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# Precise subcellular organelle-targeted analyses of the hepatotoxicity of rhubarb

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**Abstract**

**Objective:** Drug-induced liver injury (DILI) is the leading cause of acute liver failure and poses a significant challenge to human health. Rhubarb (*Rheum officinale Baill*. DaHuang) has been clinically used for its heat-clearing and diuresis-promoting effects. However, its toxic effects on different organelles in the liver require further validation.

**Methods:** We analyzed the potential targets affecting hepatotoxicity in rhubarb and the potential damage relationship with five major organelles, including microsomes, mitochondria, endoplasmic reticulum (ER), Golgi apparatus (GA), and lysosomes through Integrated Traditional Chinese Medicine (ITCM)/HERB databases and network pharmacology. We isolated and purified different organelles, incubated them with different fractions and monomers of rhubarb in an adenosine triphosphate (ATP) culture system and examined the structural and functional changes in the organelles using particle size analysis and molecular biological experiments to investigate whether rhubarb affects the damage and rupture of major organelles in the liver.

**Results:** By combining virtual predictions and experimental verification, our research confirmed that emodin isolated from the anthraquinone of rhubarb, catechin in the tannins of rhubarb, and palmitic acid in the organic acids of rhubarb caused the most significant functional and structural damage to the representative organelles. Among all the monomeric compounds, emodin caused the most damage to the microsome, mitochondria, ER, and lysosome; catechin induced microsome and GA damage; and palmitic acid caused the most damage to microsomes and GA in the liver, suggesting that rhubarb components may exert hepatotoxicity through multi-organelle injury.

**Conclusions:** Our findings revealed that rhubarb has varying degrees of damaging effects on different organelles, which in turn affects cellular life activities by impairing organelle morphology and function. This study provides a theoretical basis and technical support for a refined analysis of the toxic components and targets of rhubarb.

**Keywords:** DILI, Emodin, Organelle toxicology, Palmitic acid, Rhubarb

**Graphical abstract:** http://links.lww.com/AHM/A109.

## Introduction

Drug-induced liver injury (DILI) is a widespread liver disease caused by the direct or indirect effects of drugs or their metabolites after exposure, such as anti-tuberculosis drugs, antiepileptic drugs, nonsteroidal anti-inflammatory drugs, anti-neoplastic drugs, immunomodulators, dietary supplements (herbs and dietary supplements, HDS), and a few traditional Chinese medicines (TCM)[1]. Clinically, DILI may manifest as a variety of acute, subacute, or chronic types of liver injury, the severity of which ranges from asymptomatic elevation of liver enzymes to fulminant hepatic failure or even death[2]. According to the statistics, only the incidence of DILI happened in China is 23.8 out of 100,000, with a case fatality rate of 0.39%[3]. The incidence of DILI in retrospective studies has been shown to be ~2.4 out of 100,000 cases per year in the UK, 2.3 out of 100,000 cases per year in Sweden, and 3.42 out of 100,000 cases per year in Spain[3]. Moreover, DILI has emerged as a prevalent and serious adverse drug reaction, often leading to the discontinuation of clinical trials or the withdrawal of new drugs. Notably, the use of herbal medicines and HDS contributes to a significant proportion (26.81%) of DILI cases and is frequently observed in individuals with pre-existing liver conditions such as viral hepatitis or steatosis[4], thus posing a more serious risk of liver failure and death.

To alleviate this potential problem, various techniques are currently available for assessing hepatotoxicity, circumventing potential toxicity, and minimizing the risk of hepatotoxicity. The cellular CCK8 assay is commonly used to determine whether hepatocytes are damaged by detecting the metabolic activity of cells. Additionally,

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hepatocyte sandwich culture, three-dimensional culture, and co-culture techniques for cholangiocytes and hepatocytes have been widely used in hepatotoxicity assessments, which can stimulate the growth environment of hepatocytes *in vivo* and reflect more realistic effects of drugs *in vivo*[5,6]. Hepatobiliary organs and zebrafish models are commonly used for hepatotoxicity assessments. The former focuses on directly assessing whether the drug affects the differentiation of pluripotent stem cells into hepatocytes, whereas the latter is a well-known hepatotoxicity assessment method owing to its advantages of a short reproduction cycle, large numbers, and convenient gene editing[7–9]. In addition to multiple *in vitro* culture platforms, high-content screening can rapidly and accurately detect the effects of drugs on hepatocytes[10,11]. As relatively independent organisms, cells possess the ability to self-regulate and repair resistance to drug toxicity, which may lead to difficulties in detecting drug hepatotoxicity at the cellular level. However, compared with multiple cellular level assessment models, organelle models are more sensitive and provide a more accurate reflection of drug hepatotoxicity[12]. The effects of drugs on mitochondria, Golgi apparatus (GA), and endoplasmic reticulum (ER) cell morphology in the kidney have been studied[13]. However, there are currently no studies assessing hepatotoxicity at the organelle level. Research in this area will help gain a deeper understanding of the mechanism underlying the hepatotoxic effects of drugs and provide more comprehensive and accurate toxicity assessment data for new drug development.

As a well-known TCM, rhubarb (*Rheum officinale Baill*. DaHuang) has historically been used to treat various diseases involving different organ injuries[14,15]. However, as research has progressed, the hepatotoxicity of rhubarb has gradually surfaced, making its clinical application risky[16]. The TCM pharmacopeia lists rhubarb as a toxic Chinese herbal medicine, which further confirms the potential damaging effects of rhubarb on the liver[9]. Clinical cases indicated that rhubarb is hepatotoxic when the dose reaches an adult intake of 0.4 kg/day[15]. The hepatotoxicity of rhubarb is further supported by the fact that rhubarbic acid causes proliferation inhibition and toxic effects on HepG2 cells *in vitro* at a concentration of 67.71 M. Long-term use of palmarosa rhubarb 3 to 15 g/day for chronic hepatitis and renal failure-related diseases may lead to hepatic and renal damage[17]. Although no toxicity studies have been reported on rhubarb toxicity in the liver with respect to specific individual organelles such as mitochondria, ER, microsomes, GA, and lysosomes, the subcellular targets of rhubarb on mitochondrial and ER morphology have recently been investigated at the cellular level in the kidney. Exposure of HK-2 cells to rhubarb extract results in decreased cell viability, accompanied by either loss of mitochondrial membrane potential[18] or disruption of ER ultrastructure and ER stress[19]. Therefore, it is necessary to determine whether rhubarb triggers damage and disruption of major intrahepatic organelles in hepatocytes at lower doses than in whole cells.

In this study, we first analyzed the potential targets within rhubarb that may influence hepatotoxicity and the potentially damaging relationships with the five major organelles through Integrated Traditional Chinese Medicine (ITCM, http://itcm.biotcm.net/index.html) and HERB (http://herb.ac.cn/) database and network pharmacology, and subsequently isolated the different organelles and incubated in an *in vitro* adenosine triphosphate (ATP)-supported culture system with different rhubarb effective parts and monomers to explore whether rhubarb could induce damage and rupture of major organelles in hepatocytes. Our findings provide a theoretical basis and scientific hypothesis for early warning of rhubarb toxicity at the subcellular level and the exploration of diagnostic biomarkers and potential therapeutic targets.

## Materials and methods

### Materials

Emodin (EN) (PU0062, suitable for HPLC, ≥99.0%, CAS Number: 518-82-1), aloe-emodin (AE) (PU0816, suitable for HPLC, ≥98.5%, CAS Number: 481-72-1), (+)-Catechin (CN) (PU0722, suitable for HPLC, ≥98.5%, CAS Number: 154-23-4) were purchased from Pusi Bio-technology (Chengdu, China). Rhein (RN, suitable for HPLC, ≥98.5%, CAS Number: 478-43-3), chrysophanol (CL, suitable for HPLC, ≥99.0%, CAS Number: 481-74-3), and palmitic acid (PA, suitable for HPLC, ≥98.0%, CAS Number: 57-10-3) were gifts from Shandong University. $MgCl_2$ (gffxc1475) and Tris-HCl buffers (R21406) were obtained from Guangfu Technology (Tianjin, China). KCl (S24120), 5’-GTP (S18078), ATP-H (S30943), glutathione reduced (S20186), phosphocreatine disodium salt hydrate (S27846), and creatine phosphokinase (S10076) were purchased from Source Leaf (Shanghai, China). Sucrose (BN20004) was purchased from BioOrigin (Beijing, China). β-Glycerol phosphate disodium salt (G418953) and cytochalasin B (C113160) were purchased from Aladdin (Shanghai, China). Ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (gffxc419) and PA (P0500) were purchased from Sigma (MS USA). Antibodies against β-ACTIN (60123-1-lg), protein disulfide isomerase (PDI) (66422-1-lg), syntaxin 6 (10841-1-AP), calnexin (66903-1-lg), binding immunoglobulin protein (GRP78/BIP) (66574-1-lg), syntaxin 6 (SYN 6, 10841-1-AP), cathepsin D (55021-1-AP), mitofusin-2 (mfn2) (67487-1-Ig), and cytochrome p450 2E1 (CYP2E1) (19937-1-AP) were purchased from Proteintech Group Inc (Rosemont, IL, USA). Antibodies against NADH dehydrogenase Fe-S protein 1 (NDUFS1) (sc-271510), lysosomal-associated membrane protein 1 (LAMP-1) (sc-20011), golgi matrix protein (GM130) (sc-55591), golgin45 (GOL45) (sc-515193), cytochrome p450 3A4 (CYP3A4) (sc-53850), β-clathrin COP (COPB) (sc-393615), voltage-dependent anion channel (VDAC1) (sc-390996), and MFN1 (sc-166644) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were obtained from the Absin Biochemical Company (Absin, Shanghai, China).

### Virtual screening evaluation

Target genes specific to organelle damage were identified by reviewing the existing literature. Furthermore, genes expressed differently due to the active compounds were sourced from the Integrated ITCM database (http://itcm.biotcm.net/index.html) and the HERB database (http://herb.ac.cn/). Toxic compounds were identified by analyzing the overlap between the target genes and differentially expressed genes (DEGs).

### Network pharmacology

Compounds from the TCM Systems Pharmacology Database (https://old.tcmsp-e.com/tcmsp.php.) were imported into the Swiss Target Prediction website (http://www.swissadme.ch/index.php) for active ingredient screening as previously described[20]. Targets related to liver injury were obtained from the on-line Mendelian Inheritance in Man database (https://omim.org/) and Gene Cards (http://www.genecards.org/). Additionally, a common target was constructed for the Venn diagram. The protein-protein interaction (PPI) network was built using the STRING website (https://string-db.org/). Furthermore, gene ontology biological process (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using the Cluster Profiler software package. Finally, a chord diagram was constructed using bioinformatics software (https://www.bioinformatics.com.cn/).

### Extraction of different fractions of rhubarb

Rhubarb was purchased from the Tongrentang Pharmaceutical Company (Beijing, China) and authenticated by Dr. Bing Xu. Anthraquinones (AQ) extraction: Methanol (830 mL of methanol) was added to 5 g of rhubarb and sonicated (120 W, 40 k H2) for 30 min, followed by lyophilization after spin distillation. After mixing, the extract was filtered through 0.45 μm microporous filter membrane, and the filtrate was kept for drug administration. Organic acid (OA) extraction: 100 mL of 95% ethanol was added to 5 g of rhubarb sample, sonicated for 30 min, and then concentrated by rotary evaporation. The supernatant was filtered and lyophilized overnight. After adding 10 mL of methanol to fully dissolve the sample, 0.5 mL of concentrated sulfuric acid and 6 mL of dd$H_2O$ were further added. Subsequently, each sample was diluted to 50 mL with n-hexane and sonicated in 95% ethanol at a ratio of 1:20 for 30 min. The final extracted OAs was 5.09%. Extraction of tannins (TN): 5 g of rhubarb powder was weighed precisely, placed into a 7.5 mL volumetric flask, and 60% ethanol was added by volume. The samples were sonicated for 100 min and replenished with ethanol, and overnight at 4°C, and the concentration of supernatant was 1 g/mL.

### Extraction of different organelles

All animal studies and procedures were approved by the Institutional Animal Care and Use Committee of the Beijing University of Chinese Medicine. C57BL/6J mice were anesthetized with isoflurane and sacrificed to harvest the liver samples for further experiments. For microsome extraction, 1 g of mouse liver tissue was weighed and placed in a pre-cooled Petri dish and washed with a cleaning solution (animal cell/tissue microsomal component separation kit, Jiemei Gene, Shanghai, China, GMS18002). The washed liver tissue was transferred into a clean pre-cooled Petri dish, cut with sterilized scissors, and transferred into a 15 mL centrifuge tube. To the centrifuge tube from the previous step, 3 mL of pre-cooled equilibrium solution was added, followed by homogenization of the liver tissue using a homogenizer. The mixture was placed into a pre-cooled 4°C centrifuge and centrifuged for 30 min at 9,000×*g*. The supernatant was aspirated into two new 1.5 mL centrifuge tubes and centrifuged in a pre-cooled 4°C ultra-high-speed centrifuge for 60 min at 100,000×*g*. HepG2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco modified Eagle's medium (DMEM). Mitochondria in hepatocytes were extracted using the Cell Mitochondria Isolation Kit (Beyotime, Beijing, China). For sucrose radiant separation of the ER, a sucrose gradient (12%, 15%, 20%, 25%, 30%, 33%, 36%, and 40%) was prepared in an ultracentrifuge tube. After hepatocyte collection, 1 mL of 8% sucrose solution was added and homogenized 100 times using a pre-cooled homogenizer at −20°C. The effectiveness of centrifugation was assessed at approximately 90% cell disruption, using an inverted microscope. The cell lysate was further aspirated into a sterile 1.5 mL eppendorf (EP) tube, and the entire experiment was conducted on ice. Then, 200 μL of cell lysate was added to the upper layer of the sucrose gradient. The cells were then centrifuged at 12,000×*g* for 2 h. The upper 400 μL contained the ER. Proteins were quantified using Caulmer Brilliant Blue, followed by *in vitro* culture and drug administration. For GA extraction, fresh liver samples were cut, resuspended, and homogenized in reagent A from a GA isolation kit (HR0247, Bio Lab, Xi-an, China). After centrifugated at 1,000×*g* for 10 min, the supernatant was further centrifugated at 3,000×*g* for 10 min and subsequent 5,000×*g* for 10 min. After collecting the supernatant and adding 10 μL reagent WT for 20 min centrifugation (20,000×*g*), the pellets were added to 500 μL reagent B to centrifuge (20,000×*g* for 30 min) for collecting the pellets containing GA. For lysosome extraction, 50 to 100 mg of fresh liver tissue was collected from mouse samples and washed twice with cold phosphate buffered saline (PBS). Next, 400 μL of cold reagent A (lysosome extraction kit, Bebo, Shanghai, China, BB-3603) was added and kept on ice for 10 min. Afterward, the sample was homogenized using a Dounce homogenizer and centrifuged at 4°C, 1,000×*g* for 5 min. The supernatant was further centrifuged at various speeds for various durations. Reagent B was then added to the precipitate and mixed thoroughly. Finally, the sample was centrifuged again to collect the precipitate, which was resuspended in lysosomal conservation solution C to obtain a lysosomal sample for storage or further experimentation.

### Organelle and cell culture and treatment

HepG2 cells were cultured in DMEM (Thermo Fisher Scientific Inc, Shanghai, China). The subcellular organelle culture system was reconstituted with a mixture of Tris-HCl (50 mM), sucrose (0.2 M), KCl (50 mM), β-glycerol phosphate disodium salt (20 mM), $MgCl_2$ (10 mM), ATP solution (2 mM), 5'-GTP (1 mM), glutathione reduced (1 mM), and EGTA (15 mM). The ATP solution contained phosphocreatine disodium salt hydrate (100 mM), ATP (1 mM), cytochalasin B (0.4 mM), and creatine phosphokinase (4 mM). The components and monomers were configured and administered to different organelles in a 96-well plate at the concentration of 10 µg/mL and 10 µM, respectively, after which samples were collected for subsequent experiments.

### Particle size analyze

After warming the instrument, the particle size analysis system was launched. The diluted samples were then added to the tube up to the top edge of the detection notch before the test button was tapped, and the software entered the baseline measurement state to record the average of three baseline measurements using a laser light-scattering-based particle size analyzer (CILAS Particle Size Analyzer, NANO DS).

### Western blot

Different organelle samples were quantified using a bicinchoninic acid (BCA) protein quantification kit and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were then transferred and blocked with 5% milk, followed by incubation with different antibodies. Images were scanned with Bio-rad chemiDoc™ MP imaging system (Bio-Rad Laboratories, Hercules, CA, USA) and quantified using quantity one software v4.62 (Bio-Rad Laboratories Co., Ltd.).

### Statistical analysis

The data included in this study represent experiments that were independently repeated three times. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey multiple comparison test using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). Statistically significant differences were identified as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

## Results

### Screening of organelles-toxic ingredients of rhubarb

In this study, we established a scoring system to screen hepatotoxic ingredients in rhubarb by intersecting target genes and ingredient-related DEGs *via* ITCM and HERB databases. If the target genes that were upregulated represented increased damage or the targets that were downregulated represented decreased damage, we set a +1 score. If not, we set −1 score. Figure 1A shows that the damage patterns of the active compounds of rhubarb in different organelles were distinct. Considering that there were redundant toxic ingredients and the limited availability of sequencing data in public databases, we noticed that EN had strong toxic effects on mitochondria, GA, and lysosomes. Figure 1B shows the regulatory effects of EN on target genes related to damaged organelles. As illustrated in Figure 1C, EN was predicted to inhibit hexokinase 1 (HK1), whose lolcation is on the outer mitochondrial membrane and is implicated in cell survival, thereby impairing glucose uptake[21]. Additionally, EN exerts toxic effects on the ER by inhibiting calreticulin (CALR), a $Ca^{2+}$-binding chaperone that regulates $Ca^{2+}$ homeostasis[22]. These predicted results provide preliminary evidence that the components of rhubarb may negatively affect different organelle functions.

### Network pharmacology-based prediction of the potential toxic effects of rhubarb on the liver

Based on the results shown in Figure 1, the rhubarb monomer may mainly affect microsomes, mitochondria, ER, and other organelles. In order to further explore and validate the above results and considering that network pharmacology has become an effective way to systematically study the pharmacological effects and potential mechanisms of TCM, we then constructed a network of "drug component targets" and screened the nodes involved in the pathogenesis process. In total, 103 targets of rhubarb were obtained from the TCMSP database. Six major ingredients (RN, EN, CL, AE, CN, and PA) were selected as candidate bioactive compounds in rhubarb when referring to values of oral bioavailability (OB) and drug-likeness (DL), and a literature source (Figure 2A). Next, 1,174 liver injury-related targets were traced from the CTD database and intersected with rhubarb-related targets. As shown in Figure 2B, 33 overlapping targets were captured and the PPI network between them was constructed (Figure 2C, upper panel), showing close interactions between different targets. Furthermore, using Metascape prediction, the specific organ affected by these 33 targets was mainly the liver (Figure 2C, lower panel), which was consistent with our expectations. Subsequently, KEGG pathway analysis was performed to evaluate the relevant pathways. Overall, 33 targets were involved in 46 pathways, and the top 20 enriched pathways were selected for bubble visualization analysis. As shown in Figure 2D, it was obvious that transforming growth factor-beta (TGF-β), oxidative stress, negative regulation of cellular processes are relatively significant signaling pathways, indicating the effect of rhubarb on the liver was closely related to the organelle-related biological processes. A GO-BP network was constructed and analyzed to further identify the functions of these targets. As shown in Figure 2E (upper panel), top 20 biological processes were depicted and found to be mainly involved in inflammation, oxidative stress, and response to xenobiotic stimulus signal pathway, which were further validated by the KEGG results. In addition, the diseases associated with these targets mainly included DILI, fatty liver disease, liver failure, and hepatitis (Figure 2E, lower panel). We then identified organelles specifically regulated by representative targets through research investigation and constructed a chord diagram. The results showed that the organelles mainly regulated by these targets were microsomes, mitochondria, ER, GA, and lysosomes, which was consistent with the predictions in Figure 1 (Figure 2F). Moreover, we traced the

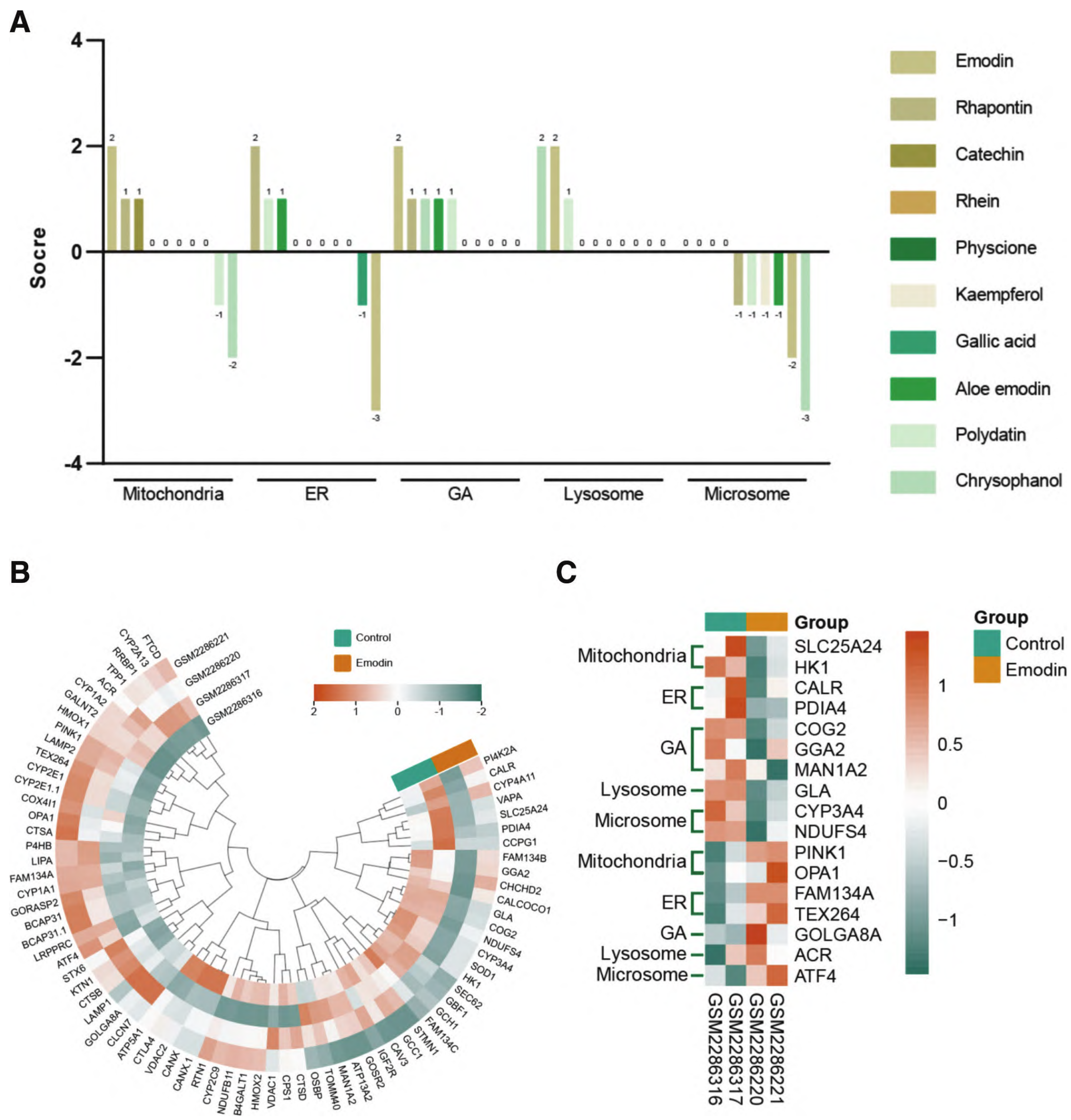


**Figure 1.** Effects of primary active ingredients of rhubarb on organelles. (A) Damage patterns of the primary active ingredients of rhubarb on organelles: Positive scores indicate damaging effects, whereas negative scores indicate protective effects. (B) Circular heatmap illustrating organelle damage-associated DEGs in cells treated with emodin, a representative toxic ingredient of rhubarb, or DMSO (GSE85871). (C) Heat map of key genes in (B). DEGs: Differentially expressed genes; DMSO: Dimethyl sulfoxide; ER: Endoplasmic reticulum; GA: Golgi apparatus; ITCM: Integrated Traditional Chinese Medicine.

drugs that regulate specific targets from Figure 2A and combined them with the results from Figure 2F, and predicted that RN, EN, CL, and AE, especially EN, might affect multiple organelles; CN and PA might mainly affect microsomes, which requires further experimental verification (Figure 2G).

### *Construction and evaluation of organelle platform for drug toxicity evaluation*

Rapid, efficient, and reliable early prediction of toxicity *in vitro* is crucial for drug development and the reduction of clinical trial risks. Considering that organelle damage is closely related to the occurrence and development of many diseases such as DILI, liver diseases, and hepatic tumors[23,24], we used multiple *in vitro* organelle platforms, including microsomes, mitochondria, ER, GA, and lysosomes, to explore the potential toxicity of rhubarb on the liver. Based on the predicted organelle targets in Figures 1 and 2, the corresponding biomarkers for different organelles were selected to reflect their functions. We first isolated different organelles according to the corresponding flowchart (Figure 3A–E, left panels) and further examined the success rate and extraction efficiency of the organelles at different steps using western blot analysis. As shown in Figure 3A–E (right panels), the protein expressions of microsome markers (CYP3A4 and CYP2E1, mainly responsible for the metabolic activity of microsome), mitochondria markers (NDUFS1, VDAC1, and MFN2, mainly responsible for mitochondrial respiration, apoptosis, and fusion processes), ER markers (CALNEXIN and GRP78, mainly responsible for ER chaperone regulation and quality control), Golgi markers (GM130 and SYNTAXIN 6, mainly responsible for vesicle delivery and membrane transport), and lysosome markers

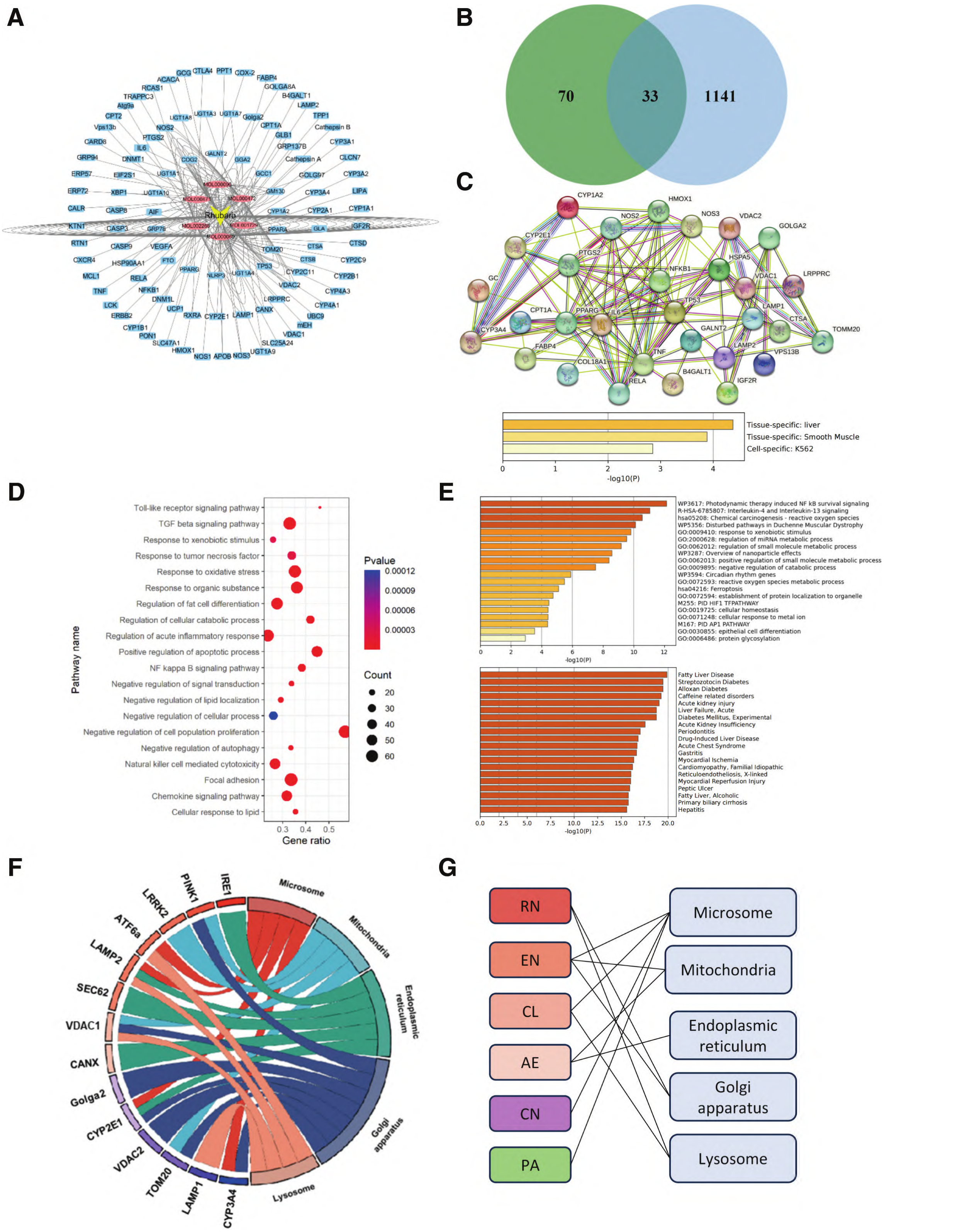


Figure 2. Network pharmacology effects of rhubarb active ingredients on liver injury. (A) Drug-compound-target network of rhubarb. (B) Venn diagram of the targets of the active ingredients of rhubarb and liver injury. (C) PPI network of common targets (upper). The tissue type in which the common target was locked (lower). (D and E) KEGG and GO analyses of rhubarb. (F) Types of organelles regulated by specific targets are shown in chord diagrams. (G) Potential toxic effects of active rhubarb ingredients on different organelles. AE: Aloe-emodin; CL: Chrysophanol; CN: Catechin; EN: Emodin; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PA: Palmitic acid; PPI: protein-protein interaction; RN: Rhein; TGF: Transforming growth factor.

(LAMP1 and CATHEPSIN D, mainly responsible for transmembrane transport and degradation of proteins) were highly expressed in the precipitation of the collectible sample step but not in the supernatant (S) or precipitate (P) discarded at each step, indicating that the isolation methods of these organelles are feasible. Therefore, these organelles were selected for subsequent experiments.

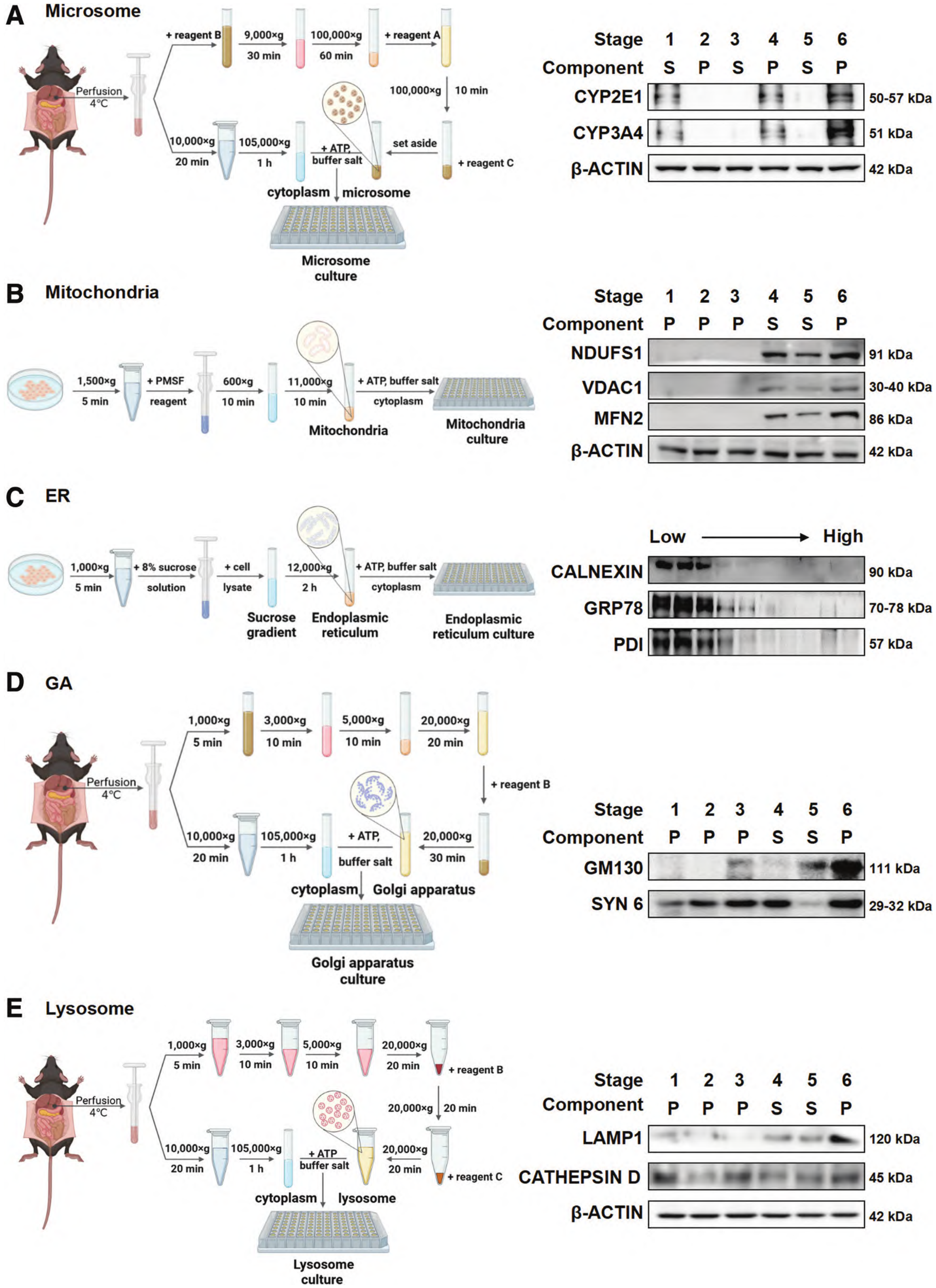


Figure 3. Extraction and identification of different organelles. Flowchart of the microsome extraction process (A, left panel), mitochondria (B, left panel), ER (C, left panel), GA (D, left panel), and lysosomes (E, left panel). Representative immunoblots against (A, right panel) microsome markers (CYP3A4, CYP2E1), (B, right panel) mitochondria markers (NDUFS1, VDAC1, MFN2), (C, right panel) ER markers (CALNEXIN, GRP78, PDI), (D, right panel) GA markers (GM130, SYNTAXIN 6), (E, right panel) lysosome markers (LAMP1, CATHEPSIN D) and β-ACTIN. ER: Endoplasmic reticulum; GA: Golgi apparatus; LAMP1: Lysosomal-associated membrane protein 1; PDI: Protein disulfide isomerase.

### *Effects of extracts and all monomers of rhubarb on different organelle systems*

After successfully acquiring the different organelles, we performed short-term administration of rhubarb extract components and their potentially toxic monomers and assayed their effects by western blotting. Microsomes are dynamic organelles that primarily function in drug metabolism. Our results showed that the microsomes were dysfunctional following EN, CL, and PA exposure, as reflected by the decreased protein expression of CYP2E1 and CYP3A4 (Figure 4A). Mitochondria produce ATP for energy and interact with other organelles

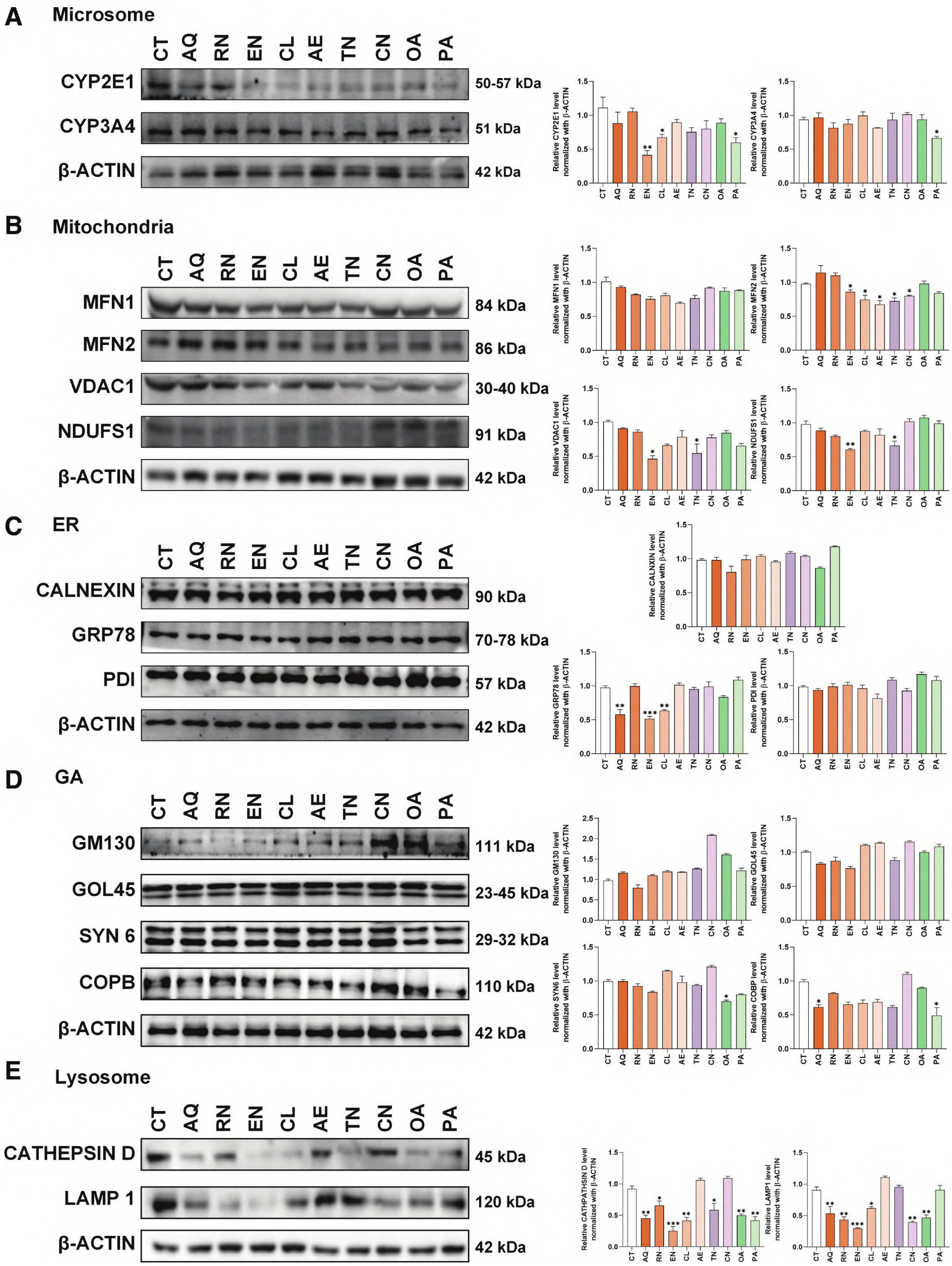


Figure 4. Effects of effective fractions and active ingredients of rhubarb on different organelles. Representative immunoblots against (A, left panel) microsome markers (CYP2E1 and CYP3A41), (B, left panel) mitochondria markers (MFN1, MFN2, VDAC1, and NDUFS1), (C, left panel) ER markers (CALNEXIN, GRP78, and PDI), (D, left panel) GA markers (GM130, GOL45, SYNTAXIN 6, and COPB), (E, left panel) lysosome markers (CATHEPSIN D and LAMP1) and β-ACTIN after administration of effective fractions (AQ (10 μg/mL), TN (10 μg/mL), and OA (10 μg/mL)) and active ingredients (RN (10 μM), EN (10 μM), CL (10 μM), AE (10 μM), CN (10 μM), and PA (10 μM)) from rhubarb. (A–E, right panel) The relative densities of above proteins normalized with β-ACTIN. Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group. AE: Aloe-emodin; AQ: Anthraquinone; CL: Chrysophanol; CN: Catechin; CT: Control; EN: Emodin; ER: Endoplasmic reticulum; GA: Golgi apparatus; LAMP1: Lysosomal-associated membrane protein 1; OA: Organic acid; PA: Palmitic acid; PDI: Protein disulfide isomerase; RN: Rhein; TN, Tannin.

that are necessary for cell maturation[25]. Next, we examined the expression of mitochondria-related markers, and the results showed that the expression of NDUFS1 and VDAC1 markedly decreased after EN and TN administration. In addition, the expression of MFN1 and MFN2 showed a decreasing trend after EN, CL, AE, TN, and CN treatments (Figure 4B). The main function of the ER is to synthesize proteins and lipids that play a role in transporting substances within the cell[26,27]. We then used the ER marker GRP78 to examine the effects of rhubarb monomers on ER organelles. Compared with the control group, the expression of GRP78 was significantly downregulated by AQ, EN, and CL intervention, while CALNEXIN showed a decreasing trend after RN and OA treatment, and PDI showed a decreasing trend after AE and CN administration (Figure 4C). Proteins synthesized in the ER must be transported to the GA and subsequently transferred to the cell surface *via* vesicular transport[27,28]. Therefore, we next examined the function of GA in organelles after treatment with the rhubarb extract and monomer. Western blotting was performed to examine the expression of GA markers. As shown in Figure 4D, GA was damaged by RN, EN, TN, and PA treatments, as evidenced by the decreased expression of GM130, GOL45, SYN6, and COPB. Additionally, compared with the control group, abnormal increases in these proteins were observed after CN treatment, indicating a protective effect of CN. As organelles are closely related to intracellular proteins, we examined lysosomes to further explore the disruptive effects of rhubarb. As shown in Figure 4E, the expression of CATHEPSIN D and LAMP1 was more functional in the AQ, RN, EN, CL, and OA groups, but not in the AE group. These results indicate that different rhubarb monomers exert diverse toxic effects on different organelles. Overall, EN and TN, which belong to the AQ category, exhibited more obvious toxic effects on these organelles.

### *Effects of extracts and selective monomers of rhubarb on different organelles*

Based on these results, EN, CN, and PA were identified as the most promising toxic compounds derived from AQ, TN, and OA. We further investigated and demonstrated the potentially toxic effects of the most toxic monomers in different components on different organellar systems. As expected, in the microsomes, the protein expression of CYP2E1 and CYP3A4 were significantly downregulated by EN (belongs to AQ), with a dramatically downregulation in CN (belongs to TN) but slightly downregulation in PA (belonging to OA) groups (Figure 5A). In the mitochondria, the expression of NDUFS1, VDAC1, and MFN1 was markedly decreased by EN (belonging to AQ), TN, and PA (belonging to OA) treatment (Figure 5B). Moreover, the expression of GRP78 was significantly decreased after EN administration, with the downregulation of CANX and PDI in the CN group (Figure 5C). In addition, the expression of GA markers was slightly decreased (GM130 and SYN6) or markedly downregulated (GOL45 and COPB) after EN, OA, or PA (OA) treatment (Figure 5D). Finally, the protein levels of lysosomal markers were also detected, and the results showed that the expression of CATHEPSIN D and LAPM1 significantly decreased after EN (belonging to AQ), CN (belonging to TN), and PA (belonging to OA) (Figure 5E). These results further validate the previous results, showing that AQ and EN might be the most potent toxic drugs targeting all organelles.

### *Particle size analysis of extracts and monomers of rhubarb on different organelles*

To further verify the damage status of rhubarb in different organelles, particle size measurement technology was applied to statistically analyze changes in particle size in different organelles after drug intervention. Defects in organelle size have been reported to result in improper cell function[29]. Under stimulus exposure, liver microsomal stability decreased[30], the mitochondrial fragmentation and rupture of the mitochondrial outer happened[31], GA integrity and dynamics were destroyed[32]. ER failed to accumulate around the spindle and was scattered in the cytoplasm, causing the condensation of lysosomes in the cytoplasm and presenting as a clustered or spindle peripheral localization pattern[33], indicating that protein modification, transport, and degradation processes were affected. Our results showed that the particle size of the microsomes was dramatically decreased by RN, EN, CL, AE, and CN in the range of 0 to 70 nm, whereas it was only significantly decreased by EN in the range of 70 to 140 nm (where the microsomes mainly existed) compared with the control group (Figure 6A). Further, RN and PA markedly decreased the mitochondrial diameter at the range of 0 to 0.5 μm, while RN and PA decreased the particle size of mitochondria at range 0.5 to 2 μm (the mitochondria mainly existed) compared with the control group (Figure 6B). Notably, RN, EN, CL, and AE were observed to significantly reduce the size of particles in the ER at the range of 40 to 60 μm (the ER mainly existed), while AQ itself only slightly decreased the diameter of ER, suggesting that other ingredients of AQ except for RN, EN, CL and AE may exert a protective effect (Figure 6C). Furthermore, the diameters of GA were dramatically declined by AQ, RN, EN, AE, TN, CN, OA, and PA administration at range of 20 to 40 μm (the GA mainly existed), while significantly downregulated by RN, EN, CL, AE, TN, CN, OA, and PA treatment at range of 40 to 60 μm (Figure 6D). Finally, the particle size of lysosomes was also tested, and the results showed that only RN significantly decreased the diameter of lysosomes in the range of 200 to 1,000 nm (where lysosomes mainly existed) (Figure 6E).

## Discussion

In this study, we predicted and analyzed the potential targets of rhubarb affecting hepatotoxicity and its potential in damaging relationships with five major subcellular organelles, including the mitochondria, ER, GA, microsomes, and lysosomes, using the ITCM and HERB databases and network pharmacology. After initially determining the key organelles damaged by the drug, we isolated different organelle systems and cultured separate organelles with different effective parts and representative monomers of rhubarb in an ATP culture system *in vitro*. Subsequently, western blot and particle size analyses were performed to verify the toxic effects of rhubarb on different organelles. By combining the above virtual

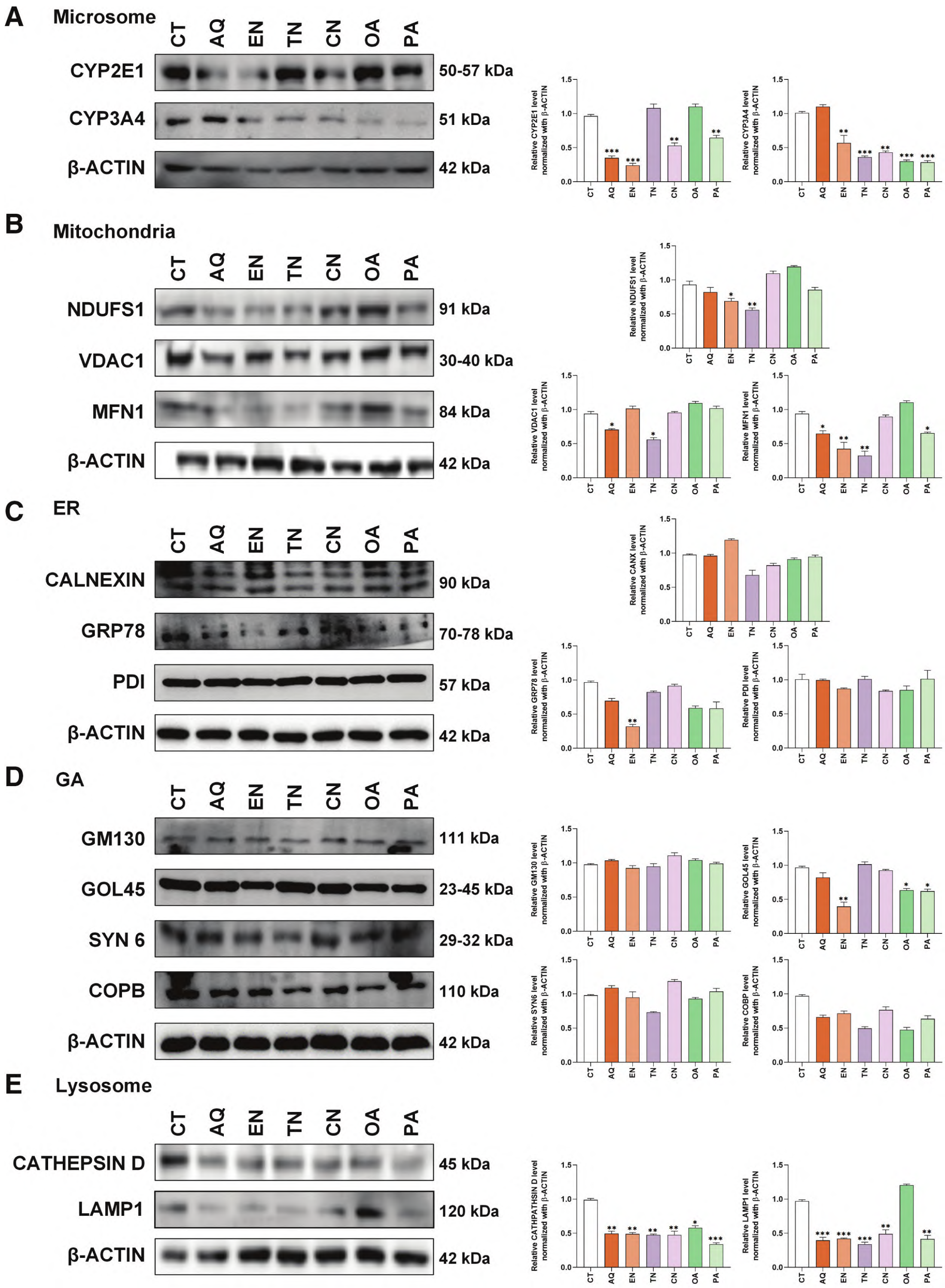

Figure 5. Effects of representative fractions and ingredients of rhubarb on different organelles. Representative immunoblots against (A, left panel) microsome markers (CYP2E1 and CYP3A41), (B, left panel) mitochondria markers (NDUFS1, VDAC1 and MFN1), (C, left panel) ER markers (CALNEXIN, GRP78, and PDI), (D, left panel) GA markers (GM130, GOL45, SYNTAXIN 6, and COPB), (E, left panel) lysosome markers (CATHEPSIN D and LAMP1) and β-ACTIN after administration of effective fractions (AQ (10 μg/mL), TN (10 μg/mL), and OA (10 μg/mL)) and active ingredients (EN (10 μM), CN (10 μM), and PA (10 μM)) from rhubarb. (A–E, right panel) The relative densities of above proteins normalized with β-ACTIN. Statistical significance: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the control group. AQ: Anthraquinone; CN: Catechin; CT: Control; EN: Emodin; ER: Endoplasmic reticulum; LAMP1: Lysosomal-associated membrane protein 1; OA: Organic acid; PA: Palmitic acid; PDI: Protein disulfide isomerase; TN: Tannin.

database prediction and molecular biology experiments, we confirmed that among the three components of rhubarb, the EN of AQ, CN of ellagitannin, and PA of OAs damaged five organelles: microsomes, mitochondria, ER, GA, and lysosomes. The most harmful toxicity of rhubarb to each organelle was as follows: EN caused the most significant damage to the microsomes, mitochondria, ER, and lysosomes; CN caused obvious damage to the microsomes and GA; and PA caused the most significant damage to the microsomes and GA.

Research on subcellular drug distribution is imperative to comprehend the effectiveness and toxicity of

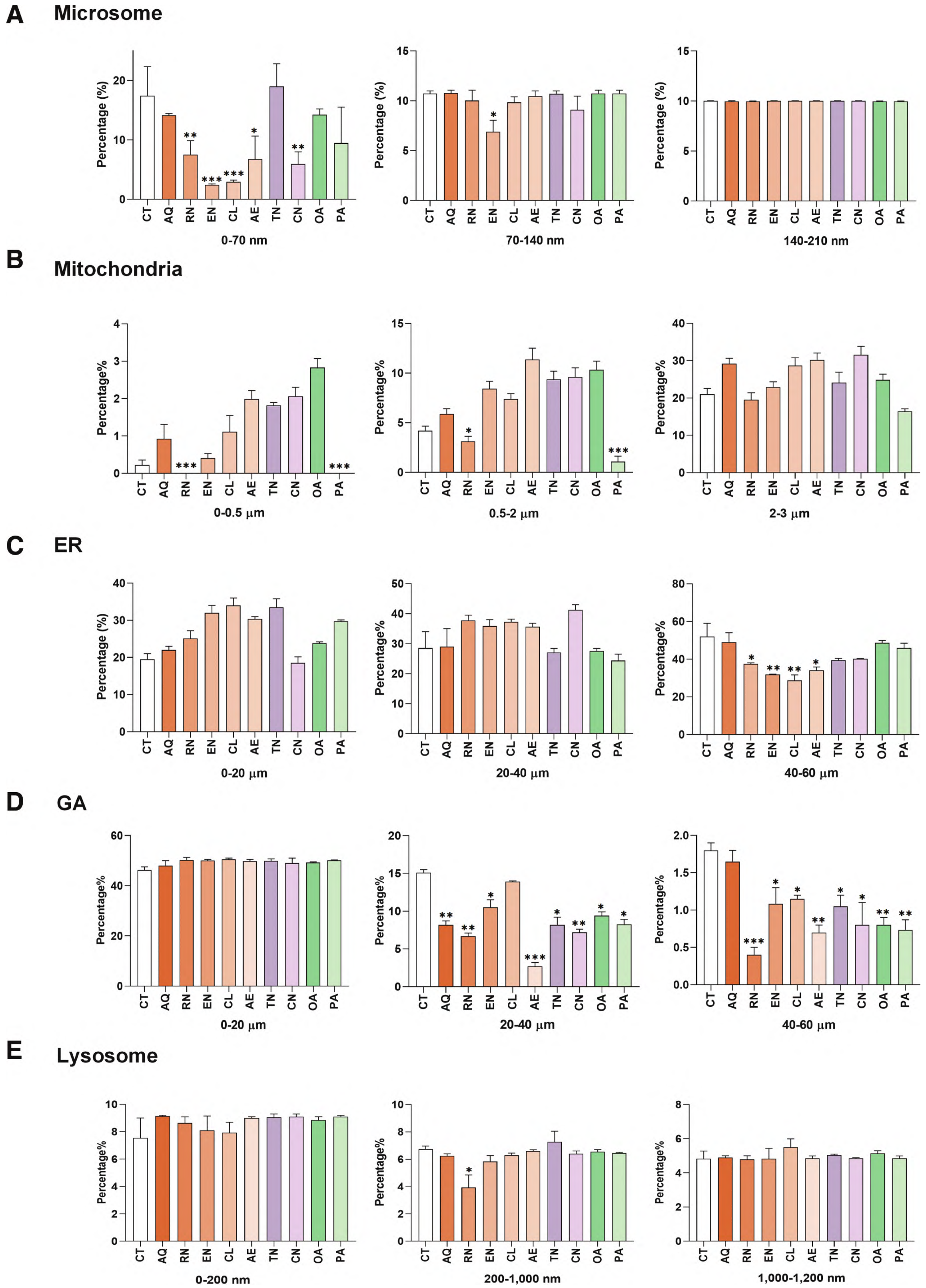


Figure 6. Particle size detection of effective fractions and active ingredients of rhubarb on different organelles. The particle size analysis results of (A) microsomes, (B) mitochondria, (C) ER, (D) GA, and (E) lysosomes after administration of effective fractions (AQ (10 μg/mL), TN (10 μg/mL), and OA (10 μg/mL)) and active ingredients (RN (10 μM), EN (10 μM), CL (10 μM), AE (10 μM), CN (10 μM), and PA (10 μM)) from rhubarb. Three representative graphs for each organelle are shown. Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group. AE: Aloe-emodin; AQ: Anthraquinone; CL: Chrysophanol; CN: Catechin; CT: Control; EN: Emodin; ER: Endoplasmic reticulum; GA: Golgi apparatus; OA: Organic acid; PA: Palmitic acid; RN: Rhein; TN: Tannin.

drugs, as well as the emergence of drug resistance in the human body. This study has significant potential for providing valuable insights into targeted subcellular drug delivery. Taking two organelles as examples, recent studies have shown that rhubarb enhances mitochondrial membrane permeability, promotes the release of pro-apoptotic factors in the mitochondria, and promotes the formation of an apoptotic complex, which results in hepatocyte apoptosis and leads to the occurrence of various diseases, such as nonalcoholic steatohepatitis (NASH)[34]. As shown in Figure 1D, the heatmap analysis indicated that EN administration might affect mitochondrial fusion through the mitochondria-related gene OPA1 and regulate mitochondrial stress by inhibiting NDUFS4. Therefore, for protein level verification, we selected the mitochondrial fusion-related proteins MFN1 and NDUFS for western blotting to investigate the effects of the active parts and representative ingredients isolated from rhubarb on mitochondria. Based on the reduced expression of MFN1 and NDUFS1 after the administration of different drugs, we hypothesized that EN, which belongs to the AQ of rhubarb, may primarily damage mitochondria at the organelle level by affecting mitochondrial membrane fusion. The ER is a prominent site for the production of reactive metabolites. Reportedly, 21 g/kg AQ can mediate cell death and inflammatory responses through the production of reactive metabolites and systemic regulation of the adaptive pathway mediated by PD1A4, which plays a significant role in drug metabolism activation and toxicity[35]. Our results have confirmed the sensitivity and precision of our organelle system since we already observed the organelle cytotoxicity caused by AQ at a lower dose of 10 μg/mL. Our particle size results showed that the ER was sensitive to all components of rhubarb mentioned in our study, which highlights the importance of isolating organelles for toxicity assessment to issue early toxicity warnings. This experimental subcellular structural system can accurately differentiate between the specific action sites of different monomers, providing a foundation for future research on specific action pathways.

Previous studies indicated that rhubarb exerts bidirectional effects. Rhubarb phenol 8-O-glucoside, the active ingredient of Rheum officinale, was found to have an antifibrotic effect by inhibiting the nuclear translocation and phosphorylation of p-STAT3, thereby blocking HSC activation[36]. Rhubarbol mixture reduced hepatocyte damage and decreased lipid peroxidation, alleviating $CCl_4$-induced liver injury in rats[37]. Similarly, the ethanol extract of Rheum officinale reduces liver injury induced by chronic ethanol administration[38]. However, owing to the potentially toxic properties of its pharmacologically active components, precise dose control is crucial for achieving low toxicity and high efficacy. For instance, rhubarb at 1 μg/mL reduced inflammation and alleviated liver and cardiovascular diseases by activating pathways such as nuclear factor κ-b, mitogen-activated protein kinase, and extracellular signal-regulated kinase. However, rhubarb extract at a concentration of 10 μM also induced adverse reactions such as irreversible liver damage *in vivo*[39]. AQ typically induces DNA damage, mutations, and genotoxicity by inducing oxidative stress[40]. One of the AQ components, EN (60 mg/kg), induces hepatic CYP450 metabolic enzymes and causes other forms of genetic damage by disrupting the integrity of the mitochondrial outer membrane, inhibiting PTEN-induced putative kinase 1 activity, and reducing mitochondrial autophagy[41]. Results from Figure 5 on mitochondrial WB and particle size demonstrated that EN at 10 μM and AQ at 10 μg/mL already cause cytotoxic mitochondrial damage, suggesting our system might be more sensitive than a typical *in vitro* cell culture environment. Current toxicological research on rhubarb has primarily focused on the effects of AQ on the gallbladder and kidney, with a lack of systematic screening and evaluation of other pharmacologically active substances. This narrow focus may have resulted in the oversight of potentially toxic components. In our toxicity assessment system, our findings revealed that besides AQ components, other active components such as PA of OA and CN of TN also exhibited significant toxicity on mitochondria, ER, GA, microsomes, and lysosomes by inhibiting the expression of representative organelle markers and disrupting organelle structure, as reflected by particle size measurement. However, research on the toxicity of these organelles, specifically at the hepatocyte level, is limited. This can be attributed to the use of insensitive detection models or the presence of complex intracellular drug metabolic pathways. Given the inconsistencies observed among human trials, animal *in vivo* studies, cellular *in vitro* experiments, and organelle-level tests, future research should focus on conducting comprehensive evaluations at multiple levels to better understand the potentially toxic effects of rhubarb.

Regarding the limitations of this study, we only evaluated the expression and size changes of typical targets by western blot and particle size analysis. However, the latter may only reflect the degree of organelle fragmentation after damage, the results of which might be affected by the superimposed combination of ruptured organelles, causing inevitable problems that affect accuracy. Therefore, the combined assessment of organelle-level toxicity of drugs by other means, such as *in vitro* mitochondrial membrane potential assays, should be considered in the future. Thus far, no studies have investigated the extracellular mixing of independently isolated organelles to replicate the toxicological effects of rhubarb on the cellular environment *in vitro*. In the current study, we attempted to isolate different organelles and incubate them with different rhubarb components to evaluate the effects of drugs on different organelles. In future studies, we plan to extract these independent organelles separately and conduct recombinant cultures *in vitro* (it is possible to first mix and culture mitochondria and ER, and then gradually add other organelles). This approach allowed us to better mimic the intracellular microenvironment and to systematically assess the toxicological damage caused by the drug. Third, cytotoxicity is possibly not caused by the raw rhubarb drug itself, but rather by its metabolites. For example, a previous study demonstrated that rhubarbic acid at a dose of 50 mg/mL, a secondary metabolite of rhubarb extract identified through high-performance liquid chromatography-mass spectrometry (MS)/MS analysis, has the potential to induce damage to renal epithelial cells[42]. Therefore, we will further consider using several new chemical/technical methods to analyze secondary metabolites after drug administration to cells and investigate the damaging effects and specific targets of action of different metabolites of rhubarb in different organelles by combining virtual screening.

In summary, we developed a novel integrated evaluation strategy to investigate the toxic effects and potential mechanisms of rhubarb on microsomes, mitochondria, ER, GA, and lysosomes within hepatocytes. Although rhubarb is widely recognized as an important TCM, it is crucial to acknowledge its potential hepatotoxicity *in vivo*, *in vitro*, or subcellular levels, which encourages the development of effective strategies to reduce liver damage through appropriate processing, compatibility, and dosage adjustment. Our study not only sheds new light on the mechanisms of drug toxicity but is also vital to ensure the safety and effectiveness of rhubarb in clinical applications.

## Conflict of interest statement

The authors declare no conflict of interest.


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## Author contributions

Xiaojiaoyang Li: Supervision, writing, review, and editing. Runping Liu: Visualization. Jianzhi Wu: Methodology, writing of the original draft. Zhi Ma: Methodology, writing of the original draft. Yinhao Zhang: Methodology. Shuni Duan: Methodology. Rong Sun: Methodology. Yijie Li: Methodology.

## Ethical approval of studies and informed consent

Not applicable.


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None.


## Data availability

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

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