly decreased and only 27.1% remained within 1 hour (Dai et al., 2019). Pharmacokinetic studies on WA showed a rapid plasma clearance in mice administered a single dose of WA via intraperitoneal injection (Thaiparambil et al., 2011; Vanden Berghe, 2012). A study reporting pharmacokinetic evaluation of WA after oral administration demonstrated a short half-life with the value of 59.9 minutes for this constituent in mice administered a single oral dose of *W. somnifera* extracts (Patil et al., 2013). A previous report found a relatively short half-life (t₁/₂ = 2.0 hours) of WA in mice after a single intraperitoneal injection of 5 mg/kg WA (Patel et al., 2019). Moreover, the observation that no detectable WA was found in plasma samples in humans orally administered a capsule formulation of WA at 72–216 mg/kg in two or four divided doses per day for at least 30 days as measured by high performance liquid chromatography with a limit of quantitation of 50 ng/mL (Pires et al., 2020). However, few studies evaluated the metabolic route of WA. Others found that WA was metabolized via hydroxylation, hydrogenation, and hydrolysis (Rosazza et al., 1978; Funska et al., 1985; Fusk et al., 1987), which was further supported by a more recent study where metabolites of WA generated from these pathways were found in rats (Dai et al., 2019). Due to the structural similarities of steroidal lactone classification between WA and endogenous steroids, it...
likely undergoes oxidative metabolism mediated by CYPs. More detailed future investigations are needed to characterize the metabolic pathway(s) as well as the major metabolizing enzymes responsible for WA metabolism in vivo.

**Pharmacokinetic Insights into Future Directions.** Poor pharmacokinetic behavior indicated that the hepatoprotective activity of WA may be attributed to its metabolites. Phase I reactions convert a parent drug to metabolites that, in some cases, could possess superior efficacy and be more biologically active and water-soluble than the parent compound by unmasking or inserting a polar functional group. The generated active metabolites, as "metabolized" molecules, can be less prone to first pass metabolism and thus have improved pharmacokinetic behaviors compared with the parent drugs (Sun and Wesolowski, 2021). Therefore, it is of great interest to compare the hepatoprotective effects of WA and its metabolites side by side to elucidate the effective substance basis of WA and explore new drug candidates in treating liver diseases.

On the other hand, it is also worth noting that decreased expression levels of phase I and II enzymes and drug transporters are often observed under pathologic states of the liver, including acute liver injury, the metabolic diseases and NAFLD, liver fibrosis, liver cancer, and systematic inflammation (Hanada et al., 2012; Wu and Lin, 2019; Bao et al., 2020; Feng et al., 2020), which may lead to reduced metabolism of WA. Thus, it is important that pharmacologists keep this in mind for designing appropriate doses of WA when using liver injury models. Whether WA is toxic is still controversial, and more toxicological evaluation is needed to determine the safety of WA. In addition, different formulations may need to be studied so that the adjusted pharmacokinetic properties (absorption, distribution, metabolism, and elimination) will yield optimal efficacy. Indeed, WA embedded in polycaprolactone implants was proposed to overcome the problems regarding its bioavailability and pharmacokinetics (Gupta et al., 2012), thereby allowing long-term systemic circulation for controlled treatment.

The exact mechanism by which WA alleviated liver diseases remains largely unknown, due in part to that fact that no receptor for this compound has been identified. Regarding the potentially extensive metabolism of WA in vivo, it is not clear whether WA, the parent drug, or its biologically active metabolite(s) may directly

### Table 2

The pharmacokinetic parameters of WA in experimental animals

<table>
<thead>
<tr>
<th>Dosing Method</th>
<th>Animal Model</th>
<th>Dose</th>
<th>Parameter</th>
<th>Value</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p.</td>
<td>Mice</td>
<td>5 mg/kg WA</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (nM)</td>
<td>14.3 ±1.8</td>
<td>(Patel et al., 2019)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 ±0.6</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AUC&lt;sub&gt;0-48h&lt;/sub&gt; (h nM/mL)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>177 ±10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AUC&lt;sub&gt;0-infinity&lt;/sub&gt; (h nM/mL)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>184 ±12</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Kel (1/h)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.38 ±0.11</td>
<td></td>
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<tr>
<td></td>
<td>Mice</td>
<td>4 mg/kg WA</td>
<td>AUC&lt;sub&gt;0-48h&lt;/sub&gt; (ng/mL hr)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>541</td>
<td>(Thaiparambil et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (hr)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CL (mL/min)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.52</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Vd (L/kg)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>14.8</td>
<td></td>
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<tr>
<td>Gavage</td>
<td>Mice</td>
<td>1000 mg/kg W. somnifera extract (0.4585 mg/kg WA)</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>16.7 ±4.02</td>
<td>(Patil et al., 2013)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Tmax (min)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>20 (20–30)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>59.9 ±15.9</td>
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<td></td>
<td></td>
<td></td>
<td>AUC&lt;sub&gt;0-infinity&lt;/sub&gt; (ng/mL min)</td>
<td>1570 ±57.8</td>
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<td></td>
<td></td>
<td></td>
<td>CL (mL/min/kg)</td>
<td>1670 ±54.5</td>
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<td></td>
<td></td>
<td></td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>274 ±10</td>
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<tr>
<td>i.v.</td>
<td>Rats</td>
<td>5 mg/kg WA</td>
<td>AUC&lt;sub&gt;0-48h&lt;/sub&gt; (ng/mL h)</td>
<td>3615 ±670</td>
<td>(Dai et al., 2019)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AUC&lt;sub&gt;0-infinity&lt;/sub&gt; (ng/mL h)</td>
<td>3685 ±685</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>MRT&lt;sub&gt;0-4&lt;/sub&gt; (h)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>4.0 ±0.4</td>
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<td></td>
<td>MRT&lt;sub&gt;0-infinity&lt;/sub&gt; (h)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>4.5 ±0.8</td>
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<td></td>
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<td></td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>4.5 ±1.1</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>CL (L/h/kg)</td>
<td>1.4 ±0.3</td>
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<td></td>
<td>V (L/kg)&lt;sup&gt;k&lt;/sup&gt;</td>
<td>9 ±2</td>
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<td></td>
<td></td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>3048 ±509</td>
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<tr>
<td>Gavage</td>
<td>Rats</td>
<td>10 mg/kg WA</td>
<td>AUC&lt;sub&gt;0-48h&lt;/sub&gt; (ng/mL h)</td>
<td>2345 ±345</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AUC&lt;sub&gt;0-infinity&lt;/sub&gt; (ng/mL h)</td>
<td>2789 ±683</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MRT&lt;sub&gt;0-4&lt;/sub&gt; (h)</td>
<td>6.8 ±1.1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MRT&lt;sub&gt;0-infinity&lt;/sub&gt; (h)</td>
<td>10.8 ±4.5</td>
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<td></td>
<td></td>
<td></td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>7.6 ±3.3</td>
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<td>Tmax (h)</td>
<td>0.11 ±0.07</td>
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<td></td>
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<td></td>
<td>CL (L/h/kg)</td>
<td>3.9 ±1.1</td>
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<td></td>
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<td></td>
<td>V (L/kg)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>41 ±15</td>
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<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>619 ±125</td>
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<td></td>
<td>F (%)&lt;sup&gt;l&lt;/sup&gt;</td>
<td>32.4 ±4.8</td>
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</tr>
</tbody>
</table>

**a**T<sub>1/2</sub>, half life.

**b**AUC<sub>0-48h</sub>, area under the concentration versus time curve from time 0 to 48 h.

**c**AUC<sub>0-infinity</sub>, area under the concentration versus time curve from time 0 to infinity.

**d**Kel, elimination rate constant.

**e**AUC<sub>0-t</sub>, area under the concentration versus time curve from time 0 to the last measurable time point.

**f**CL, clearance.

**g**V<sub>d</sub>, volume of distribution.

**h**T<sub>max</sub>, time to peak concentration.

**i**MRT<sub>0-t</sub>, mean residence time from time 0 to the last measurable time point.

**j**MRT<sub>0-infinity</sub>, mean residence time from time 0 to the last measurable time point.

**k**V, volume of distribution.

**l**F, bioavailability.
contribute to its hepatoprotective effects. Due to the short half-life of WA after gastric uptake, the pharmacological effects achieved after oral intake of WA may be due in part to modulation of the host intestinal targets, gut microbiota, or other factors involved in the gut-liver axis. The specific intestinal targets with which WA interacts to produce its pharmacological effects on liver diseases after oral gastric warrant further investigation. Various methodologies, including such as RNA sequencing, proteomics, metagenomics, and untargeted metabolomics, are needed to flush out mechanistic clues. Pharmacokinetic studies are also needed to be performed to clarify the distribution of WA and its potential active metabolites, which could direct future mechanistic research to better select a target organ/cell for analyses.

Conclusion

This review summarizes WA therapeutic potential in liver diseases, including acute liver injury, the metabolic diseases and NAFLD, liver fibrosis, liver cancer, and systemic inflammation. WA exerts its hepatoprotective effects mainly through antioxidant and anti-inflammatory activities in part by modulating NRF2 and NFXB signaling and the NLRP3 inflammasome. The antitumor effects are efficient at the safe doses of WA employed in rodent models, suggesting a selectivity of WA toward killing liver tumor cells compared with its activity in normal hepatocytes. WA is known to protect against liver cancer both in rodent models and in HCC cell lines in vitro.

The pharmacological and toxicological properties of WA, especially in humans, have been poorly studied. Structure modifications to make it circulate longer and yet retain its biologic activity may be of value in drug discovery. However, it should be noted that WA is found to be absorbed at low levels in WA-containing natural products used to treat humans, and thus the potential hepatoprotective effects of WA metabolites as well as the other components deserve further study. WA holds a great potential for novel drug discovery in the treatment of both acute liver injury, chronic liver injury, metabolic diseases as well as liver tumor, which deserves extensive future studies.

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Wrote or contributed to the writing of the manuscript: Xia, M. Yan, Wang, Hamada, N. Yan, Hao, Gonzalez, T. Yan.

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Hepatoprotective Effects and Metabolism of Withaferin A


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