Acupuncture and Herbal Medicine

Research progress on the toxicity of *Asari Radix* et *Rhizoma*

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Abstract

Asari Radix et *Rhizoma* (ARR), also known as Xixin, has been broadly used as a traditional herbal medicine in East Asia and is an important component of classic prescriptions, including *Mahuang Fuzi Xixin* decoction. It was initially classified as a "top grade" herb in ancient Chinese Pharmacopeia, *Shennong's Materia Medica*. Volatile oils, lignans, fatty acids, flavonoids, and nitrogen-containing compounds are the main ARR components. Previous pharmacological studies have shown that ARR exerts beneficial effects in humans for treating headaches, toothaches, and several inflammatory diseases by dispelling wind and cold, alleviating pain, and eliminating phlegm. However, "the dosage of ARR should not exceed one coin (approximately 3.75 grams)," as stated in Shizhen Li's *Compendium of Materia*, which emphasized the considerable ARR toxicity and significantly constrained its clinical application. This review aimed to consolidate recent advancements in the understanding of the toxic ARR components. Additionally, we provide an overview of the hepatotoxicity, genotoxicity, neurotoxicity, and pulmonary toxicity of ARR and discuss the underlying molecular mechanisms. This study reviews the limitations of current studies and enhances our understanding of the toxic effects of ARR from the perspective of its toxic components and mechanisms, thereby providing a theoretical basis for the rational clinical practice of ARR-based medications.

Keywords: *Asari Radix* et *Rhizoma*, Powder, Toxicity, Volatile oil, Xixin

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Introduction

Asari Radix et *Rhizoma* (ARR, Xixin) is derived from the dried roots and rhizomes of *Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag, *A. sieboldii* Miq., and *A. sieboldii* Miq. var. *seoulense* Nakai, was originally listed as a "top grade" herb in *Shennong's Materia Medica* （神农本草经）, approximately 2,000 years ago. ARR is credited with dispelling wind and cold, relieving pain, opening orifices, and warming the lungs to resolve fluid retention. Clinically, it is commonly used to treat the common cold due to cold wind, headache, toothache, nasal congestion with a runny nose, sinusitis, rhinitis, rheumatic pain, and cough with phlegm. The earliest record of the dosage limit and toxicity of ARR is found in *Bencao Bie Shuo* （本草别说） during the Song Dynasty stating that "If ARR is used alone in powder form, the dose should not exceed 0.875 grams, as higher doses may induce suffocation and death," which has led to increased attention on the dosage and toxic side effects of ARR. Shizhen Li's *Compendium of Materia Medica* （本草纲目） during the Ming Dynasty also mentioned that, for solo use in powdered form, the ARR dose should not exceed one coin (approximately 3.75 g)[1]. *Yi Yi Bing Shu* (医医病书) noted that in the transmission of Chen and Li's classic books through generations, the prerequisite condition of "solo use in powdered form" was overlooked, resulting in a general rule that the ARR dose should not exceed "one coin" in all medical practices[2]. Moreover, the daily limit of ARR consumption has been restricted to 1 to 3 g in various editions of the *Chinese Pharmacopoeia*[3] without explicitly restricting its use. This deviates significantly from the original intention of the "Guidelines," which recommends the dosage restriction only for ARR use in powder form, and to some extent, limits its clinical use.

Modern toxicological researchers, both clinical and fundamental, tends to consider the current dosage restrictions on ARR unreasonable. Since 1965, seven cases of acute adverse reactions due to ARR overdose have been reported. None of the cases resulted in fatalities, and all individuals recovered well. Notably, one individual experienced acute heart failure after consuming a prescription containing 6 g of ARR[4]. Another case

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involving arrhythmia was triggered by the consumption of Xiaoqinglong decoction, containing 8 g of ARR, to manage cough and asthma symptoms[5]. Furthermore, a 70-year-old patient developed respiratory paralysis after ingesting a decoction containing 8 g ARR[6]. Notably, high levels of volatile oil residues due to the short decoction time were suspected to be the cause of these cases. This evidence shows that proper processing or decoction, rather than direct application of the powder, can greatly increase the safe ARR dose used clinically to exert stronger medicinal effects.

Another concern limiting the clinical use of ARR may be related to aristolochic acid (AA). AA, a well-known toxic substance associated with traditional Chinese medicine, is a nitrophenanthrene compound derived from plants of the Aristolochiaceae family, including ARR. AA is highly nephrotoxic, carcinogenic, and mutagenic[7,8] and is classified as a class I carcinogen. The main pathogenic components are AA I and AA II, among which AA I is the most toxic. However, researchers[9] once measured 18 ARR samples and found that AA I was not detected in the roots and stems of ARR, whereas the content in ARR leaves was 0.0313 to 0.0873 mg/kg, which was far lower than the pharmacopoeia regulations (the limit requirement was under 0.001%). Additionally, since 2005, the *Chinese Pharmacopoeia* has required that the medicinal part of ARR be changed from the whole plant to the roots and rhizomes. Therefore, the risk posed by AA in medicinal ARR can be ignored. Therefore, in this review, we do not discuss the mechanism of AA toxicity.

The present research approach to ARR toxicity, strictly adhering to traditional toxicological principles, resulted in overstated or incorrect interpretations of its toxic effects. This review aimed to provide a comprehensive and in-depth analysis of ARR in terms of toxic substances, toxic phenotype classification, and toxic mechanisms. This study aimed to guide future research on the potential toxicity of ARR with the ultimate goal of promoting its rational clinical application, including broadening the range of dosages used to achieve better therapeutic effects while avoiding toxicity.

Bioactive and potentially toxic ingredients in ARR

Previous studies have demonstrated that the main chemical ARR components include volatile oils, lignans, fatty acids, flavonoids, nitrogen-containing compounds, and so on[4], among which volatile oil is one main active components. To date, research has separated and identified various monomer components from the volatile oil of ARR, which can be divided into terpenoids, aromatics, and aliphatics. Wang et al.[10] identified a total of 66 chemical components such as phenylpropanoids, ethers, and terpenes in the volatile oil of ARR extracted by headspace injection method, mainly including camphor (11.27%), methyleugenol (10.37%), diethyl phthalate (8.49%), n-hexadecane (7.60%), 3,5-dimethoxytoluene (7.51%), 2,4,6-trimethoxytoluene (5.41%), 3,4,5-trimethoxytoluene (4.19%), D-borneol (3.74%), β-Pinene (3.30%), α-Pinene (3.18%), and eugenin (2.37%), accounting for 87.16%. Li et al.[11] extracted volatile oil using steam distillation and identified 55 compounds from seven different ARR sources using gas chromatography-mass spectrometry. They found that the main active components of ARR volatile oils are methyleugenol, safrole (SFO), 3,5-dimethoxytoluene, 1,2,3-trimethoxy-5-methylbenzene, myristin, and elemicin. Furthermore, it has been observed that the volatile oil content was variable in ARR from different origins, with methyl eugenol, SFO, and 3,5-dimethoxytoluene varied greatly (8.89%–47.58%, 9.61%–29.1%, and 2.02%–16.39%, respectively). Studies in animals have indicated that ARR volatile oils are the primary toxic components. For instance, intraperitoneal injection of 0.2 mL/kg of ARR volatile oil in mice resulted in reduced movement and retention of the righting reflex. Injection of 0.5 mL/kg led to mice lying still, whereas doses of 0.6 and 1.2 mL/kg ARR volatile oil resulted in instant death.

Lignans comprise the second largest group of components in the ARR, most of which are non-volatile or weakly volatile. The antiviral, antibacterial, and anti-inflammatory effects of lignans extracted from ARR have been well described[12]. ARR also contains numerous fatty acids, with the root and rhizome containing the most fatty acids. Among these, ferulic acid has good anti-oxidant effects, whereas linoleic acid has anti-inflammatory effects. However, linoleic acid has recently been reported to increase the risk of the exacerbation of respiratory disease asthma[5]. Flavonoids in ARR primarily exist in the form of glycosides and exhibit pharmacological properties such as anti-inflammatory, analgesic, antibacterial, antiviral, and immune-enhancing activities. Several nitrogen-containing compounds in ARR have anti-inflammatory, asthmatic, and anti-oxidant effects. Based on their structural diversity, these ingredients can be further divided into amide alkaloids, phenanthrene alkaloids, and nitrophenanthrene carboxylic acids according to their structural diversity[13]. In addition to the compounds mentioned above, other compounds have been identified in ARR, including steroids such as beta-sitosterol[14], amides such as n-isobutyl-dodecatetraenoylamide, and phenolic acids such as 4-hydroxybenzoic acid[15]. Other researchers have extracted small amounts of essential amino acids such as isoleucine[16]. However, there are few toxicity reports or toxicological studies of these compounds.

ARR hepatotoxicity

ARR, as a natural herbal medicine, is generally considered safe and effective at therapeutic doses; however, multiple studies have found that long-term (28 d) or excessive use (over 3 g/d/person) of ARR can lead to liver dysfunction and possibly develop into severe liver injury[17–20], including different degeneration forms, varying degrees of inflammation in the lobules or around the central vein, and even necrosis. Different ARR components may induce hepatotoxicity through diverse mechanisms. These results indicate that ARR powder may cause liver damage by interfering with the redox status inside liver cells, damaging cell membranes, and inhibiting the detoxification enzyme system. Specifically, volatile oil, the main active ingredient of ARR, has been confirmed

to possess hepatotoxicity, primarily by inducing oxidative stress through the disruption of the anti-oxidant enzyme system, which will be thoroughly discussed in the following section[21]. Other compounds account for a relatively small proportion of ARR and are not the primary contributors to its pharmacological and toxicological effects; there are few studies on the hepatotoxic effects of these compounds, and it is not entirely clear whether they have a definite hepatotoxic mechanism. Nonetheless, studies have shown that compounds such as eucalyptol, sesamin, and ferulic acid have been shown to exert protective effects against liver damage[22–26], providing a reference for future research (Figure 1).

Hepatotoxic mechanism of ARR powder

The hepatotoxic mechanism of ARR powder is a complex process involving various aspects such as bile acid metabolism, amino acid metabolism, and carbohydrate metabolism. In a comprehensive study, Cao et al.[27,28] employed solid-phase microextraction and gas chromatography-mass spectrometry to analyze the volatile ARR components, predicted the bioactive targets of these components using Swiss Target Prediction, and identified potential liver damage targets using DisGeNET. The *in vivo* study indicated that high-dose ARR (1.35 g/kg) administration for 28 d caused severe liver injuries, evidenced by balloon degeneration and punctate necrosis of hepatocytes. Metabolomic analysis revealed that the metabolic pathways affected by ARR mainly included the metabolism of phosphoinositol, galactose, and taurine. Transcriptomic sequencing results of liver tissues from the control and high-dose ARR-treated rats further revealed 434 genes with significant alterations, which were involved in the circadian rhythm, p53 signaling, steroid biosynthesis, and other pathways. However, the interpretation of metabolomic and transcriptomic data may be subject to potential bias by researchers, and the long-term or delayed toxic effects may not be fully revealed within a specific experimental period. Hence, more independent studies are needed to verify or supplement the results obtained.

Hepatotoxic mechanisms of volatile oil components in ARR

The hepatotoxic effects of the volatile oil components of ARR are diverse and include cytological and histological changes, oxidative stress promotion, cellular damage, cytochrome P450 (CYP450) enzyme inhibition, and toxic metabolite activation.

Cytological and histological alterations

In several studies on the hepatotoxic effects of methyleugenol and α-asarone, particular attention was paid to the cytological and histological alterations. Abdo et al.[29] conducted a 14-week study on rodents to observe the effects of methyleugenol and found that methyleugenol (300 and 1,000 mg/kg) induced significant hepatic pathological changes, including bile duct proliferation, inflammatory infiltration, increased liver enzyme activities, and bile acid concentrations, indicating hepatocyte damage and functional changes. López et al.[30] investigated the effects of α-asarone on long-term cultures of adult rat hepatocytes, which were particularly cultured on a 3T3 cell support layer, finding that the exposure to α-asarone (25–50 μg/mL) resulted in accumulation of lipid droplets, enlargement and vacuolization of the mitochondria, and suppression of protein synthesis and secretion. These observations suggested that α-asarone significantly affected the functional and survival capabilities of liver cells. It is worth noting that most hepatotoxicity studies on ARR have mainly focused on parenchymal hepatic cells, while the changes occurring in non-parenchymal cells that also play important roles in liver physiology and pathology, such as bile duct epithelial cells, intrahepatic bile duct epithelial cells, hepatic stellate cells, Kupffer cells, and sinusoidal endothelial cells, have not yet been thoroughly explored. The response of these cells to the ARR components may provide novel insights into the complex mechanisms of hepatotoxicity.

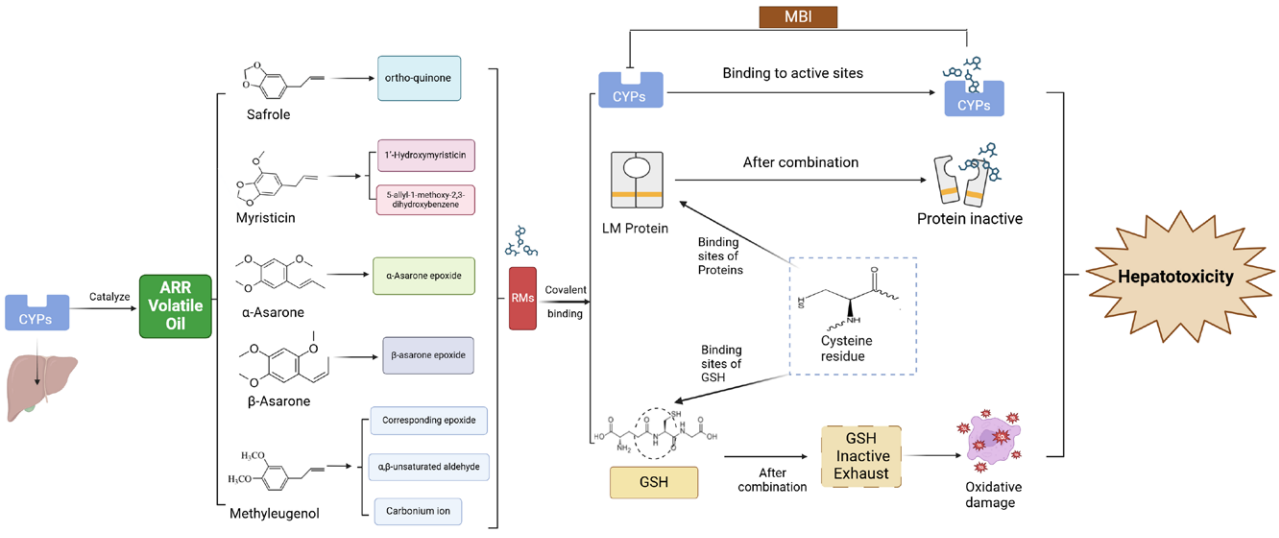

Figure 1. Molecular mechanisms underlying ARR hepatotoxicity. ARR: *Asari Radix* et *Rhizoma*; CYP: Cytochrome P450; GSH: Glutathione; LM: Liver microsomes; MBI: Mechanism-based inactivation; RMs: Reactive metabolites.

Formation of reactive metabolites (RMs)

A key factor affecting the toxicity of natural substances is their transformation into RMs during metabolic biotransformation. CYP450 plays a crucial role in this process by converting xenobiotics into chemically reactive intermediates and stable metabolites. The resulting product, RMs, can bind to the catalytic site of the enzyme when it is highly reactive, leading to its inactivation. This process is known as mechanism-based inactivation (MBI)[31]. The reactive compounds formed covalently combine with liver macromolecules and are often associated with liver toxicity, as highlighted by Masubuchi et al.[32]. Fontana et al.[33] summarized the most frequent substructures responsible for the MBIs of CYPs, including those containing methylenedioxyphenyl. Compounds such as SFO and myristicin have substructures similar to those of methylenedioxyphenyl and, therefore, have been studied extensively. Ueng et al.[34] evaluated the inhibitory effect of SFO using *Escherichia coli*-expressed human P450 and human liver microsomes (HLM) and determined the content and activity of CYPs in the presence of SFO *via* fluorescence and high performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection. The results showed that SFO had inhibitory effects on human CYPs (CYP1A2, CYP2D6, CYP2E1, CYP3A4, and CYP2C19), especially on CYP1A2 (half maximal inhibitory concentration [IC_{50}] values: 5.7 ± 0.9 μM). Yang et al.[35] conducted a CYP cocktail screening assay on metabolically active HLM in the presence of SFO, and found that SFO inhibited CYP enzymes in a concentration-dependent manner. Among these, CYP1A2 exhibited the strongest inhibitory effect. The researchers further identified the RMs (quinones and their tautomers) of SFO in HLM, indicating that SFO may undergo a CYP-mediated MBI process to produce RMs, resulting in the subsequent inhibition of enzyme activity and hepatotoxicity. Another similar study[36] explored the hepatotoxic mechanism of SFO in HepaRG cells, finding that SFO's toxicity was increased when CYP1A2 was activated by omeprazole whereas decreased upon CYP1A2 inhibition by a-naphthoflavone (α-NAP), which was linked to the formation of RMs. Moreover, the depletion of cellular glutathione (GSH) increased SFO toxicity, confirming that SFO hepatotoxicity is likely mediated by ortho-quinone RMs, in which CYP1A2 plays a central role.

Similarly, researchers[37] found that compared with other CYP enzymes in HLMs, myristicin (5 and 50 μM) showed a more significant inhibitory effect on CYP1A2 (IC_{50} = 1.796 μM), suggesting it may be an MBI of CYP1A2. Moreover, the relative activity of CYP1A2 recovered from 48.6% to 91.2% when the GSH concentration was close to 1.796 μM, confirming the role of GSH in rescuing the inhibition. Yu et al.[38] also employed the "Cocktail" probe substrate method to screen the inhibitory effects of SFO, myristicin, methyleugenol, and α-asarone on CYPs and investigated their binding capacities with CYP1A2 enzyme using semi-flexible molecular docking. Among them, the active metabolic intermediates produced by SFO and myristicin had a significantly higher binding capacity with the CYP1A2 enzyme than the other four volatile oil components, indicating that the active intermediates and the CYP1A2 enzyme were more likely to form a structurally stable complex, resulting in accumulation in the body and the production of toxic effects. Besides reacting with CYPs, RMs also bind to specific proteins and induce hepatotoxicity. A study showed that RMs produced by methyleugenol, including epoxide, α,β-unsaturated aldehyde, and carbonium ion, modified cysteine residues of hepatic protein and facilitated the adduction of protein, indicating a correlation between increased protein covalent binding and hepatotoxicity of RMs[39]. Notably, the IC_{50} value varies with the experimental context, such as the cell type and the particular biochemical pathway under investigation; therefore, it is difficult to judge toxicity by directly comparing the IC_{50} values of volatile oils and their metabolites.

It is reported[40] that a single intraperitoneal injection of SFO (1,000 mg/kg) in rats induced the hepatic accumulation of markers of oxidative damage, lipid hydroperoxides (LHP) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG), and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, confirming its hepatocellular toxicity. Additionally, we assessed the protective roles of vitamin E, deferoxamine, and N-acetylcysteine against oxidative injury induced by SFO. Among the three anti-oxidants tested, N-acetylcysteine, a GSH, showed the strongest protective effect. In contrast, buthionine sulfoximine, an inhibitor of GSH synthesis, enhanced the oxidative damage caused by SFO, as confirmed by increased LHP and 8-OH-dG levels. Nakagawa et al.[41] investigated the effects of hydroxychavicol (HC), a catechol component that is a major SFO metabolite, in rats and humans. They found that exposure of freshly isolated rat hepatocytes to HC (0.25–1.0 mM) for 0 to 3 hours resulted in cell death and the depletion of cellular ATP, reduced GSH, and protein thiols, as well as the accumulation of GSH disulfide and malondialdehyde in a dose- and time-dependent manner, suggesting the occurrence of lipid peroxidation. Further mechanistic research indicated that mitochondria were the target organs of HC and that the cytotoxic effects of HC were more pronounced than those of SFO, providing novel insights into the hepatotoxicity of SFO.

The configuration of double bonds in the side chain of α-asarone and β-asarone is different. β-Asarone had been proven to be cytotoxic to human liver cells (THLE-2 cells, IC_{50} = 40.0 ± 2.0 μg/mL), which was associated with notable lipid peroxidation and GSH depletion[42]. Zhao et al.[43] compared the oxidative damage capacity caused by α-asarone (50 μM) and β-asarone (50 μM) through *in vivo* and *in vitro* studies, finding that the primary metabolic pathway of β-asarone is mainly involved in the epoxidation at C-1′ and C-2′, while α-asarone is mainly metabolized into the corresponding alcohol produced by C-3′ hydroxylation, evidently indicating greater metabolic activation for β-asarone. In addition, CYP1A2 dominated the metabolism of α-asarone and β-asarone, and the orientation of β-asarone in the active site of CYP1A2 is more conducive to its epoxidation than that of α-asarone. Such metabolic processes can lead to

the production of reactive oxygen species (ROS) in liver cells, thereby triggering oxidative stress and resulting in cell damage and liver dysfunction.

Synthesizing the above studies, we found one commonality in the hepatotoxicity mechanisms induced by different monomers of ARR essential oil, which is mainly induced by RM-mediated MBI and oxidative stress. However, there are variations in the specific manifestations of liver toxicity caused by these compounds. For instance, they differ in selectivity toward specific CYP450 enzymes, as well as metabolic products and toxicity levels, which may be related to the structural characteristics of the compounds and the individual's metabolic capacity for these compounds. Considering that the current research mainly focuses on acute and high-dose exposure, future studies should concentrate on long-term exposure and low-dose effects to comprehensively assess the liver toxicity of these compounds. Moreover, the variability in genetic susceptibility and metabolic abilities among individuals is a crucial aspect to consider in future research.

Genetic toxicity and carcinogenic mechanism of ARR

In 2010, it was reported that ARR decoction induces mouse polychromatic erythrocyte micronuclei and sperm abnormalities[44], indicating that ARR has potential mutagenic effects. Several studies have demonstrated the genetic toxicity and carcinogenicity of alkenylbenzene compounds derived from rodent spices and herbs[45,46]. Herein, we discuss the relationship between ARR-induced genetic toxicity and structural diversity (Figure 2).

Genotoxicity and carcinogenicity caused by the formation of DNA adducts

Genotoxicity and carcinogenicity typically occur because of DNA adduct formation, which involves the binding of external compounds (such as carcinogens) to specific sites on DNA molecules. This binding can disrupt the normal DNA replication and repair processes, leading to genetic variation or damage. Notably, the specific mechanism may vary depending on the chemical substance and the reaction mechanism of the organism. DNA adduct formation is widely recognized as a key factor in genotoxicity and carcinogenesis.

A previous study indicated that DNA adducts were observed in human hepatoma cells ($HepG_2$ cells) exposed to six aforementioned allylbenzenes (SFO, estragole, methyleugenol, myristicin, dill apiol, and parsley apiol) at 50, 150 and 450 μM, as assayed by the monophosphate ^{32}P-post-labeling, with estragole, methyleugenol, SFO, myristicin, dill apiol, and parsley apiol ordered in decreasing potency[47]. These findings demonstrate that these alkenylbenzene compounds may directly contribute to human carcinogenesis by promoting DNA transformation. Recent studies have investigated the DNA-damaging potential of various alkenylbenzene compounds using the Turkey Egg Genotoxicity Assay. Specifically, turkey eggs aged 22- to 24-day-old fetuses were injected three times using alkenylbenzene compounds: SFO (1, 2 mg/egg), methyleugenol (2, 4 mg/egg), estragole (20, 40 mg/egg), myristicin (25, 50 mg/egg), elemicin (20, 50 mg/egg), anethole (5, 10 mg/egg), methyl isoeugenol (40, 80 mg/egg), eugenol (1, 2.5 mg/egg), and isoeugenol (1, 4 mg/egg). Fetal liver samples were collected to measure DNA strand breaks using the comet assay and DNA

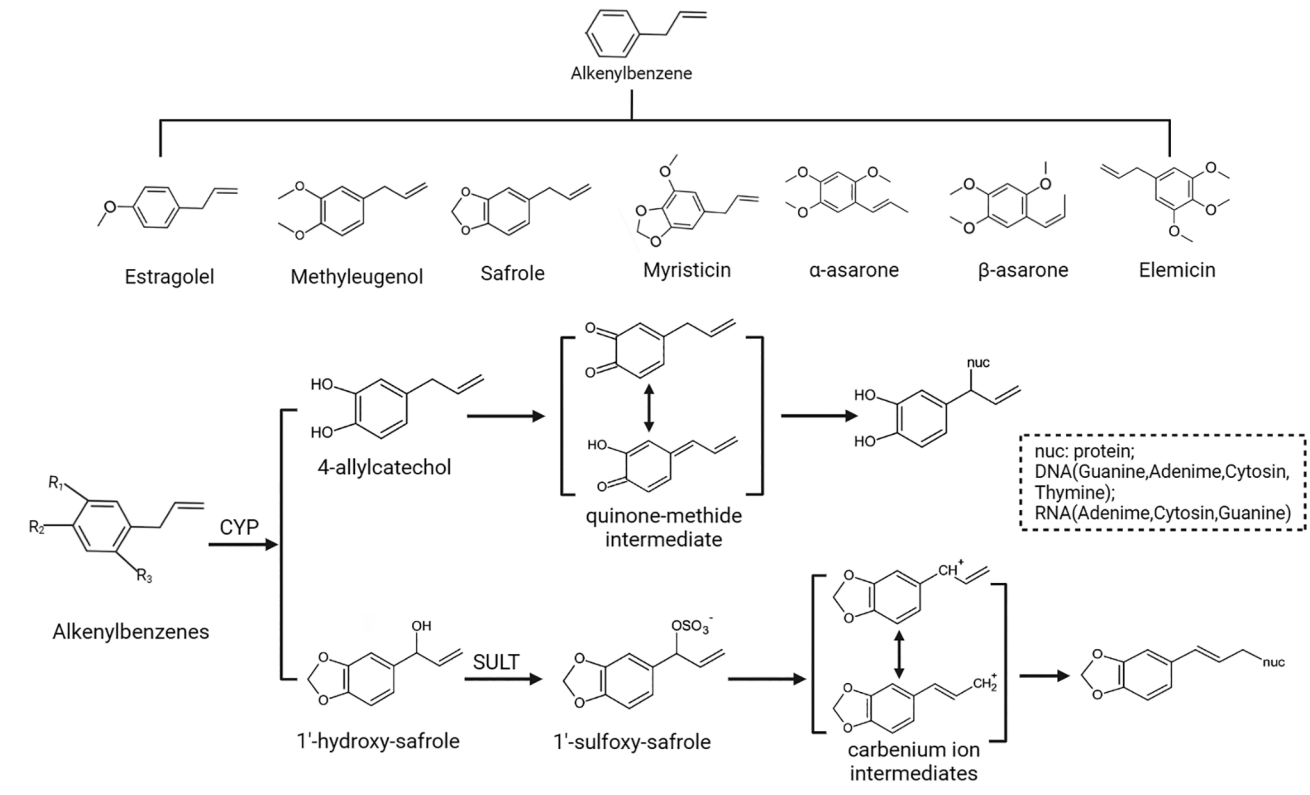

Figure 2. Molecular mechanisms underlying AEO genotoxicity. AEO: *Asari Radix* et *Rhizoma* essential oil; CYP: Cytochrome P450; SULT: Sulphotransferase.

adduct formation using the nucleotide ^{32}P-post-labeling assay after the last injection. These results showed that alkenylbenzene compounds at the highest dose induced the formation of DNA adducts, demonstrating that these natural volatile components have significant mutagenic and carcinogenic effects[48,49]. However, despite these studies confirming that the genotoxicity of ARR volatile oil is closely related to the characteristic structure of the compounds and providing effective experimental methods and possible test standards for evaluating genotoxicity and carcinogenic potential, experimental evidence and systemic investigation of the underlying biochemical mechanisms remain unsatisfactory.

Genotoxic effect of SFO

In 1983, a bioactivation pathway for SFO was proposed, involving hydroxylation of the benzyl carbon, conjugation with sulfate, and subsequent alkylation of DNA with displacement of the sulfate group[50]. Martati et al.[51] found that the hepatogenic and genotoxic effect of SFO was meditated by the formation of a DNA-reactive 1'-sulfooxysafrole in rodents at high dose levels (eg, 50 mg/kg), and further found that malabaricone C as a major constituent to inhibit SFO DNA adduct formation by sulfotransferase-mediated bioactivation, indicating that sulfotransferase was essential in the process of SFO-induced genotoxicity and providing a potential method to reduce the carcinogenic risk of SFO. Another study suggested that 1'-hydroxysafrole of SFO was converted into unstable sulfates and formed adducts with DNA, and the main P450 enzymes involved in the liver 1'-hydroxysafrole metabolism pathway were CYP2C9 and CYP2E1, with CYP2E1 showed three times of intrinsic clearance to CYP2C9, further demonstrating that CYP450 was a key enzyme involved in the 1'-hydroxysafrole metabolism[52]. Moreover, a previous study demonstrated[53] that another metabolite 2', 3'-oxide of SFO was an electrophilic metabolite that also can react with DNA bases to form DNA adducts, inducing the formation of N7-(3-benzo[1,3] dioxol-5-yl-2-hydroxypropyl) guanine (N7γ-SFO-Gua). Subsequently, Wu et al.[54] elucidated the genotoxic mechanisms of safrole oxide SAFO (30, 60, 90, and 120 mg/kg) in mice. They observed a significant N7γ-SAFO-G production in the liver and urine samples of SAFO-treated mice, by applying the isotope-dilution ultraperformance liquid chromatography coupled with tandem mass spectrometry (ID-HPLC-ESI-MS/MS) method, demonstrating that SFO-induced genotoxicity may be in part mediated by its metabolic activation to SAFO.

Genotoxic effect of methyleugenol

Mutagenic effects and underlying mechanisms have been investigated in several studies. 1'-hydroxymethyleugenol is the main oxidative metabolite of methyleugenol and undergoes phase I metabolism catalyzed by CYP450[55]. In humans, the activity of sulfotransferase 1A1 (SULT1A1) is affected by genetic polymorphisms, such as single-nucleotide polymorphisms and copy number variations. Tremmel et al.[56] used the ID-HPLC-ESI-MS/MS method to quantify methyleugenol DNA adducts in 121 human surgical liver samples and detected methyl eugenol DNA adducts in all samples studied, and their levels were significantly correlated with both mRNA and protein levels of SULT1A1 (r_s = 0.43, and 0.44, respectively). Besides, Herrmann et al.[57] further demonstrated that methyleugenol in low-dose level (0.05 mg/kg) could form DNA adducts (eg, N6-(trans-methylisoeugenol-3′-yl)-2′-deoxyadenosine and N2-(trans-methylisoeugenol-3′-yl)-2′-deoxyguanosine) that was enough to be detected in SULT1A1 knockout and human SULT1A1/2 transgenic (tg) mice. It is worth noting that the DNA adducts formed by methyleugenol in SULT1A1 knockout mice were significantly reduced, while they were significantly increased in human SULT1A1 transgenic mice, indicating that 1′-hydroxymethyleugenol predominantly catalyzed *via* SULT1A1. However, this intermediate is unstable and spontaneously decomposes upon sulfate cleavage into a highly reactive carbocation that can interact with DNA. Carlsson et al.[58] demonstrated that methyleugenol induces DNA adducts to trigger DNA replication. It also induces caspase-dependent mitochondrial apoptosis by activating the p53-Bax pathway in hepatocytes, ultimately leading to DNA fragmentation. While the relationship between methyleugenol metabolism, genotoxicity, and carcinogenicity is well-established, research remains limited on how methyleugenol-induced DNA adducts interfere with DNA replication, trigger DNA damage responses, and modulate downstream signaling pathways that govern cellular responses.

Genotoxic effect of estragole

Estragole, which is often used as a food additive in cosmetics, has been shown to possess genotoxic properties that can cause liver cancer in rodents[59–60]. The mechanism of estragole-related carcinogenicity is similar to the SFO and methyleugenol, which is meditated through hydroxylation and subsequent sulfoconjugation catalyzed by CYP450 and SULT1A1, leading the transformation of estragole to the proximate carcinogen 1'-hydroxyestragole and 1'-sulfoxyestragole[59]. A previous study[61] verified that the levels of estragole-DNA adducts and 1'-hydroxyestragole glucuronide detected by LC-MS/MS in the liver were significantly higher than those in the kidneys and lungs. Another study[62] further found sex differences in SULT1A1 activity in the mouse liver[62]. In this study, we assessed the frequency of micronuclei in polychromatic erythrocytes and the mutant frequency of reporter genes in B6C3F1 gpt delta mice treated with estragole at doses of 0, 37.5, 75, 150, and 300 mg/kg by gavage for 13 weeks; the highest dose in female mice was reduced to 250 mg/kg. The levels of estragole-specific DNA adducts in females were higher than those in males at all doses except the highest dose. These data demonstrate that estragole-induced hepatocarcinogenesis is significantly affected by SULT1A1-mediated carbocation formation and the resultant genotoxicity. In addition, another study conducted in-depth research, which detected the expression of sulfotransferases in the liver and bile ducts in a simulated liver and bile duct cancer model using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry

techniques, and found that SULT2B1, rather than SULT1A1, was expressed in both rat and human bile duct cells, suggesting that cholangiocarcinoma in rats fed estragole might partly rely on the activation of the 1'-hydroxysterol metabolite SULT2B1, and DNA damage in bile duct cells may be related to their metabolic activity toward 1'-hydroxyestrogens, providing new insights into the mechanisms by which estragole promoted cholangiocarcinoma[63]. The hepatocarcinoma and cholangiocarcinoma models applied in these studies provide an effective framework for researching the impact of compounds on specific types of cancer, helping us better understand how compounds affect physiological environments.

Genotoxic effect of asarone

Asarone has two configurations, α-asarone and β-asarone, both of which are classified as carcinogens in rodents and possess genotoxic properties[64,65]. Stegmüller et al.[66] studied the reactivity of these isomers with nucleosides using ultra-high-performance liquid chromatography-ultraviolet/visible detection (UPLC-UV/VIS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and nuclear magnetic resonance (NMR) spectroscopy. They identified epoxide derivatives of DNA adducts of 2'-deoxyadenosine and 2'-deoxyguanosine, named N^6-1'-hydroxy-dihydroasarone-da and N^2-1'-hydroxy-dihydroasarone-dg, respectively. The chemical synthesis of these adducts, along with the preparation of isotopically labeled standards and the development of sensitive and specific isotope dilution mass spectrometry techniques, enabled the quantitative analysis of DNA adducts formed in primary rat hepatocytes incubated with α-asarone or β-asarone for 48 hours. DNA adduct formation was almost linearly related to the concentrations of the compounds. Following research by Uebel et al.[67] and Hermes et al.[68] suggested that the observed genotoxicity and carcinogenicity may be related to Phase I metabolites and epoxide products of asarone isomers. Further research conducted by Zhao et al.[43] demonstrated that the primary metabolic pathway of β-asarone was meditated by the epoxidation at C-1′ and C-2, while that of α-asarone was meditated by the hydroxylation at C-3′ to form corresponding alcohols. Furthermore, they found that CYP1A2 dominated the metabolism of α-asarone and β-asarone, and β-asarone's orientation at the CYP1A2 active site facilitates its epoxidation showed more effect than that of α-asarone. These findings provide important information for understanding the metabolic pathways and pathogenic mechanisms of the asarone isomers.

Genotoxic effect of elemicin and myristicin

A previous study that administered elemicin to male F344 *gpt* delta rats *via* gavage at various doses (0, 25, 100, or 400 mg/kg) for 13 weeks[69] found that rats receiving 100 mg/kg or higher doses showed a significant increase in liver weight; at a dose of 400 mg/kg, there was a significant increase in serum parameters, indicating liver toxicity. Besides, a previous study[47] compared the capacity of allylbenzenes (myristicin, SFO, estragole, and methyleugenol) for forming DNA adducts in $HepG_2$ cells *via* ^{32}P-post-labeling, finding that all allylbenzenes could form DNA adducts in cultured human hepatocytes, with estragole > methyleugenol > SFO ≈ myristicin in decreasing order, demonstrating that elemicin and myristicin may contribute to carcinogenesis by altering the cellular DNA.

In summary, the mechanism of genotoxicity of alkenylbenzene compounds in ARR volatile oil is mostly related to its characteristic structure (Figure 2). The presence of double bonds in the alkenyl side chain at the terminal 2', 3'-position facilitates the formation of approximately carcinogenic 1'-hydroxyl metabolites or terminal epoxides, or carbocation ion formation that can mediate DNA adduct formation, or the absence of free phenolic hydroxyl groups that are essential for the formation of non-toxic gluconic acid conjugates. In addition, most of these compounds are activated by the metabolic processes of CYP450 and sulfotransferases and are converted into DNA adducts.

Genotoxicity induced by oxidative DNA damage pathways

In contrast to the genotoxicity and carcinogenicity associated with DNA adduct formation, oxidative DNA damage, including base oxidation, single-strand breaks, and cross-linking, is typically induced by ROS or compounds that directly attack DNA molecules and impair their bases and sugar backbones. Oxidative damage can lead to incorrect base pairing, resulting in point mutations or DNA damage during replication. Chronic oxidative damage causes genomic instability in cells, potentially leading to tumorigenesis. Therefore, oxidative DNA damage is a crucial pathway for genotoxicity and carcinogenicity.

SFO can transform into hydroxychavicol (4-allyl-1,2-dihydroxybenzene) through the cleavage of the methylenedioxy ring and further biotransform to o-quinone through 2-electron oxidation, which may induce oxidative damage *in vitro*[70]. Another study further demonstrated hydroxychavicol-induced 8-OH-dG formation and found that the GSH precursor, N-acetylcysteine, had the strongest protective effect in examining the protective role of anti-oxidants in rats (eg, vitamin E, deferoxamine, and N-acetylcysteine) in SFO-induced oxidative damage. However, the GSH synthesis inhibitor enhanced SFO-induced oxidative damage, as evidenced by the elevation of LHP and 8-OH-dG levels on day 3 ($P < 0.05$)[40]. By measuring oxidative damage using human endonuclease III and 8-oxoguanine-DNA-N-glycosylase-modified comet assays, Ding et al.[71] found hepatic DNA damage in estragole (300, 600, and 1,000 mg/kg)-and SFO (125, 250, and 450 mg/kg)-treated rats without digestion or with either endonuclease III or human 8-oxoguanine-DNA-N-glycosylase digestion, indicating that oxidative DNA damage induced by SFO and estragole may contribute to their genotoxicity. Similarly, another study found that digestion with Endo III led to increases in DNA damage in enzyme-modified comet assays in rats administered methyleugenol (400, 1,000, and 2,000 mg/kg), demonstrating that methyleugenol can also induce oxidative DNA damage, which may be partly responsible for its genotoxicity[72].

Genotoxicity induced by RNA adducts formation

Genotoxicity induced by RNA adduct formation is a relatively new area of research that primarily involves the combination of RNA molecules with certain chemicals or metabolic products, thereby affecting normal RNA function and gene expression in cells. The formation of RNA adducts can have various effects on cellular functions, leading to genotoxicity. Wang et al.[73] explored the potentially toxic effects of SFO and its metabolites on RNA. In this study, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify the RNA adducts formed in the mouse liver S9 fraction, primary liver cells, and liver tissues following SFO exposure. Three guanosine adducts (G1–G3), two adenosine adducts (A1–A2), and two cytosine adducts (C1–C2) were also detected. These adducts were chemically synthesized, and the structural characteristics of one guanosine adduct were identified through proton nuclear magnetic resonance (^{1}H-NMR). Results showed that SFO was oxidized by CYP450 into respective 1'-hydroxy metabolites that were further metabolized by sulfotransferases to form allyl sulfate esters. Subsequently, these intermediates react with RNA bases, resulting in RNA adduction. This study indicated that RNA adduct formation could partially contribute to the toxicity of SFO by altering the biochemical characteristics of RNA and interrupting its function, providing new insights into the toxicological mechanisms of SFO and emphasizing the importance of considering its potential effects on RNA when evaluating the toxicity of SFO and its metabolites.

Overall, the study of RNA adduct formation-induced genotoxicity not only reveals the toxicity and RNA metabolic mechanisms of compounds but also expands our understanding of their genotoxic mechanisms. This contributes to a more accurate assessment of the risks of traditional Chinese medicine compounds in drug safety evaluation and toxicological research. However, the current understanding of RNA adduct formation and the resulting genotoxicity remains limited. Future studies should investigate how RNA adducts affect cellular functions and health. Moreover, research in this area may provide new insights into the pathological mechanisms and potential therapeutic methods for several diseases.

Neurotoxicity of ARR

Wang et al.[74] found that ARR essential oil (AEO) (1.05 g/mL) mainly contained methyleugenol, SFO, and 3,5-dimethoxytoluene, which retarded insect behavior, suggesting its potent biotoxicity and repellency to insects. Liu et al.[75] further analyzed the active substances in AEO and found that the heavy fraction had high SFO and methyleugenol concentrations, while the light fraction contained high α- and β-pinene concentrations. Subsequent *in vitro* toxicity tests revealed that AEO-stimulated PC12 cells exhibited significantly abnormal nuclei, accelerated apoptosis, and oxidative damage, including increased ROS formation and decreased superoxide dismutase generation. Consistent with this, *in vivo* experiments showed that the heavy fraction had the smallest LD_{50} (1.9566 g/kg), which resulted in the highest mortality in mice, indicating that AEO has significant biotoxicity and may cause neurological damage. Guo et al.[76] investigated the acute neurotoxicity of methyleugenol in the hippocampus and found that methyleugenol (0.1 mM) decreased the phosphorylation of the glutamate AMPA receptor subunit 1, which caused learning disabilities and potentially anxious behavior in mice. The nervous system, including the medulla oblongata, regulates respiratory behavior by affecting skeletal muscles and vague nerves. Zhou et al.[77] found that rabbits experienced a process of respiratory excitation followed by inhibition after oral ARR administration (7.325 g/rabbit) and that the changes in phrenic nerve electrical activity and respiratory movement waveforms were consistent. Subsequently, they observed the expression of neurotransmitter-related proteins in hypothalamic neurons of rats. The experimental results showed that ARR significantly reduced the acetylcholinesterase and tyrosine hydroxylase levels in the hypothalamus, suggesting that the inhibitory effect of ARR on the respiratory center may be mediated by interference with catecholamine and cholinergic transmitter levels. Another *in vitro* experiment assessed the excitatory effect of ARR-containing serum on isolated dorsal respiratory group (DRG) neuronal cells of the medulla oblongata (MRG), showing that ARR-activated voltage-gated Na^+ channels in the medulla oblongata DRG inner membrane caused a subsequent massive inward flow of Na^+, which led to continuous inspiratory neuron excitation[78]. Furthermore, Yang et al.[79] found that ARR-containing serum had a significant activating effect on the delayed rectifier potassium current of respiratory neurons in the DRG, which led to the enhancement of the outward potassium current and respiratory paralysis. In addition, β-asarone (1, 10, and 100 μM), the volatile component of ARR, was shown to induce the blockade of the hypoglossal respiratory-like rhythm and activity of the airway preganglionic parasympathetic motoneurons, suggesting that β-asarone inhibited the central nervous system and thus caused acute respiratory disorders[80] (Figure 3).

Lung toxicity of ARR

Besides causing toxic effects on the respiratory system through interventions in respiratory centers, studies have shown that ARR can directly cause pathological changes in the lungs. Nie et al.[17] evaluated the pulmonary toxicity of an ARR decoction in rats and found that 6 to 24 g/kg ARR decoction caused lung damage in rats after 28 days of oral administration. Specifically, the ARR decoction (6 g/kg) caused slight thickening of the alveolar wall in rats, accompanied by a small amount of inflammatory cell infiltration, whereas high doses (12 and 24 g/kg) significantly thickened the alveolar wall, recruited numerous inflammatory cells, and damaged the alveolar epithelial cells, suggesting that ARR exposure may be toxic to the lungs. Furthermore, Cai et al.[81] gave ARR (0.18, 0.95, and 1.72 g/kg) to rats for 4 weeks, finding the occurrence of pulmonary alveolar dilatation accompanied by alveolar septal rupture occurred in the lung, and arterial partial oxygen pressure (PaO_2) and arterial oxygen saturation (SaO_2) were significantly reduced, while alveolar-arterial partial oxygen pressure difference ($AaDO_2$) were significantly increased. These results suggested that the pulmonary

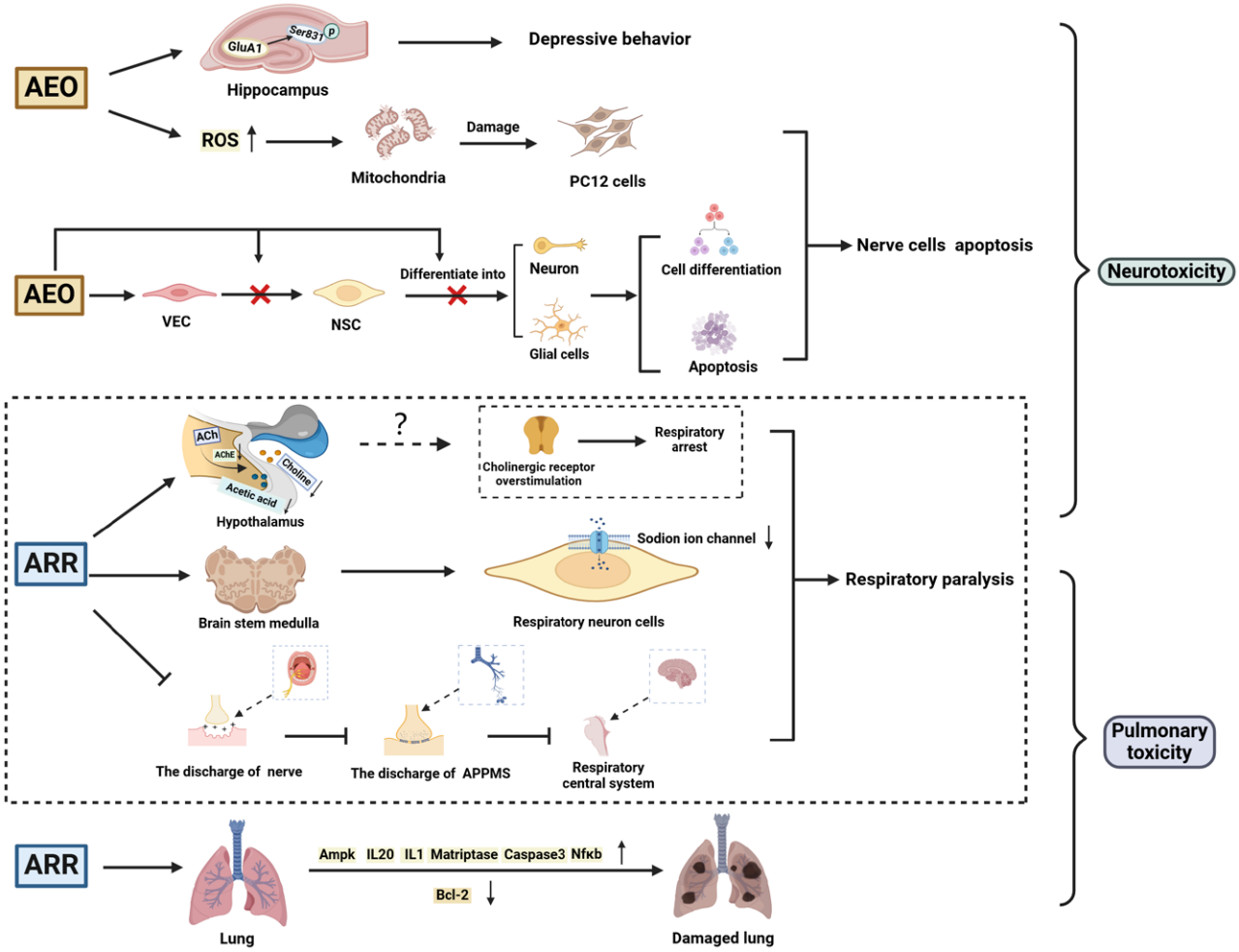

Figure 3. Molecular mechanisms underlying the neurotoxicity and respiratory toxicity of ARR. ACh: Acetylcholine; AChE: Acetylcholinesterase; AEO: ARR essential oil; APPMs: Air way preganglionic parasympathetic motoneurons; ARR: *Asari Radix* et *Rhizoma*; Bcl-2: B-cell lymphoma-2; GluA1: AMPAR subunit glutamate receptor 1; IL: Interleukin; Nfκb: Nuclear factor-κB; NSC: Neural stem cell; ROS: Reactive oxygen species; VEC: Vascular endothelial cell.

toxicity of ARR mainly causes type I chronic respiratory failure, with lung ventilation dysfunction as the main pathological feature. Then, 2 weeks after ARR elimination, lung tissue lesions and arterial blood gas abnormalities in the rats were relieved, indicating that the lung tissue damage caused by ARR was reversible. However, the mechanism by which ARR causes pulmonary toxicity remains unclear. A recent study explored the potential toxicological mechanisms through transcriptomic sequencing and found that differentially expressed genes were predominantly enriched in signal transduction pathways such as the olfactory transduction pathway, mitogen-activated protein kinase (MAPK) signaling pathways, calcium signaling pathways, and signaling molecules such as neuroactive ligands. Quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry were used to verify sequencing results. ARR may affect the MAPK-nuclear factor-κB (NFκB) and B-cell lymphoma-2 (BCL-2) signaling pathway, and inflammation-associated proteins to promote lung toxicity[82]. The vapor of α-pinene is one of the specialty ARR components, showing persistent sensory irritation effect on the upper respiratory tract. It also induces anesthesia and/or pulmonary irritation and sudden death at concentrations above 2,600 ppm[83]. Collectively, ARR causes definite pulmonary toxicity; however, its toxicological mechanism requires further study (Figure 3).

Conclusion and prospects

ARR is one of the most widely used Chinese medicinal herbs for treating colds, headaches, toothaches, and rheumatism. However, undesirable reactions have limited its clinical application. In this review, we summarize the main active ingredients of ARR and discuss their potential toxicity to the liver, lungs, and nervous system. ARR contains numerous chemical ingredients, including volatile oils, lignans, fatty acids, flavonoids, and nitrogen-containing compounds, with volatile oils and lignans being the main active ingredients. Various mechanisms have been identified to be involved in the toxic effects of ARR[84]: Volatile oils were found to be the prominent toxic components in ARR, among which methyleugenol and SFO showed hepatotoxicity and may induce tumorigenesis and respiratory paralysis. Evidence has shown that the volatile oil-induced toxic effects were associated with disturbances of key metabolic pathways and signaling transduction. The hepatotoxicity of volatile oil is mediated by metabolic pathway interference,

especially the inhibition of CYP enzymes, the production of toxic metabolites, and oxidative stress, while its genotoxicity is mainly attributed to the formation of DNA adducts or direct oxidative damage. Furthermore, volatile oil-induced pulmonary toxicity involves histological alterations resulting from alterations in multiple molecular pathways. Neurotoxicity is caused by the disruption of neurotransmitter levels and receptor agonism.

The saying "not more than a coin's worth of ARR" was first mentioned in Bencao Bie Shuo during the Song Dynasty: "When used solely as a powder, it must not exceed half a coin's worth, as excessive use could result in suffocation and death." In the Ming Dynasty, Shizhen Li slightly modified this in the *Compendium of Materia Medica*, changing the dosage of ARR from half a coin's worth to one. Since then, this statement has been passed down; however, it does not specify the dosage of ARR in decoctions. However, medical practitioners in later generations have overlooked the premise of "using only the powder"[85], emphasizing only the dosage and not the form. Clinical practice has shown that strictly following the aforementioned principle when using ARR products, especially in the decoction, does not achieve the expected therapeutic effects. In fact, certain reasons make us believe that the clinical application of ARR should not be overly restricted owing to toxicity concerns, but rather should be flexibly adjusted according to the specific characteristics of different diseases and various factors. First, modern toxicological studies have extensively explored the toxic threshold dose of ARR at both animal and *in vitro* levels. Based on the toxic manifestation dose involved in the studies included in our review, the calculated equivalent clinical human dose was approximately 6 g/70 kg body weight[86], which is significantly greater than the ancient records of toxic doses. Second, previous research has confirmed that toxic components of ARR, including methyl eugenol and SFO, could substantially volatilize during decoction (decreased to 2%–5% after 30 m decoction)[87], and it is recommended to cook with the lid open for an extended period to reduce toxicity, while the boiling time should not be too long to avoid excessive volatilization of the therapeutic ingredients. Consistently, some studies have shown that the LD_{50} of ARR volatile oil is 1.2 ± 0.04 g/kg, the LD_{50} of ARR powder is 4.8 g/kg; the LD_{50} of ARR raw product decoction is 145.45 g/kg, and the LD_{50} of ARR processed product decoction is 846.16 g/kg[88]. This evidence potentially allows for safe ARR use in amounts exceeding those restricted by the *Chinese Pharmacopoeia*. Finally, ARR is usually combined with other medicinal herbs to form a complex formula for clinical use. This intricate combination may enhance ARR safety by reducing the content of potentially toxic substances in the final medication through physical and chemical mechanisms, such as the formation of self-assembled nanostructures, precipitation, and chelation. However, the specific upper limit of the safe application of ARR in decoctions remains unclear, and further clinical and experimental studies are needed.

In conclusion, ARR has garnered considerable attention over the past few decades because of its potential therapeutic effects. This review summarizes the recent progress in the identification of toxicological substances, comprehensive evaluation of threshold toxic doses, and in-depth exploration of the underlying targets and mechanisms, and is expected to provide valuable insights for future research. This will guide safer and more effective ARR use in clinical practice and promote more rational regulation, thereby enabling ancient traditional Chinese medicine to play a more significant role in the battle against human diseases.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Xin Li: Writing of the original draft and visualization. Ranyun Chen: Writing the original draft and visualization. Shuni Duan: Writing-original draft, reviewing, and editing. Yijie Li wrote the original manuscript. Huiping Zhou: writing–review and editing, funding acquisition. Runping Liu: Conceptualization, writing–review and editing, funding acquisition. Rong Sun: Conceptualization, review, editing, funding acquisition, and supervision.

Ethical approval of studies and informed consent

Not applicable.

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Data availability

All data generated or analyzed during this study are included in this published article.

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