

Mini-Review

WITHA FERIN A IN THE TREATMENT OF LIVER DISEASES: PROGRESS AND PHARMOCOKINETIC INSIGHTS

Yangliu Xia, Mingrui Yan, Ping Wang, Keisuke Hamada, Nana Yan, Haiping Hao, Frank J. Gonzalez, Tingting Yan

School of Life Science and Medicine, Dalian University of Technology, Panjin, 124221, China (Y.X., M.Y); Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA (Y.X., K.H., F.J.G., T.Y.); Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai, 201203, China (P.W.); State Key Laboratory of Natural Medicines, Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China (N.Y., H.H.); and Laboratory of Clinical Biochemistry, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan (K.H.).

Address corresponding to: Haiping Hao, Email: haipinghao@cpu.edu.cn; State Key Laboratory of Natural Medicines, Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China. Frank J. Gonzalez, Email: gonzalef@mail.nih.gov or Tingting Yan, Email: Tingting.yan@nih.gov; Laboratory of

Metabolism, Center for Center for Cancer Research, National Cancer Institute, National
Institutes of Health, Bethesda, MD 20892, USA.

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ABBREVIATIONS:

APAP, acetaminophen; CCl₄, carbon tetrachloride; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; GalN, D-galactosamine; HCC, hepatocellular carcinoma; HFD, high-fat diet; HFHC, high-fat and high-cholesterol diet; I κ B, inhibitor of nuclear factor κ B; IL1 β , interleukin 1 β ; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; MCD, methionine-choline-deficient; NRF2, NF-E2-related factor-2; NLRP3, NLR family, pyrin domain containing 3; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF- κ B, nuclear factor κ B; SIRT3, sirtuin 3; TNF α , tumor necrosis factor α ; WA, withaferin A; *W. somnifera*, *Withania Somnifera*.

ABSTRACT

Withaferin A (WA) is a natural steroidal compound used in Ayurvedic medicine in India and elsewhere. While WA was used as an anti-cancer 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 *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 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 (Gillessen and Schmidt, 2020), licorice-derived glycyrrhizin to treat viral hepatitis (Li et al., 2019), and berberine to treat nonalcoholic fatty liver diseases (Runbin Sun, 2017; Runbin Sun, 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 eighth century (Mirjalili et al., 2009) . Traditionally, the berries and leaves of *W. somnifera* were used as a local treatment for ulcers and tumors (Vanden Berghe et al., 2012). In addition, several reports have linked the health benefits of *W. somnifera* to its antidiabetic, anti-epileptic, anti-inflammatory, anti-depressant and anti-arthritic activities (Dutta et al., 2019; Huang et al., 2020). The clinical trials

registered at **clinicaltrials.gov** using *W. somnifera* extract, also known as “ashwagandha” or “sensoril” as 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 anti-cancer reagent and earlier reviews have documented the effects of WA in treating cancer (W. Vanden Berghe, 2012; Behrouz Hassannia, 2020). Beyond its potential anti-cancer 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 the 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). While 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* (Sun et al., 2016). The major withanolides contained in *W. somnifera* are listed in **Figure 1**.

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 min 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 et al., 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, and hepatic levels of malondialdehyde and total nitrate/nitrite, and increase 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 h prior to APAP administration through alleviating inflammatory and oxidative stress, accompanied by an inhibitory effect on hepatic levels of proinflammatory cytokines including tumor necrosis factor α (TNF α), interleukin-1 β (IL1 β), cyclooxygenase 2 (COX2) and inducible nitric oxide

(iNOS) (Devkar et al., 2016). Among the components of *W. somnifera* extracts, WA was the most extensively studied among the natural compounds isolated from *W. somnifera*, while other abundant chemical constituents of *W. somnifera* have been studied to a much less 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 (Figure 1, Compound 3) was recognized as the most important bioactive component isolated from *W. somnifera*. Beyond the classical anti-tumor 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 (Tekula et al., 2018) and systematic 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 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 **Figure 2** for acute liver injury, **Figure 3** for chronic liver injury and **Figure 4** for liver cancer.

GalN/LPS-Induced Fulminant Hepatitis. Fulminant hepatitis is a life-threatening clinical syndrome worldwide. WA was recently reported to attenuate GalN/LPS-induced hepatotoxicity in mice, associated with attenuating the inflammatory response via targeting macrophage and NLRP3, while largely independent of NRF2 signaling, autophagy induction,

and hepatic AMPK α 1 and I κ B signaling (Xia et al., 2021). In this GalN/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 h before or 2 h after GalN 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 NLRP3-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 AMPK α 1, NRF2 and hepatocyte I κ β (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 I κ β , were suspected to mediate the hepatoprotective effects of WA in this model.

The GalN/LPS-induced acute liver injury model is characterized with systematic inflammation and hepatocyte apoptosis, while WA was found to have no direct role in TNF α -induced hepatocyte apoptosis (Xia et al., 2021). Decreasing LPS-induced systematic inflammation, also known as “cytokine storms” (Mahboubeh S. Noori, 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 hepatocyte protective 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 (i.p.) at 1 h after APAP dosing, significantly rescued APAP-induced hepatotoxicity, compared with the control vehicle ethanol (2 μ L/g) (Jadeja et al., 2015), which suggests an efficient therapeutic effect. APAP-induced Jun N-terminal kinase (JNK) activation, mitochondrial BCL2-associated X protein (BAX) 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 non-transformed 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 h or longer after APAP dosing in order to evaluate the effects on late-stage liver injury (Abdullah-Al-Shoeb et al., 2020). And, comparing the hepatoprotective activity of a drug candidate to the clinical drug *N*-acetyl cysteine (Dear et al., 2021) could help further assess the translational significance. Therefore, whether WA has a superior hepatoprotective effect against APAP-induced acute liver injury compared to *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 activity towards 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 KEAP1-independent and PTEN/PI3K/AKT-dependent manner *in vitro* as revealed by using NRF2/KEAP1 double-knockout mouse embryonic fibroblasts (Palliyaguru et al., 2016). More importantly, this study employed a hepatocyte-specific *Nrf2*-null mouse strain to confirm that the effect of WA against 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 μ L of dimethylsulfoxide (DMSO) or 7 mg/kg of WA was administered to mice via oral gavage 22 h before APAP dosing. Given that WA showed a short retention time *in vivo* in previous pharmacokinetic studies (Thaiparambil JT, 2011; Berghe WV, 2012; S. Devkar, 2015; Dai et al., 2019), it is doubtful that WA could still achieve a hepatoprotective effect if only dosed once via gavage 22 h 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 strong hepatoprotective effect against APAP-induced liver injury even when used at a very low dose (Mi Young YOON, 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.

While the parent drug APAP is not hepatotoxic, APAP is metabolized into a toxic metabolite N-acetyl-p-benzoquinone (NAPQI) in the liver, a reaction catalyzed by CYP2E1 (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). Thus, any factors that inhibit the generation of NAPQI could contribute to decreasing APAP-induced hepatotoxicity. How *W. somnifera* and WA affects 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 CYP isoforms, which are associated with 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 towards 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 study is 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 (Vedi et al., 2014). More recently, pretreatment with WA provided a significant protection against bromobenzene-induced liver damage by preventing mitochondrial dysfunction through increasing activities of mitochondrial enzymes and balancing the expression of B-cell lymphoma-2 (Bcl-2) associated X (Bax)/Bcl-2 in liver (Vedi and Sabina, 2016). Thus, WA in *W. somnifera* at least partially contributes to the

hepatoprotective effect of *W. somnifera* in this bromobenzene-induced liver injury model.

WA in the Metabolic Diseases and NAFLD. As one of the most prevalent chronic liver diseases worldwide, nonalcoholic fatty liver disease (NAFLD) affects 20-25% of the adult population (Thomas G Cotter, 2020). NAFLD is a typical type of obesity-associated metabolic syndrome, that is frequently is associated with, but is not limited to, obesity (Younes and Bugianesi, 2019; Thomas G Cotter, 2020). NAFLD includes simple nonalcoholic fatty liver (NAFL) characterized with hepatic steatosis and mild inflammation and the progressive nonalcoholic steatohepatitis (NASH) characterized by severe liver inflammation and fibrosis, which could further progress to end-stage liver diseases including cirrhosis and hepatocellular carcinoma (HCC) (Schwabe et al., 2020; Sheka et al., 2020; Francque et al., 2021). The role of WA in treating diet-induced NAFL and progressive NASH is diagrammed in **Figure 3A**.

An initial study demonstrated that WA alleviated obesity-associated metabolic syndrome and hepatic steatosis by acting as a leptin sensitizer (Lee et al., 2016). In this study, 12-week WA (1.25 mg/kg/day, i.p.) treatment reduced the levels of pro-inflammatory cytokines including toll-like receptor (TLR4), nuclear factor κ B (NF κ B), tumor necrosis factor- α (TNF α), chemokine (C-C motif) ligand-receptor, and cyclooxygenase 2 (COX2) in both the serum and liver of high-fat diet (HFD)-fed obese mice, along with protective activity against obesity, oxidative stress and insulin resistance (Lee et al., 2016). Moreover, WA (6 mg/kg, 3 times per week, i.p.) was found to therapeutically improve insulin sensitivity, downregulate the inflammatory response and decrease body weight and hepatic steatosis in established HFD-fed obese mice, which is suggested to be partly by upregulating peroxisome

proliferator-activated receptor gamma (PPAR γ) phosphorylation-related downstream genes including carbonic anhydrase 3 (*Car3*), selenium binding protein 1 (*Selenbp1*), amyloid beta (A4) precursor-like protein 2 (*Aplp2*), thioredoxin interacting protein (*Txnip*), and adiponectin (*Adipoq*) (Khalilpourfarshbafi et al., 2019). The anti-hepatic inflammation effect of WA was also observed and described in pre-existing HFD-induced obese mice in a therapeutic manner when mice were fed a HFD for 12 weeks and then dosed by oral gavage with WA at 1.25 mg/kg/day for 12 weeks (Abu Bakar et al., 2019). These studies support the view that WA has a therapeutic effect in treating obesity-induced metabolic syndrome that includes hepatic steatosis, inflammation and diabetes. However, these HFD-induced obesity models may only represent the mild fatty liver phenotype, NAFL, while still the early stage of NAFLD.

The effect of WA on the later stages of NAFLD, NASH, has also been examined. WA was demonstrated to have a potent therapeutic effect in decreasing NASH-associated symptoms in both obese and lean NASH models. In several widely-used NASH models, WA had both preventive and therapeutic effects in improving NASH, with reduced serum aminotransferase levels, liver steatosis, inflammation, endoplasmic reticulum (ER) stress and fibrosis in the WA-treated group compared to the vehicle-treated group, and this was correlated with normalized serum ceramides, hepatic ceramide catabolism and ER stress (Patel et al., 2019). In this study, methionine-choline-deficient (MCD) diet-fed wild-type C57BL/6N mice were used to induce a lean NASH model, the 40% high-fat and high-cholesterol (HFHC) diet-fed wild-type C57BL/6N mice were used to establish an obese NASH model, while 40% HFHC-fed ob/ob mice were employed to generate a leptin signaling-deficient NASH model

(Patel et al., 2019). Beyond the preventive effects, WA improved all NASH symptoms in a therapeutic manner when dosed at the later stage of the established NASH models. Given that WA was found to improve MCD-induced NASH, a lean NASH model, the hepatoprotective effect of WA against NASH was inferred to be at least partially independent of its anti-obesity 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 are summarized in **Figure 3B**. In a bile duct ligation (BDL)-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 SNAIL1, 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 (PDGF-BB) and CCl₄, WA attenuated the liver fibrosis by inhibiting oxidative stress in a SIRT3-dependent manner as revealed by using SIRT3 knockout mice and *Sirt3* silencing in JS1 cells, an immortalized mouse hepatic stellate cell line (Gu et al., 2020). These studies further extend the anti-fibrosis 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 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 the liver tumors and vessels by decreasing the cell migration, tumor vascular endothelial cell damage and inducing tumor necrosis through inhibiting the expression of pyruvate kinase PYK2 (PYK2), rho-associated coiled-coil containing protein kinase 1 (ROCK1) protein and vascular endothelial growth factor (VEGF) (Wang et al., 2015). In another study, WA was found to suppress the proliferation, migration, invasion and anchorage-independent growth of HCC by inhibiting NF κ B signaling through liver X receptor α (LXR α) activation in HCC cells including Hep3B, HepG2, Huh7 and QGY-7703 cells (Shiragannavar et al., 2020). In addition, WA sensitized tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in several human HCC cell lines including Huh7, SK-Hep1 and Hep3B cells by death receptor protein-5 (DR5) up-regulation and CASP8 and FADD like apoptosis regulator (c-FLIPR) down-regulation (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 (Sivalingam Murugan, 2015). Oral administration of WA effectively inhibited HepG2-xenograft growth and diethylnitrosamine-induced HCC by concomitantly increasing p90-ribosomal S6 kinase (RSK) phosphorylation and activating ETS-like transcription factor-1 (ELK1) and death receptor protein-5 (DR5) through increasing phosphorylation of ERK (Kuppusamy et al., 2017). The roles of WA in the

treatment of liver tumor are summarized in **Figure 4**.

WA in Systematic Inflammation

While inhibiting inflammation potentially decreases liver injury (Zhang et al., 2019), WA was reported to have potent anti-inflammation activities. WA inhibited the expression of iNOS 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 and extracellular signal-regulated kinases (ERK) via inhibition of I κ B phosphorylation and subsequent NF κ B activation (Oh et al., 2008). WA was found to exert its anti-inflammatory effects by inhibiting NF κ B activation through targeting I κ B 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 TNF α -induced NF κ B activation via inhibition of I κ B kinase activity *in vitro* in the mouse fibrosarcoma 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 IL1 β secretion as well as the cleavage of IL1 β and caspase 1 (CASP1) in LPS-primed macrophage via inhibiting NLRP3 inflammasome activation (Kim et al., 2015). WA also reduced the release of IL1 β and IL18 via inhibition of NF κ B 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 pro-inflammatory cytokines, TNF α 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 (MAPKs) including ERK, JNK and p38 as well as NF κ B activation, whereas withanone regulated only ERK and JNK signaling pathways (Purushotham et al., 2017). One withanolide 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 (*Hmox1*) (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 NF κ B pathways, inhibiting LPS-induced nitric oxide production and stimulating the NRF2/heme oxygenase 1 pathway, but this anti-oxidative and anti-inflammatory effect was tenfold 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 sub-acute 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 to 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), observed (Pires et al., 2020). Toxicity studies with pure WA has not been reported and only two studies suggest WA to be toxic to mice with 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 B, 1967; Batia S, 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 **Figure 5** and **Table 2**. WA was found to be impermeable in an *in vitro* absorption model using MDCK cells derived from canine kidney (S. Devkar, 2015). WA was found to be readily transported across Caco-2 cell plasma membranes *in vitro*, while WA was found to have an oral bioavailability of $32.4 \pm 4.8\%$ *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, with WA rapidly decreased and only 27.1% remained within 1 h (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 JT, 2011; Berghe WV, 2012). A study reporting pharmacokinetic evaluation of WA after oral administration demonstrated a short half-life with the value of 59.9 min for this constituent in mice administered a single oral dose of *W. somnifera* extracts (Patil D, 2013). A previous report found a relatively short half-life ($t_{1/2}$ =2.0 h) of WA in mice after a single intraperitoneal injection dose of 5 mg/kg WA (Patel et al., 2019). Moreover, the observation that no detectable of WA was found in plasma samples in humans orally administered a capsule formulation of WA at 72-216 mg/kg in 2 or 4 divided doses per day for at least 30 days as measured by HPLC 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 JP, 1978; Fuska J, 1985; Fuska J, 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 which, 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 to 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 pathological 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 RC, 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 contribute to its hepatoprotective effects.

Due to the short half-life of WA after gavage *in vivo*, 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 gavage 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 systematic inflammation. WA exerts its hepatoprotective effects mainly through antioxidant and anti-inflammatory activities in part by modulating NRF2 and NF κ B signaling, and the NLRP3 inflammasome. The anti-tumor effects are efficient at the safe doses of WA employed in rodent models, suggesting a selectivity of WA towards killing liver tumor cells compared to 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 the humans, have been poorly studied. Structure modifications to make it circulate longer and yet retain its biological 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.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Y.X., M.Y, P.W., H.K., N.Y., H.H., F.J.G., T.Y..

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Figure legends:

Figure 1. Representative withanolides contained in *Withania Somnifera* (*W. somnifera*).

Compound 1, withaferin A; Compound 2, withanone; Compound 3, withanolide A; Compound 4-7, withanolide D-G; Compound 8-12, withanolide I-M.

Figure 2: The role and mechanism of withaferin A (WA) in the treatment of acute liver

injury. In D-galactosamine/ lipopolysaccharide (GalN/LPS)-induced acute liver injury model, macrophage depletion using clodronate liposome were used to demonstrate that WA decreases the GalN/LPS-induced fulminant liver failure depending on its inhibitory effect towards macrophage activation, while global NLR family, pyrin domain containing 3 (NLRP3) knockout mice (*Nlrp3*^{-/-} mice) were used to demonstrate that WA decreased GalN/LPS-induced liver injury partially by inhibiting the NLRP3 inflammasome activation. In the acetaminophen (APAP)-induced acute liver injury model, WA attenuates APAP-induced hepatotoxicity accompanied by hepatic NRF2 activation. Although hepatocyte-specific *Nrf2*-null mice were used to determine the contribution of NRF2 to the hepatoprotective effect of WA in APAP model, whether the hepatoprotective effect of WA in treating APAP-induced liver injury is dependent on NRF2 signaling still warrants further study due to the questioned experimental process (dashed line).

Figure 3: The role and mechanism of withaferin A (WA) in the treatment of chronic

liver injury. (A), In the high-fat diet (HFD)-induced obesity model, WA decreases obesity-associated metabolic syndrome and fatty liver as a leptin sensitizer. In the nonalcoholic steatohepatitis (NASH) model, WA both prevents and therapeutically alleviates

the obese and lean NASH is accompanied by decreasing ceramides and ER stress. In HFD and high-fat and high-cholesterol diet (HFHC)-fed ob/ob mice, WA therapeutically improves the obesity and NASH independent of the presence of leptin signaling. (B), In the non-NASH-associated fibrosis model induced by carbon tetrachloride (CCl₄), bile duct ligation (BDL) or PPDF-BB, WA was demonstrated to activate sirtuin 3 (SIRT3) to decrease the oxidative stress and liver fibrosis by using *Sirt3*^{-/-} mice.

Figure 4: The role and mechanism of withaferin A (WA) in the treatment of liver cancer.

In hepatocellular carcinoma (HCC), WA inhibits proliferation, migration and invasion of HCC cells (Hep3B, HepG2, Huh7, QGY-7703, SK-Hep1, MHCC97H and MHCC97L cells) by elevating the levels of p90-ribosomal S6 kinase (RSK), ETS-like transcription factor-1 (ELK1), death receptor protein-5 (DR5) and extracellular-signal-regulated kinase (ERK), and activating liver X receptor α (LXR α) to inhibit NF- κ B transcriptional activity. In orthotopic liver tumor, WA inhibits liver tumor invasion and angiogenesis by downregulating the expression of pyruvate kinase PYK2 (PYK2), rho-associated coiled-coil containing protein kinase 1 (ROCK1) and vascular endothelial growth factor (VEGF).

Figure 5: The proposed metabolism routes of withaferin A (WA). Seven metabolites, M1-M7, were identified via hydroxylation, hydrogenation and hydrolysis of WA in rat or human liver microsomes, with M2, M3 and M7 specifically found in human liver microsomes.

Table 1. Clinical trials for Withania Somnifera registered in ClinicalTrials.gov.

Drugs/ Supplements	Dietary	Study title	Status	Identifier
Drug: WSE ^a	Adjunctive	Withania Somnifera	Recruiting	NCT03437668
Drug: Placebo oral tablet	(Ashwagandha)	for Persistent Symptoms in People with Schizophrenia		
Drug: Ashwagandha ^b	Ashwagandha	for Cognitive Dysfunction	Not Yet Recruiting	NCT04092647
Drug: Placebo				
Drug: Sensoril ^{®c}	Withania Somnifera:	an Immunomodulator and Anti-inflammatory Agent for Schizophrenia	Completed	NCT01793935
Drug: Placebo				
Drug: Ashwagandha		Effect of Ashwagandha on Salivary Antioxidant and Serum c Reactive Protein in Chronic Generalized Periodontitis	Completed	NCT03533972
Other: Placebo				
Dietary Supplement: Ashwagandha extract		The Effects of Ashwagandha in Endurance Exercise Performance	Unknown	NCT03596307
Dietary Supplement: Placebo				
Dietary Supplement:		Pilot Study of Curcumin Formulation	Unknown	NCT00689195

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Curcumin powder	and Ashwagandha Extract in		
Dietary Supplement:	Advanced Osteosarcoma		
Ashwagandha extract			
Dietary Supplement:	Ashwagandha: Effects on Stress,	Completed	NCT00817752
Ashwagandha	Inflammation and Immune Cell		
	Activation		
Dietary Supplement: Smart	Treatment of Fibromyalgia and CFS	Recruiting	NCT04598243
Energy System (A nutritional	With Ribose, Ashwagandha,		
/herbal combination)	Rhodiola, Licorice, Schisandra and		
	Green Tea Extract		
Drug: Sensoril	Sensoril (Ashwagandha) for Bipolar	Completed	NCT00761761
Other: Placebo	Disorder		
Drug: Withania somnifera	NF- κ B Inhibition in Amyotrophic	Not yet	NCT05031351
Drug: Placebo	Lateral Sclerosis	Recruiting	
Dietary Supplement:	Effects of an Adaptogenic Extract on	Completed	NCT03780621
Andrographis and Withania	Electrical Activity of the Brain in		
Dietary Supplement: Placebo	Elderly Subjects with Cognitive		
	Impairment		

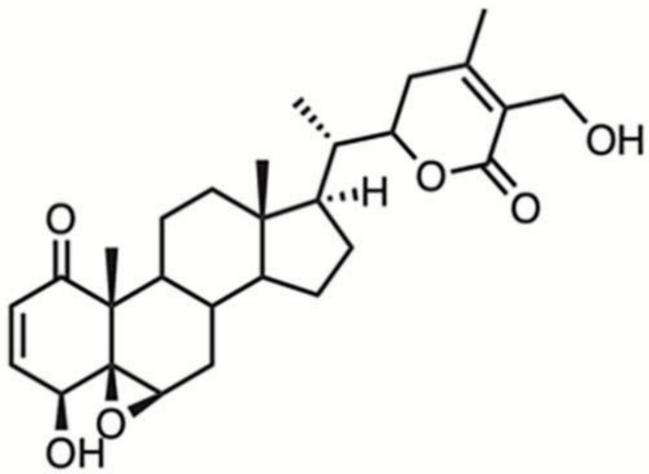
^aWSE, Withania Somnifera extract. ^bAshwagandha, Withania Somnifera. ^cSensoril® is a proprietary extract of Withania Somnifera. Each Sensoril® capsules will contain 250 mg of standardized extract of Withania Somnifera.

Table 2. The pharmacokinetic parameters of WA in experimental animals.

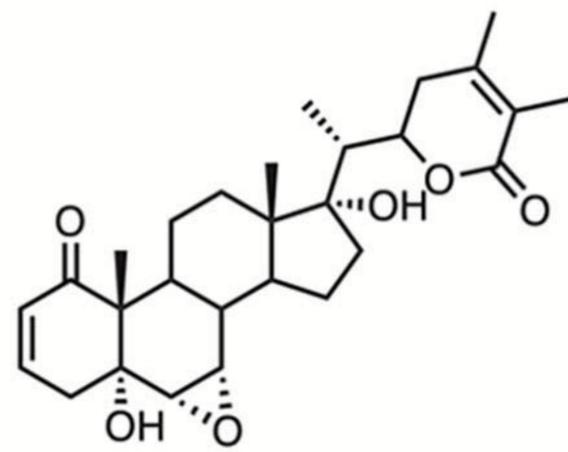
Dosing method	Animal model	Dose	Parameter	Value	Ref.
i.p.	mice	5 mg/kg WA	C_{max} (nM)	14.3 ± 1.8	(Patel et al., 2019)
			$T_{1/2}$ (h)	2.0 ± 0.6	
			AUC_{0-48h} (h nM/mL)	177 ± 10	
			$AUC_{0-infinity}$ (h nM/mL)	184 ± 12	
			K_{el} (1/h)	0.38 ± 0.11	
	mice	4 mg/kg WA	AUC_{0-t} (ng/mL hr)	541	(Thaiparambil JT, 2011)
			$T_{1/2}$ (hr)	1.3	
			CL (mL/min)	2.52	
			V_d (L/kg)	14.8	
gavage	mice	1000 mg/kg W. somnifera extract (0.4585 mg/kg WA)	C_{max} (ng/mL)	16.7 ± 4.02	(Patil D, 2013)
			T_{max} (min)	20 (20-30)	
			$T_{1/2}$ (min)	59.9 ± 15.9	
			AUC_{0-t} (ng/mL min)	1570 ± 57.8	
			$AUC_{0-\infty}$ (ng/mL min)	1670 ± 54.5	
			CL (mL/min/kg)	274 ± 9.10	
i.v.	rats	5 mg/kg WA	AUC_{0-t} (ng/mL h)	3615 ± 670	(Dai et al., 2019)
			$AUC_{0-\infty}$ (ng/mL h)	3685 ± 685	
			MRT_{0-t} (h)	4.0 ± 0.4	

			MRT _{0-∞} (h)	4.5 ± 0.8
			t _{1/2} (h)	4.5 ± 1.1
			CL (L/h/kg)	1.4 ± 0.3
			V (L/kg)	9 ± 2
			C _{max} (ng/mL)	3048 ± 509
gavage	rats	10 mg/kg WA	AUC _{0-t} (ng/mL h)	2345 ± 345
			AUC _{0-∞} (ng/mL h)	2789 ± 683
			MRT _{0-t} (h)	6.8 ± 1.1
			MRT _{0-∞} (h)	10.8 ± 4.5
			t _{1/2} (h)	7.6 ± 3.3
			T _{max} (h)	0.11 ± 0.07
			CL (L/h/kg)	3.9 ± 1.1
			V (L/kg)	41 ± 15
			C _{max} (ng/mL)	619 ± 125
			F (%)	32.4 ± 4.8

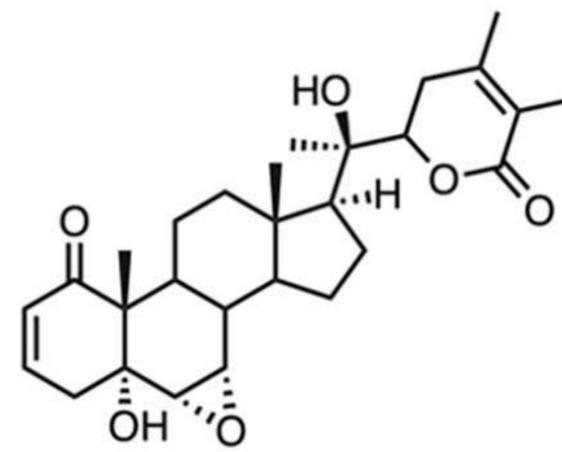
Figure 1



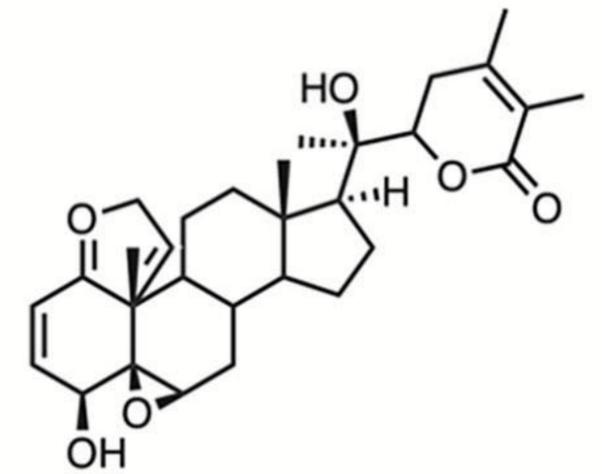
1. Withaferin A



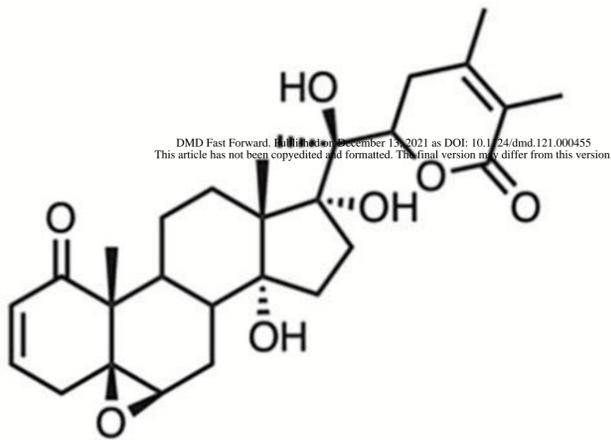
2. Withanone



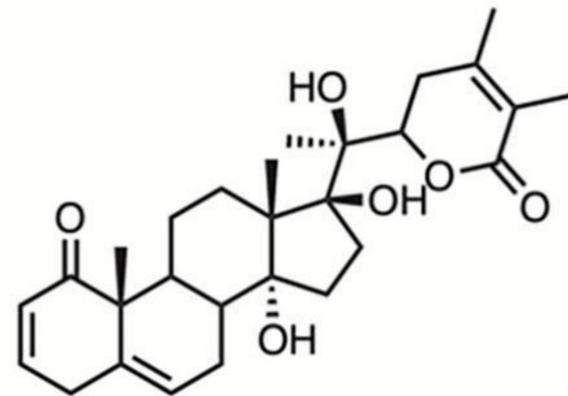
3. Withanolide A



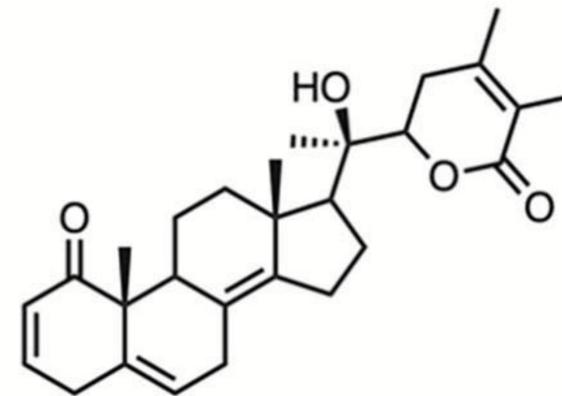
4. Withanolide D



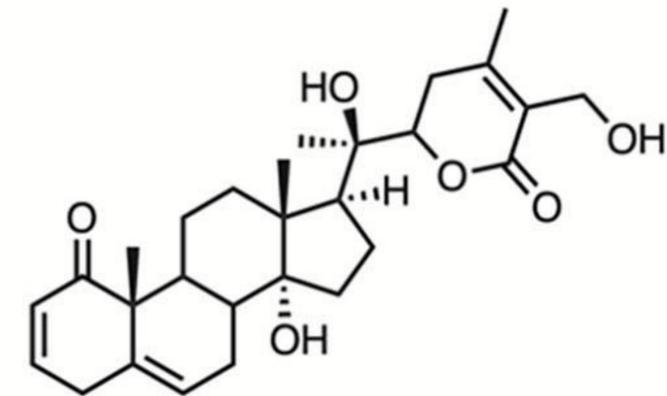
5. Withanolide E



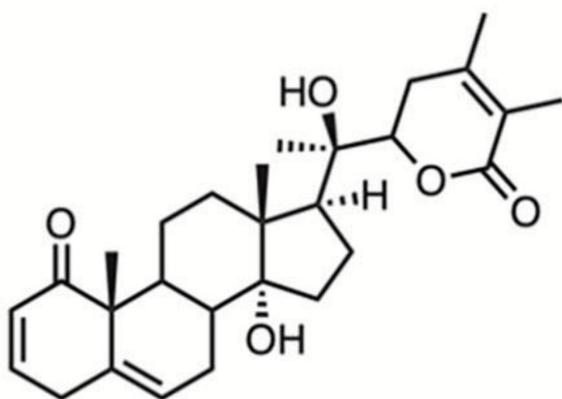
6. Withanolide F



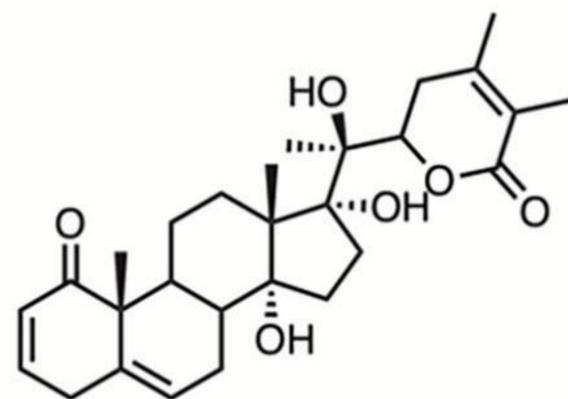
7. Withanolide G



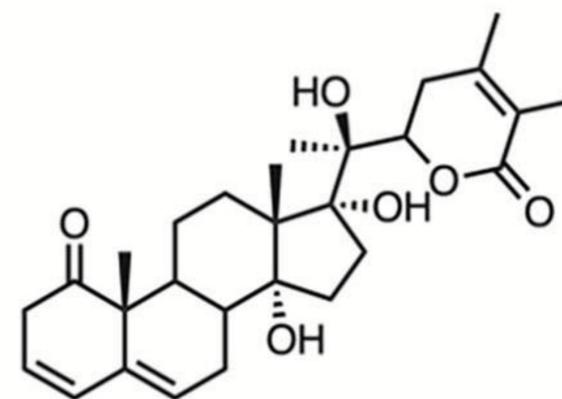
8. Withanolide I



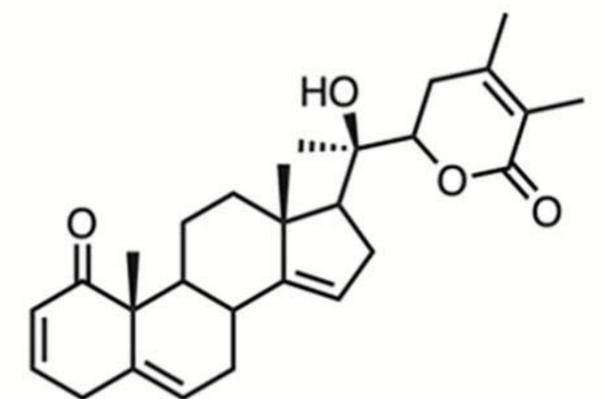
9. Withanolide J



10. Withanolide K



11. Withanolide L



12. Withanolide M

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Figure 2

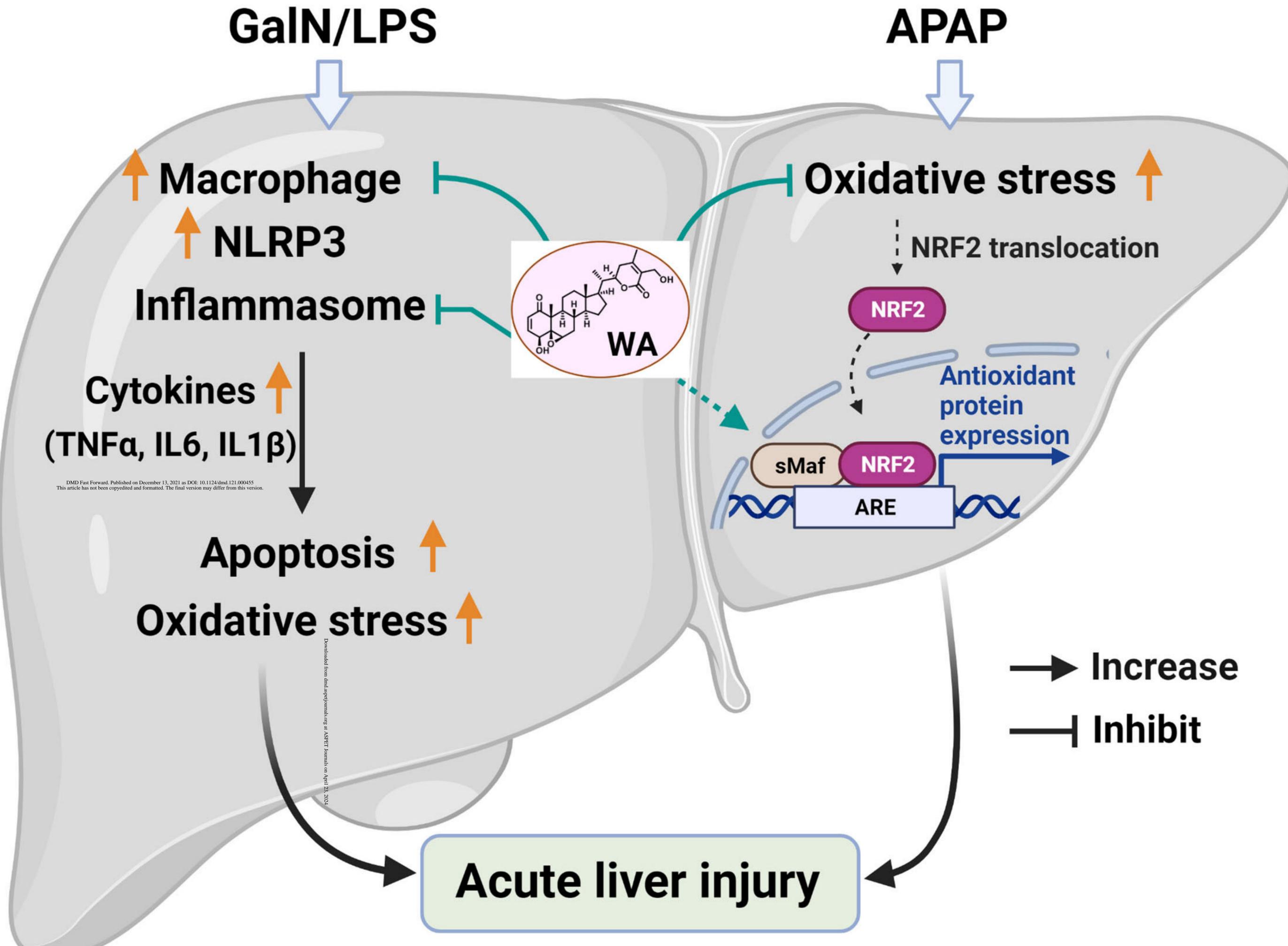


Figure 4

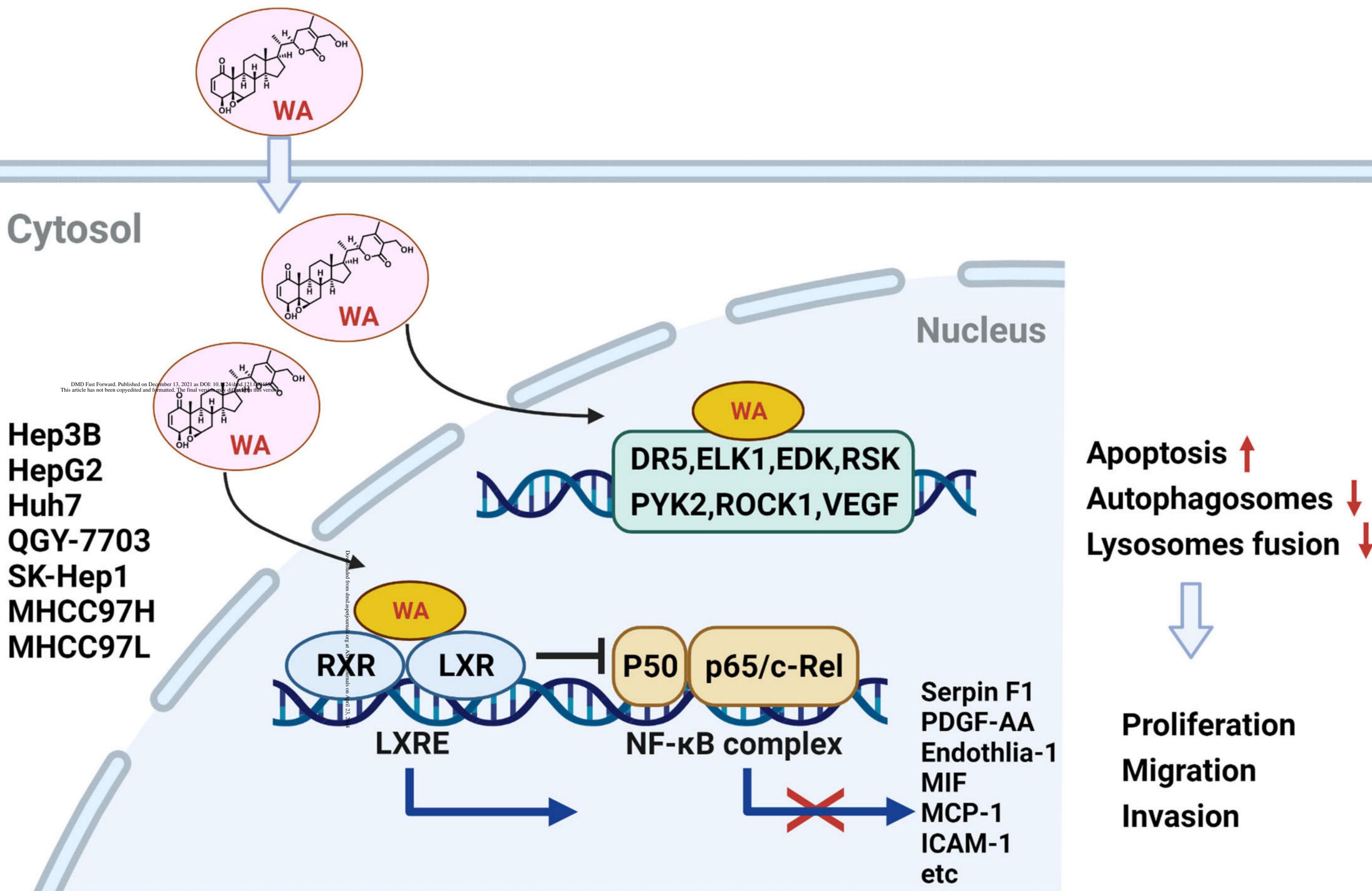
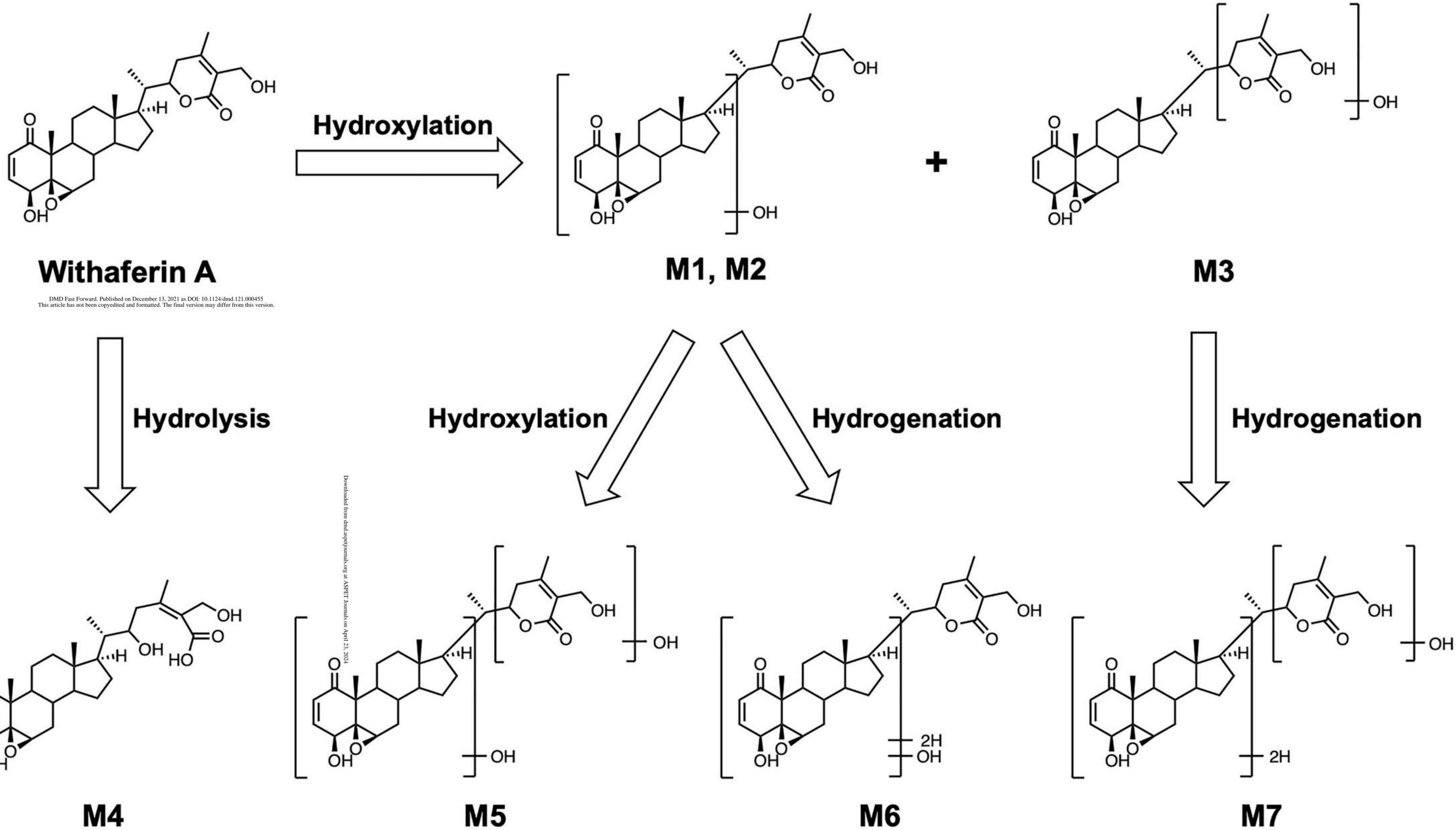


Figure 5



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