

Discovery of key lipids from *Panax quinquefolius* against doxorubicin-induced cardiotoxicity based on a zebrafish model

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Research Article

Keywords: *Panax quinquefolius*, Doxorubicin, Cardiotoxicity, Zebrafish, Lipid

Posted Date: January 10th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2436228/v1>

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Abstract

Objective

To discover novel pharmacodynamic substances from *Panax quinquefolius* against doxorubicin (Dox)-induced cardiotoxicity using a zebrafish model.

Methods

AB line zebrafish embryos at 30h post-fertilization (hdf) were exposed to Dox (30 μ M) for 42h and the heart rate, stroke volume, cardiac area, and fractional shortening of larval zebrafish were used to assess cardiotoxicity. The lipid sample from *Panax quinquefolius* (PQL) was evaluated the protection of doxorubicin-induced cardiotoxicity compared with the lipids from soybean (SOL) and egg yolk (YOL). The three lipids were analysed using lipidomics techniques based on Q Exactive LC-MS/MS to screen differential lipids. The key lipid was verified the activity against doxorubicin-induced cardiotoxicity using the zebrafish model.

Results

PQL could significantly alleviate the Dox-induced the decreased heart rate, decreased stroke volume, and decreased fractional shortening (%) on the zebrafish model. 216 differential metabolites were identified, among which the unsaturated fatty acids were the crucial difference components between the three lipid samples. The 18 carbon fatty acids with four carbon-carbon double bonds (FA (18:4)) had been identified and be as a remarkable active compound with protection of Dox-induced cardiotoxicity on the zebrafish model.

Conclusion

In this research, PQL was discovered firstly to exhibit notable activity against Dox-induced cardiotoxicity in zebrafish, and FA (18:4) was identified as a novel key active component from PQ.

Introduction

Doxorubicin (Dox), an anthracycline antitumor antibiotic, is the first-line drug for the treatment of breast cancer, pediatric solid tumors, soft tissue sarcomas, leukemia, and aggressive lymphomas [1]. However, it exerts adverse effects on normal cells/tissues, particularly cardiotoxicity on cardiac tissue, mainly in the form of electrocardiographic abnormalities, arrhythmias, irreversible degenerative cardiomyopathy, and congestive heart failure [2], which brings us to a conundrum that how to reduce the side effects of Dox [3].

Panax quinquefolius (PQ) is an edible medicinal herb and gradually increasing research reveal its various pharmacological activities, including antitumor[4], cardiovascular protection [5], immunomodulation [6], metabolic modulation [7], and anti-inflammatory [8]. In addition, many researches has shown its cardioprotective effects, such as alleviating chemotherapy drug-related complications and improving quality of life of patients with cancer [9]. In animal model, PQ showed cardioprotective effects by regulating reactive oxygen species (ROS) levels and depleted cardiac contractile function to inhibit hypertrophy in heart failure [5]. PQ also exhibited cardioprotective effects through pathological cardiac remodeling by inhibiting oxidative stress and oxidative stress-induced cardiomyocyte death through activation of the Nrf2 pathway [10], and can attenuate cisplatin-induced renal damage [11]. Lipids from PQ are a large group of plant primary metabolites whose functions have not been widely studied, which was about 0.89% by weight in PQ [12]. In recent years, the cardiovascular protective effects of marine-derived polyunsaturated lipids (e.g., deep-sea fish oils) have been increasingly recognized [13], however, the data on the effects of plant-derived lipids are lacking, especially for those of medicinal origin. Therefore, investigating the protective effects of lipids of PQ against Dox-induced cardiotoxicity is important for us.

Zebrafish are characterized by their small size, high yield, *in vitro* fertilization, body transparency, and genetic ease of handling, which are particularly useful for studies involving therapeutic drug screening [14] and have been successfully and increasingly used in the clinical studies of diseases, such as epilepsy [15], liver injury [16], kidney injury [17], and diabetes [18]. Due to the similarities between the zebrafish and mammalian hearts, the rapid development of embryonic heart, and easy method to observe the cardiac function zebrafish models are suitable for studying human heart diseases [19]. Liquid chromatography-tandem mass spectroscopy (LC-MS/MS) based lipidomics is widely used for lipid analysis of a wide range of samples. Lipidomics generally determines the structure and content of lipid molecules from MS data and systematically analyzes changes in the lipid composition and levels in organisms during various biological processes [20].

In our study, zebrafish model was developed to investigate the cardioprotective effects of PQL, and lipidomics was used to screen the difference compositions among PQL, SOL (a common plant-derived lipid), and YOL (an animal-derived lipid). Furthermore, the key active components of PQL that intervened in Dox-induced cardiotoxicity were confirmed in a zebrafish model.

Materials And Methods

Animals and materials

The wild AB line zebrafish were provided by the Zebrafish Centre of Shandong First Medical University. PQ (Wendeng, Weihai, China) was identified by Associate Researcher Liwen Han as PQ of the Araliaceae family, and soybean(SOYA)and egg yolk (YOLK) were purchased from the market. Doxorubicin hydrochloride (Sigma-Aldrich, 98–102.0%, Lot No: WXBD6966V) and FA (18:4) (Macklin, Lot No. C14432948) were obtained from their respective seller. Ibuprofen, CuSO₄, anhydrous ethanol, and

ammonium acetate were obtained from Sinopharm. Methanol, acetonitrile, and isopropanol were obtained from Thermo Fisher Scientific. Other reagents included ROS kit (Solaibao), n-hexane and acetone (Tianjin Fuyu), chloroform (Greagent), purified water, and dimethyl sulfoxide (Maclean's).

Instruments and equipment

Instruments and equipment used were as follows: Zebrafish culture system (Shanghai Haisheng Biological Experimental Equipment Co., Ltd), fluorescence microscope-ICX41RFL series (Ningbo Sunyu Instruments Co., Ltd. No: 2202923K), stereo microscope (Chongqing Photoelectric), high-resolution mass spectrometer (Thermo Fisher Scientific), high-performance liquid chromatograph (Thermo Fisher Scientific), and chromatographic column (ACQUITY UPLC BEH C8 [100 mm×2.1 mm×1.7 μ m], Waters Technology Co. [Waters]).

Preparation of lipid samples

The crude lipids were extracted from PQ, SOYA, and YOLK by the previous method with some modification [21]. 25 g each of PQ, SOYA, and YOLK (six batches each) were crushed and passed through a 60-mesh sieve, and extracted 3 times with 95% ethanol for 4 hours. The mixtures were filtered, evaporated, and then extracted with n-hexane 3 times. The n-hexane layers were concentrated and mixed with 4 times volume of acetone for 12 hours. The n-hexane layer was collected, dried, the lipids of PQ, SOYA, and YOLK were obtained, named PQL, SOL, and YOL. All lipids samples were stored at -20°C until use.

Preparation of zebrafish embryos

The wild AB line adult zebrafish were reared in a semi-static system at 28°C under 14 h light/10 h dark and fed with artemia twice a day. Before the night, adult male and female zebrafish were placed in the mating tank at a ratio of 2:2. The next day, the zebrafish were stimulated by light to mate and spawn naturally. The embryos were collected within 1 h of spawning and washed with fish water. The clean embryos were transferred into the E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl_2 , 0.16 mM MgSO_4) and placed in a light incubator at 28.5°C for subsequent experiments. After 24 h, 0.2 mM N-phenylthiourea was added to the E3 medium to prevent the embryos from forming melanin. The animal study was reviewed and approved by the Ethics Committee of Shandong First Medical University & Shandong Academy of Medical Sciences.

Development of Dox-induced cardiotoxicity in the zebrafish model

Zebrafish embryos (AB) at 30 hpf were randomly placed in a 6-well cell culture plate with 20 larvae in each well. The exposure components were divided into five groups: the control group (left untreated), the Dox treated group (10, 20, 30, and 40 μ M Dox). The samples were mixed, covered, and placed in a constant temperature and light incubator at 28°C. After 42 h of administration in dark, observed and photographed under a fluorescence microscope. Four parameters (heartbeat, stroke volume, cardiac area, fractional shortening) were calculated to evaluate the cardiac functions. Measurements of cardiac

functions were performed as previously described [22]. Briefly, the lengths of longitudinal axis (a) and lateral axis (b) were measured and ventricular volume was calculated with the formula $V = 4/3\pi ab^2$. Stroke volume was the difference between end-diastolic volume and end-systolic volume. Fractional shortening (%) was calculated by formula: $(\text{diastolic diameter} - \text{systolic diameter}) / (\text{systolic diameter}) \times 100\%$. All zebrafish samples were quantitatively analyzed with the Image J software.

Protective effect of lipid samples against Dox-induced cardiotoxicity

Zebrafish embryos (AB) at 24 hpf were transferred to a 6-well culture plate, 20 larvae per well. Three kinds of lipid samples (PQL, SOL and YOL) dissolved with culture water were added to the different drug groups with concentrations of 1.25, 2.5, and 5 $\mu\text{g/mL}$. All groups were placed in the light incubator (28°C) for the embryos to continue to develop. After 6 h, 30 μM of Dox was used to treat the zebrafish in each group for 42h except for the control group. Then, observed and photographed under a fluorescence microscope. The cardiac area, stroke volume, and Fractional shortening (%) of all zebrafish samples were quantitatively analyzed with the Image J software. **Metabolomics analysis**

Pre-treatment: The three lipid samples including PQL, SOL and YOL, were weighed 20 mg into 1.5-mL Eppendorf (EP) tubes. 20 μL internal standard (Lyso PC-17:0, 0.1 mg/mL, methanolic preparation) was added, 400 μL isopropanol-methanol ($v:v = 1:1$) was added, followed by a 30 s vortex and sonication for 3 min, and the solution was transferred to 1.5 mL EP tubes, which were left to stand at -20°C for 2 h. The samples were centrifuged for 10 min at 13000 rpm, and 150 μL the collected supernatant was loaded into LC-MS injection vials with liner tubes for LC-MS analysis. The quality control samples (QC) were prepared by mixing equal volumes of extracts from all samples, with the volume of each QC being the same as the sample.

LC-MS conditions: The chromatographic conditions were set as follows: column temperature = 55°C ; acetonitrile: water = 6:4 (v/v with 10 mM ammonium acetate, mobile phase A); isopropanol: acetonitrile = 9:1 (v/v with 10 mM ammonium acetate, mobile phase B); flow rate = 0.26 mL/min; and injection volume = 2 μL .

The solvent gradients were set as follows: 32% B, initial; 32% B, 1.5 min; 85% B, 15.5 min; 97% B, 15.6 min; 97% B, 18.0 min; 32% B, 18.1 min; and 32% B, 20.0 min.

MS conditions were as follows: heated-electrospray ionization (HESI-Positive) in positive and negative ion modes, respectively. The samples were separated using Dionex U3000 UHPLC (Thermo Scientific™) and then analyzed by MS using a Q Exactive Plus (Thermo Scientific™).

MS primary full scan + data-dependent acquisition (DDA) secondary sub-ion scan- positive ion mode conditions were set as follows: spray voltage = + 3.5 kV; capillary temperature = 300°C ; aux gas heater temperature = 350°C ; sheath gas flow rate = 45 Arb; aux gas flow rate = 10 Arb; S-lens radio frequency (RF)

level = 50; mass range (m/z) = 150–1500, full MS resolution = 70000; MS/MS resolution = 17500; TopN = 10; and normalized collisional energy (NCE)/stepped NCE = 25, 35, 45.

Mass spectrometry primary full scan + DDA secondary sub-ion scan- negative ion mode conditions were set as follows: spray voltage = -3.0 kV; capillary temperature = 300°C; aux gas heater temperature = 350°C; sheath gas flow rate = 45 Arb; aux gas flow rate = 10 Arb; S-lens RF level = 50; mass range (m/z) = 150–1500, full MS resolution = 70000; MS/MS resolution = 17500; TopN = 10; and NCE/stepped NCE = 25, 35, 45.

Model discrimination

The matrix was imported in R to carry out Principle Component Analysis (PCA) to observe the overall distribution among the samples and the stability of the whole analysis process. Orthogonal Partial Least-Squares-Discriminant Analysis (OPLS-DA) and Partial Least-Squares-Discriminant Analysis (PLS-DA) were utilized to distinguish the metabolites that differ between groups. To prevent overfitting, 7-fold cross-validation and 200 Response Permutation Testing (RPT) were used to evaluate the quality of the model.

Identification of differential lipids:

Variable Importance of Projection (VIP) values obtained from the OPLS-DA model were used to rank the overall contribution of each variable to group discrimination. A two-tailed Student's T-test was further used to verify whether the metabolites of difference between groups were significant. Differential metabolites were selected with VIP values greater than 1.0 and p-values less than 0.05.

Evaluation of the anti-Dox cardiotoxicity of the FA (18:4) using zebrafish model

Zebrafish embryos (AB) at 24 hpf were transferred to a 6-well culture plate, (20 larvae per well). FA (18:4) at concentrations of 0.125, 0.25, 0.5 $\mu\text{g/mL}$ were added to the different drug groups. All groups were placed in the light incubator (28°C) for the embryos to continue to develop. After 6 h, 30 μM Dox was used to treat the zebrafish in each group for 42h except for the control group. Then, observed and photographed under a fluorescence microscope. The cardiac area, stroke volume, and Fractional shortening (%) of all zebrafish samples were quantitatively analyzed with the Image J software.

Following the above experiments, the staining was performed using the ROS kit, and the fluorescence staining of the zebrafish cardiac area was observed and recorded under a fluorescent microscope, and the fluorescence intensity was statistically analyzed with the Image J software.

Data processing and analysis

The original Q Exactive LC-MS/MS data in raw format were processed by software Lipid Search for MSⁿ and the exact mass-to-charge ratio (m/z) of parent ions. The molecular structure of lipids and the additive mode of its positive and negative ions were identified. According to the parent ions and multi-

stage mass spectrometry data of each individual sample. The results were aligned according to a certain retention time range and combined into a single report to sort out the original data matrix. In each sample, all peak signals were normalized (that is the signal intensity of each peak is converted to the relative intensity in the spectrum, and then multiplied by 10000). The extracted data were then further processed by removing any peaks with a missing value (ion intensity = 0) in more than 50% in groups and by replacing the zero value by half of the minimum value. A data matrix was combined from the positive and negative ion data.

The activity experiments were subjected to a one-way analysis of variance (ANOVA) using GraphPad Prism for each group of data, and the results are expressed as the mean $\pm$ standard error of the mean (SEM).

Results And Analysis

Construction of Dox-induced cardiotoxicity model in zebrafish

After Dox (30 and 40 μ M) treatment, the zebrafish heart shape had been elongated, with distorted heart morphology (Fig. 1-e). These morphological defects led to significant and pronounced reductions in the stroke volume (** $p < 0.01$), cardiac area (** $p < 0.01$) and fractional shortening (FS) (* $p < 0.05$), as well as a moderate decrease in heart rate (** $p < 0.01$) (Fig. 1-a–d), indicating abnormal ventricular filling and systolic dysfunction. Dox (20 μ M) caused obvious reduction in heart rate (* $p < 0.05$) and stroke volume (* $p < 0.05$) compared with the blank group, however, no significant differences were observed in cardiac area and fractional shortening (Fig. 1-a–d). Dox (10 μ M) did not caused any significant changes in cardiac morphologies or functions when compared to the control (Fig. 1-a–d).

Evaluation of the anti-Dox cardiotoxicity of PQL

The heart distorted into an elongated shape and was significantly smaller after Dox (30 μ M) treatment compared with the blank group (Fig. 2-e). These morphological alterations resulted in significant reductions in stroke volume (## $p < 0.01$), cardiac area (## $p < 0.01$), and Fractional shortening (%) (# $p < 0.05$) (Fig. 2-b–d) and a moderate reduction in heart rate (# $p < 0.05$) (Fig. 2-a), indicating abnormal ventricular filling and systolic dysfunction with successful molding. PQL protected the heart and maintained normal ventricle and atrium morphologies, and improved cardiac performance impaired by Dox, significantly increasing the Dox-induced decreases in stroke volume (* $p < 0.05$), heart rate (* $p < 0.05$), and fractional shortening (* $p < 0.05$) (Fig. 2-a–d). In contrast, did not have any protective effects in the SOL and YOL groups (Fig. 2-a–d).

Lipidomic assays

The TIC is shown in Fig. 3. A total of 1664 lipids were detected. The three samples mainly comprised fifteen types of lipids, with 414 TG, 291 PE, 265 PC, 102 DG, 84 PG, 68 PS, 67 SM, 63 LPC, 60 PI, 55 Cer, 31 OAHFA, 24 LPE, 15 PA, 14 MG, and 6 FA. The relative quantification of lipids was calculated by comparison with the total peak area of lipids. PQL was rich in TGs, accounting for 34.09%, the FA content of 23.72%, the OAHFA content of 14.07%, the DG content of 8.45%; SOL was rich in PC, accounting for 28.65%, the TG content of 21.32%, the PI content of 9.77%; YOL was rich in PC, accounting for 33.01%, the PE content of 18.49%, the TG content of 13.49%.

Multivariate statistical analysis

Multivariate statistical analysis was performed to differentiate the lipids from PQL, SOL, and YOL using PCA analysis. As shown in Fig. 4-a, these three groups were distributed in different areas, indicating that the three kind of lipids were significantly different from each other. PLS-DA and OPLS-DA were employed for variance analysis and lipid screening to highlight the differences in lipids. There was a significant separation in the PLS-DA (Fig. 4-b) and OPLS-DA scatter point diagram (Fig. 5). The R2 (R2 < 0.1) and Q2 (Q2 < 0) values of the model showed good robustness and reliability of the model, which was not overfitted (Fig. 6-a).

The S-plot of OPLS-DA was used to determine the differential lipids among PQL, SOL, and YOL. Differential metabolites were screened and identified according to S-plot and VIP values (Fig. 6-b).

Identification of differential lipids

The lipid screening used VIP > 1.0 from the OPLS-DA model and a p-value < 0.05 from the t-test to identify the differences in lipids among the groups. In final, 182 lipids showed significant differences between the PQL and SOL groups, 198 differential lipids between the PQL and YOL groups were observed, 202 differential lipids between the SOL and YOL groups were observed, and 216 differential lipids between these three lipid groups were observed. The top five most discriminating lipids of these three lipids are listed in Table 1.

Table 1
Top five most discriminating lipids in PQL, SOL, and YOL, representing the most significant differences among the lipid extracts.

No.	Lipid Group	Formula	VIP value	P-value
1	FA (18:4)-H	O ₂ H ₂₈ C ₁₈	12.99076098	4.99092E-14
2	OAHFA (36:3)-H	C ₃₆ H ₆₄ O ₄	7.520443922	1.54652E-14
3	PI (34:2)-H	C ₄₃ H ₇₉ O ₁₃ N ₀ P ₁	6.4383657	8.05777E-09
4	TG (54:6) + NH4	C ₅₇ H ₁₀₂ O ₆ N ₁	5.798802401	1.5238E-17
5	PC (36:4) + H	C ₄₄ H ₈₁ O ₈ N ₁ P ₁	5.622513514	8.56963E-22

Figure 7 shows a heat map of the top 50 total differential metabolites. The longitudinal axis shows the clustering of samples, while the transverse is the clustering of metabolites. The shorter the clustering branches, the higher the similarity. The clustering of metabolite content between groups can be seen by the horizontal comparison. It indicated that the lipid compositions of each group were clearly different.

Categorization of differential metabolite

As shown in Fig. 8a-c, the percentage of FAs in the PQL was about 28% of that in the differential metabolites, and all of them were polyunsaturated fatty acids (PUFA), which has proved beneficial for humans, the proportion of it in SOL and YOL was 0.01% and 5%, respectively. Besides, triglyceride (TG) accounted for 33%, 21%, and 13% of the PQL, SOL, and YOL, respectively; (O-acyl) ω -hydroxy FAs (OAHFA) accounted for 15%, 0%, and 1% of the PQL, SOL, and YOL, respectively; and PC accounted for 2%, 30%, and 34% of the PQL, SOL, and YOL, respectively. Phosphatidic acid (PA) accounted for 3%, 1%, and 0% of the PQL, SOL, and YOL, respectively, and diacylglycerol (DG) accounted for 9%, 4%, and 1% of the PQL, SOL, and YOL, respectively.

FAs in PQL were mainly FA (18:4), accounting for 98.97%, followed by FA (20:4), accounting for 1.019%, and FA (22:4) and FA (22:5), accounting for 0.007% and 0.002%, respectively (Fig. 9-c). FA (18:4) showed a VIP value of 12.99076098, was the differential metabolite with the highest VIP value. Its relative contents were 27.265%, 0.005%, and 0.002% in PQL, SOL, and YOL, respectively (Fig. 9-a).

As shown in Fig. 8a-c, in the differential metabolites, TG accounted for 33% in the PQL, which was the highest proportion, whereas TG accounted for 21% and 13% in SOL and YOL, respectively. Medium-and long-chain triacylglycerol (MLCT) is the few examples of the "new generation" custom-made healthful lipids, showed to acquire multiple physiological and functional properties in managing and reversing certain health disorders [23]. The highest relative content of MLCT was approximately 1.1383% in the TG of PQL, 0.7431% and 0.0035 in TG of SOL and YOL (Fig. 9-b). Therefore, PQL has more advantages than SOL and YOL.

Cardiotoxicity of the FA (18:4) against Dox and ROS determination

Omega-3 PUFAs can exert a positive effect on cardiovascular diseases [24]. FA (18:4), one of PUFAs, the cardioprotective effect of it was verified in the present study. Heart rate ($^{##}P < 0.01$) cardiac area ($^{##}P < 0.01$) stroke volume ($^{##}P < 0.01$) fractional shortening (%) ($^{##}P < 0.01$) were significantly lower in Dox-treated only group (30 μ M) compared with the control group (Fig. 10a-d), which indicated abnormal ventricular filling, systolic dysfunction in model group. Compared with the model group, FA (18:4) administration protected the heart, maintaining the normal ventricular and atrial morphology and improving Dox-induced alterations in cardiac functions, and significantly improved the Dox-induced reduction in the heart rate ($^{*}P < 0.05$), stroke volume ($^{*}P < 0.05$) and fractional shortening (%) ($^{**}P < 0.01$) (Fig. 10a-d).

Myocardial cell death induced by ROS is thought to be the molecule mechanism by which Dox damages the myocardium, leading to life-threatening cardiomyopathy [25]. The ROS-staining results (Fig. 11a-b) indicated that the zebrafish heart region fluorescence intensity in the model group increased significantly compared with in the blank group, indicating that Dox (30 μ M) treatment induced ROS production in the heart region ($^{\#}P < 0.05$). After the treatment of FA (18:4) at a dose of 0.125 μ g/mL, the fluorescence intensity decreased significantly compared with the model group ($^*P < 0.05$), indicating a reduction in the production of ROS and an ameliorative cardiotoxic effect.

Discussion

The accumulation of the antineoplastic drug Dox causes irreversible impairment of the heart, which limits its use [26]. The clinical cardiotoxic effects of Dox are manifested by the deterioration of cardiac functions, including reduced left ventricular ejection fraction, ROS accumulation, fibrosis, calcium overload, and apoptosis. The mechanism is mainly attributed to nuclear and mitochondrial dysfunction, resulting in apoptosis, necrosis, fibrosis, and autophagy [27]. Zebrafish are highly similar to humans in terms of cardiovascular functions and morphology and are a well-established *in vivo* model for studying ventricular dysfunction and congestive heart diseases. The hearts of zebrafish embryos can be directly observed using a simple bright-field microscope, which facilitates the study of morphological changes in the heart induced by Dox cardiotoxicity. Thus, zebrafish embryos were used to establish a model of Dox-induced heart injury.

Lipids perform various important biological functions, including energy conversion, material transport, cell development and differentiation, information recognition and transmission, and apoptosis. Furthermore, polyunsaturated lipids possess diverse biological activities, such as cardiovascular protective activity, pro-angiogenic activity, anti-inflammatory activity [28], and hepatoprotective activity [29]. FAs, the key components of the cell membrane and complex lipids, exert cardioprotective effects, and studies performed at the National Heart, Lung, and Blood Institute have shown that alpha-linolenic acid and linoleic acid can reduce the risk of heart disease by 40% and 70%, respectively [30], and endogenous n-3 PUFAs prevents dilated cardiomyopathy via orchestrating gene expression, protein phosphorylation, and lipid metabolism [31].

Lipids have been extensively studied, however, the function of lipids in Chinese herbs has been understudied, and fewer studies have been performed. The lipid component of forsythia has been reported as a basis for active plant growth, development, and metabolism. Lipids in natural products are mainly concentrated in pregnenolone and polyethylene glycol [32]. Lipids in egg yolk contain platelet-activating factor inhibitors, which increase their nutritional value in the prevention of cardiovascular diseases and have anti-atherosclerotic properties [33]. Soybean lipids can effectively inhibit platelet aggregation in the vascular endothelium and improve hemodynamic levels, reduce blood viscosity, lower the levels of inflammatory factors in the body, and improve the activity of regulatory factors, thereby improving blood irrigation in infarcted lesions [34]. Therefore, PQ, SOYA, and YOLK lipids were extracted in the present study. Based on the traditional efficacy and modern pharmacological characteristics of PQ,

FA (18:4) standards, PQL, SOL, and YOL were systematically evaluated and compared for anti-Dox cardiotoxicity, and the results showed that the lipid extracts of PQ exerted positive effects, whereas the SOL and YOL did not produce marked effects. Furthermore, differences among the PQL, SOL and YOL were investigated. According to the lipidomics results, the differential metabolites between these three lipid groups showed similar PA and DG contents, with PA accounting for 3%, 1%, and 0% in the PQL, SOL, and YOL, respectively, whereas DG accounted for 9%, 4%, and 1% in the PQL, SOL, and YOL, respectively. FA content varied greatly, accounting for 28%, 0%, and 5% in the PQL, SOL, and YOL, respectively. The screening result indicated that 216 differential metabolites with VIP > 1 were obtained. The VIP value of the FA (18:4) was the highest among the differential metabolites, which accounted for 27.265% in the PQL and 0.005% and 0.002% of the SOL and YOL, respectively.

In addition to ginsenosides and polysaccharides, FAs in PQ may play an essential role in the overall beneficial effects. The FA (18:4) has been previously identified in algae [35] and the leaves of *Tabebuia aurea* (Silva Manso) [36]. However, a few studies have explored its activity. Based on the lipidomics analysis and the results of this activity assay, it is evident that the highest PUFA content was found in the PQL, which exerted an anti-Dox cardiotoxic effect. The contents of SOL and YOL were low in PUFAs and had no significant anti-Dox cardiotoxic effects. Thus, it is evident that PQ PUFAs have great potential in drug development. The anti-Dox cardiotoxic activity of the differential metabolite FA (18:4) was validated and found to have significant anti-Dox cardiotoxic effects, which reduced cardiac ROS production caused by Dox cardiotoxicity.

The high content of PUFAs in the PQL has unique advantages in terms of physiological functions and biological activities, and these PUFAs have been confirmed to have cardioprotective effects. However, their production is still rare. Their exploitation is promising and has rich research value. However, their activity was not studied in detail in the present study, and their mechanism of action needs in-depth exploration.

Conclusions

The present study is the first report to show the protective effects of the lipid components of PQ against Dox-induced cardiotoxicity, and the FA (18:4) was found to be critical among them. This study may provide a reference for the use of PQ to develop anti-Dox cardiotoxic drugs and nutraceuticals and may offer a theoretical basis for the development of drugs for treating Dox-induced cardiotoxic diseases.

Abbreviations

PQ: *Panax quinquefolius*; PQL: lipid extracts of *Panax quinquefolius*; SOL: lipid extracts of soybean; YOL: lipid extracts of egg yolk; Dox: doxorubicin; hdf: hour post-fertilization; dpf: day post-fertilization; ROS: reactive oxygen species; FA: fatty acid; PUFA: polyunsaturated fatty acids; FA (18:4): fatty acid with 18 carbons and four double bonds; TG: triglyceride; DG: diacylglycerol; PA: Phosphatidic acid; OAHFA: (O-acyl) ω -hydroxy FAs; MLCT: medium-and long-chain triacylglycerol; Cer: ceramide; PI:

phosphatidylinositol; PE: phosphatidylethanolamine; LPE: lysophosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; PS: Phosphatidylserine; PG: phosphatidylglycerol; MG: monoglyceride;

Declarations

Acknowledgements

Not applicable.

Author contributions

KH performed the research, analyzed the data, and wrote the paper. HW, HW, TL were supportive during the experiment. SW, LH designed and performed the study. ZL analyzed lipids. All authors read and approved the final manuscript.

Funding

This work was financially supported by the Funding of the Key Project at Central Government Level: the ability establishment of sustainable use for valuable Chinese medicine resources (2060302), the Natural Science Foundation of Shandong Province (ZR2019MH037), and Academic Promotion Program of Shandong First Medical University (No. 2019LJ003).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal study was reviewed and approved by the Ethics Committee of Shandong First Medical University & Shandong Academy of Medical Sciences.

Consent for publication

Competing interests

The authors declare no competing interests regarding the publication of this manuscript.

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Figures

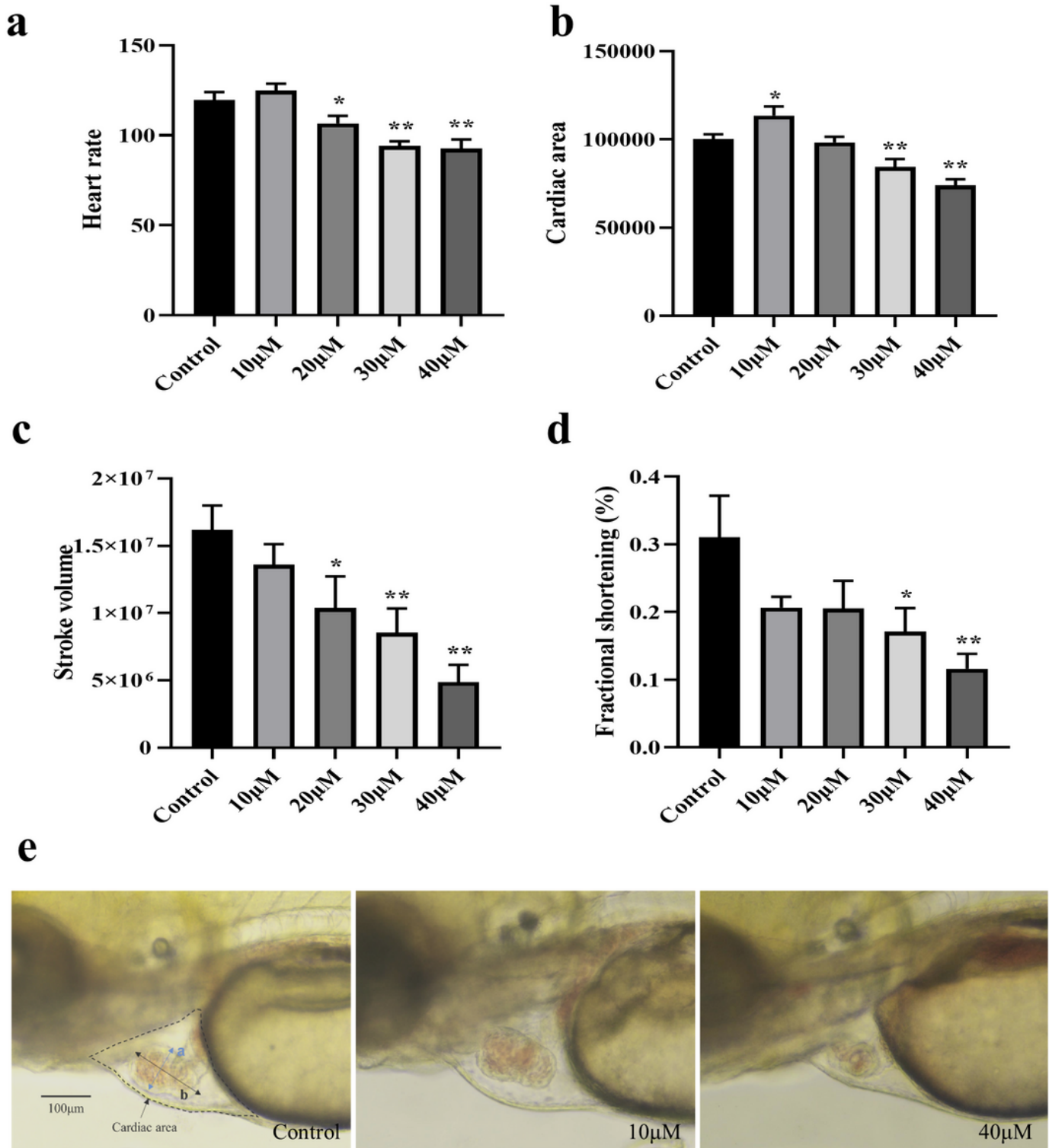


Figure 1

Construction of a doxorubicin (Dox) cardiotoxicity model in zebrafish. Zebrafish at 30 hpf were treated with different concentrations of 10, 20, 30, and 40 μM Dox for 42h. Dox-induced impairment of cardiac function in zebrafish(a–d). Heart rate (a), cardiac area (b), stroke volume (c), and fractional shortening (%) (d). Representative microscope images of zebrafish (e). Data are presented as the mean ± S.D. *, $P < 0.05$, **, $P < 0.01$.

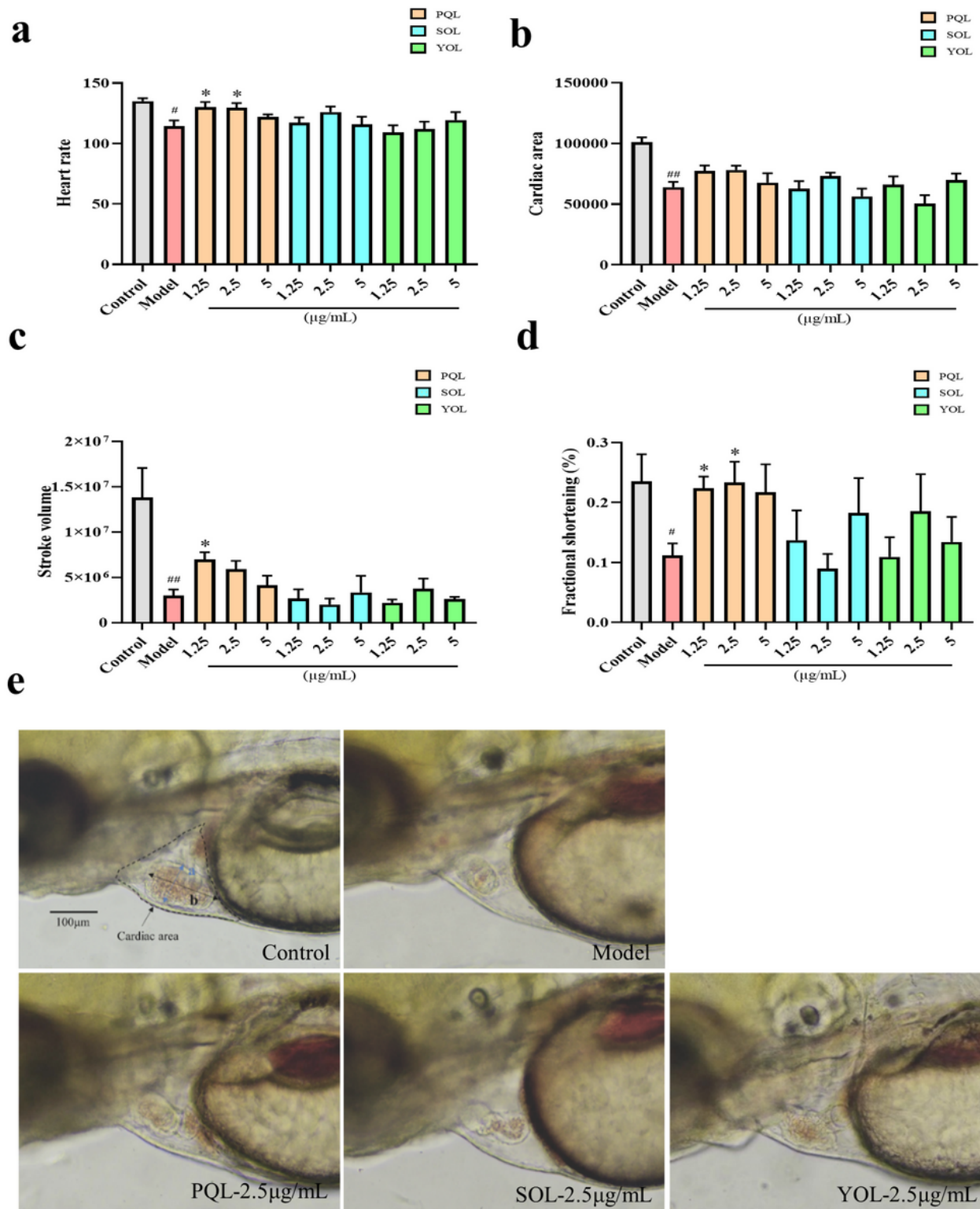


Figure 2

Protective effect of three lipid extracts against Dox-induced cardiotoxicity in zebrafish. Zebrafish at 24 hpf were treated with different concentrations (1.25, 2.5, and 5μg/mL) of PQL, SOL, YOL for 6 h, followed by the addition of 30 μM Dox for 42 h. Effects of PQL, SOL, YOL on Dox-induced impairment of cardiac function in zebrafish(a–d). The heart rate (a), cardiac area (b), stroke volume (c), and fractional

shortening (%) (d) are shown. Representative microscope images of zebrafish (e). Data are presented as mean $\pm$ SD. #P < 0.05 and ##P < 0.001 vs. control group, *P < 0.05 and **P < 0.01 vs. model group.

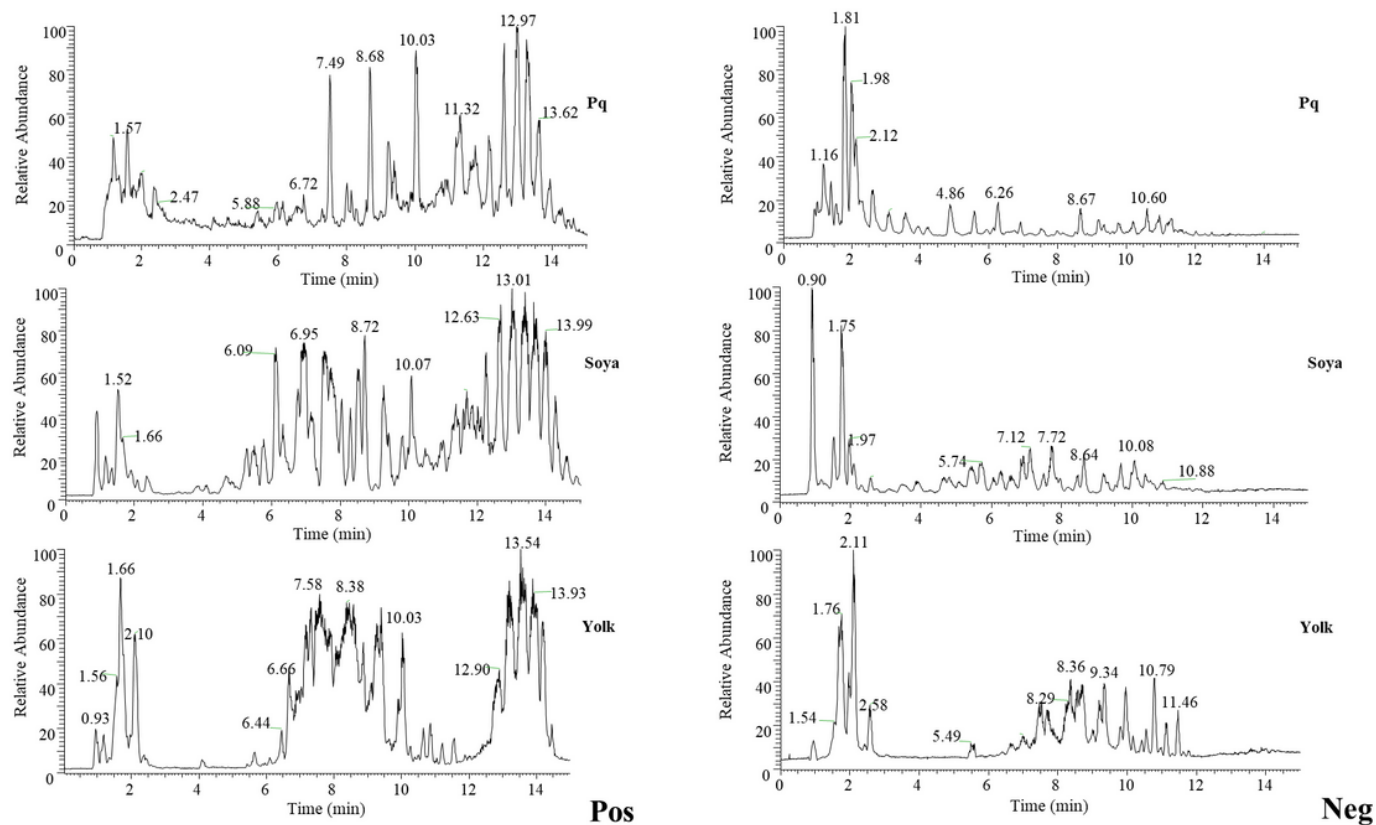


Figure 3

Total ion chromatogram of PQL, SOL, and YOL. Left: positive ion mode, right: negative ion mode.

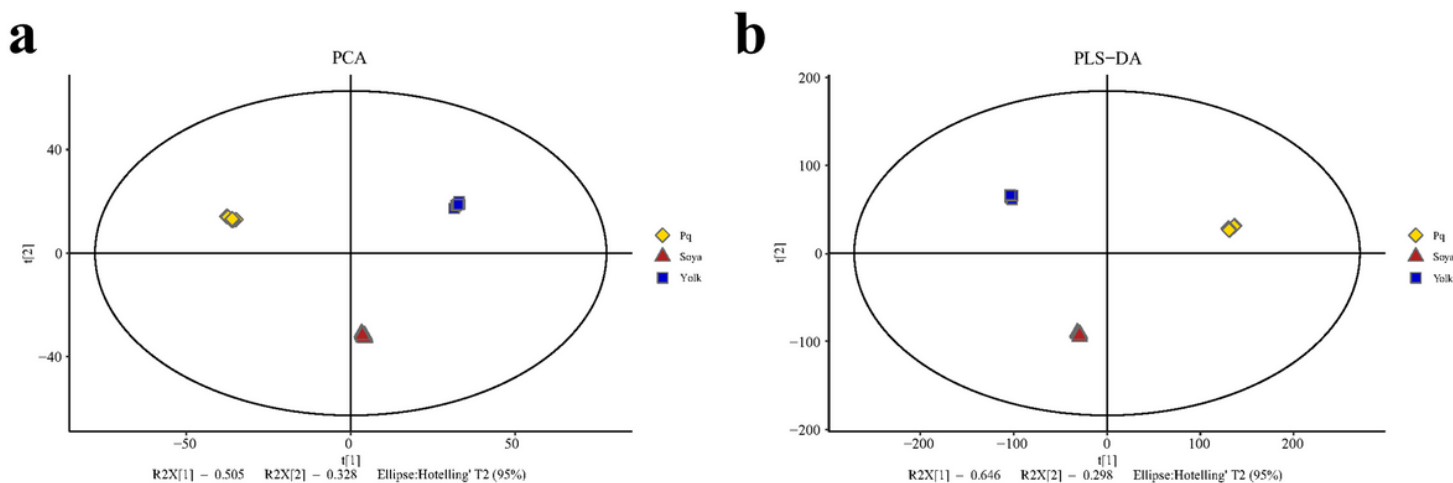


Figure 4

(a) PCA scores of PQL, SOL, and YOL. (b) Plots of PLS-DA scores of PQL, SOL, and YOL.

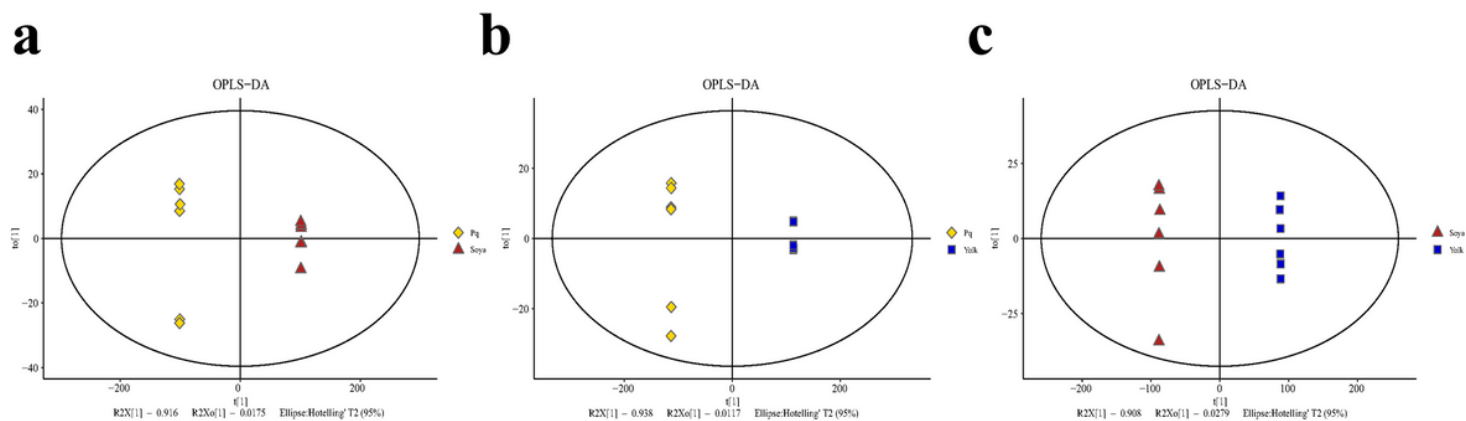


Figure 5

(a) OPLS-DA scores of PQL and SOL. (b) OPLS-DA scores of PQL and YOL. (c) OPLS-DA score of SOL and YOL.

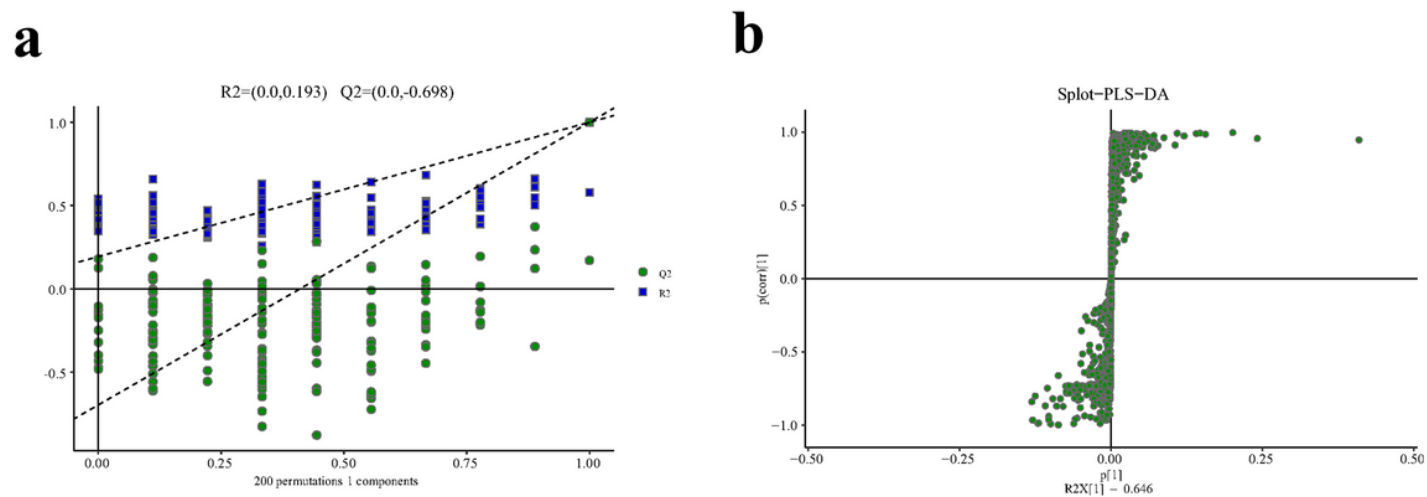


Figure 6

(a) Permutation plots of PQL, SOL, and YOL. (b) S-plots of PQL, SOL, and YOL.

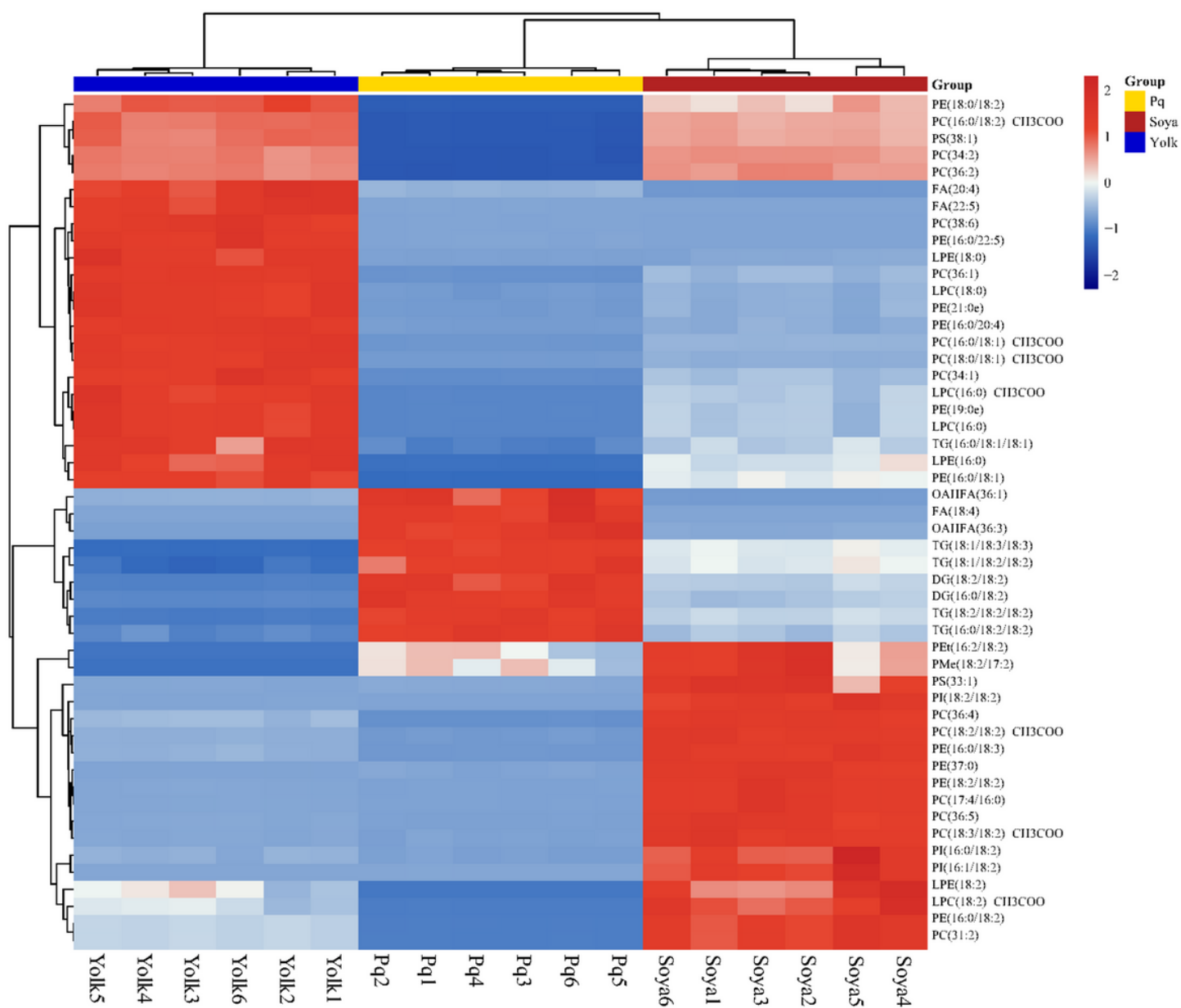


Figure 7

Clustering heat map of the top 50 total differential metabolites

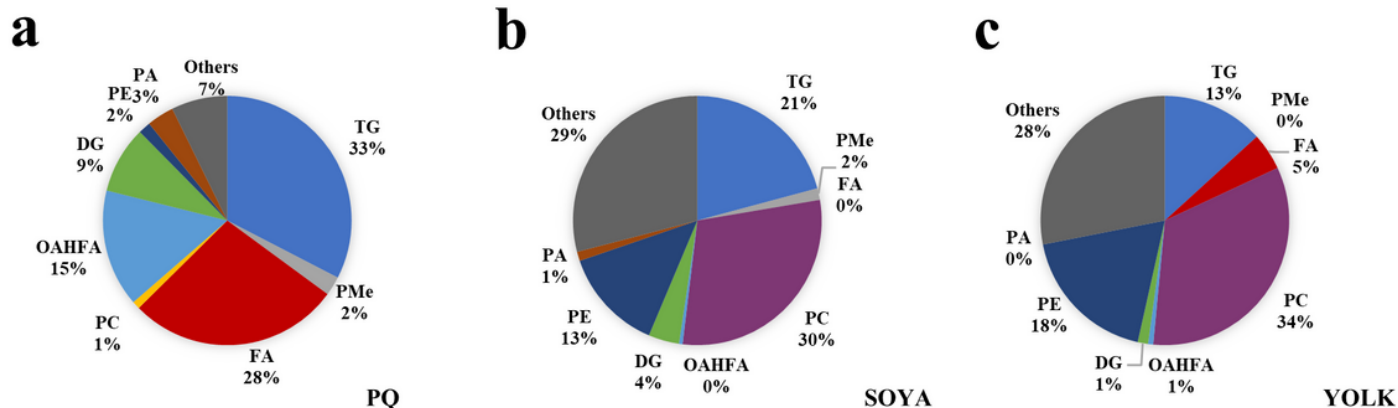


Figure 8

Lipid profiles of PQL, SOL, and YOL (percentage of of the top 50 total differential metabolites). (a) Proportion of each type of lipid in PQL in the differential metabolites. (b) Proportion of each type of lipid in SOL in the differential metabolites. (c) Proportion of each type of lipid in YOL in the differential metabolites.

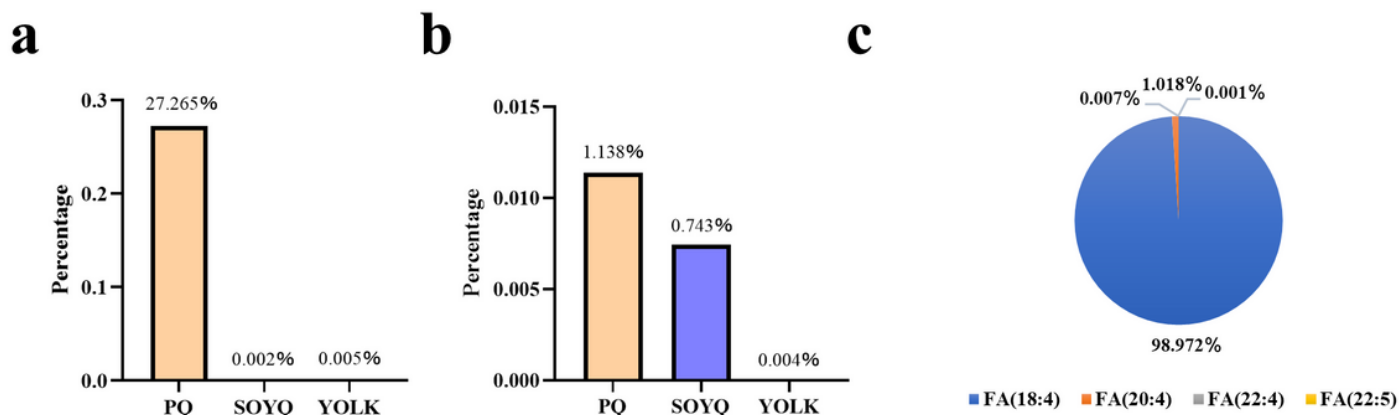


Figure 9

Advantageous lipid profiles of PQL, SOL, and YOL (percentage of of the top 50 total differential metabolites). (a) Proportion of the FA (18:4) in the differential metabolites of PQL, SOL, and YOL. (b) Proportion of MLCT in the differential metabolites of TG of PQL, SOL, and YOL. (c) Proportions of various polyunsaturated FAs in the differential metabolites of the lipids in PQL.

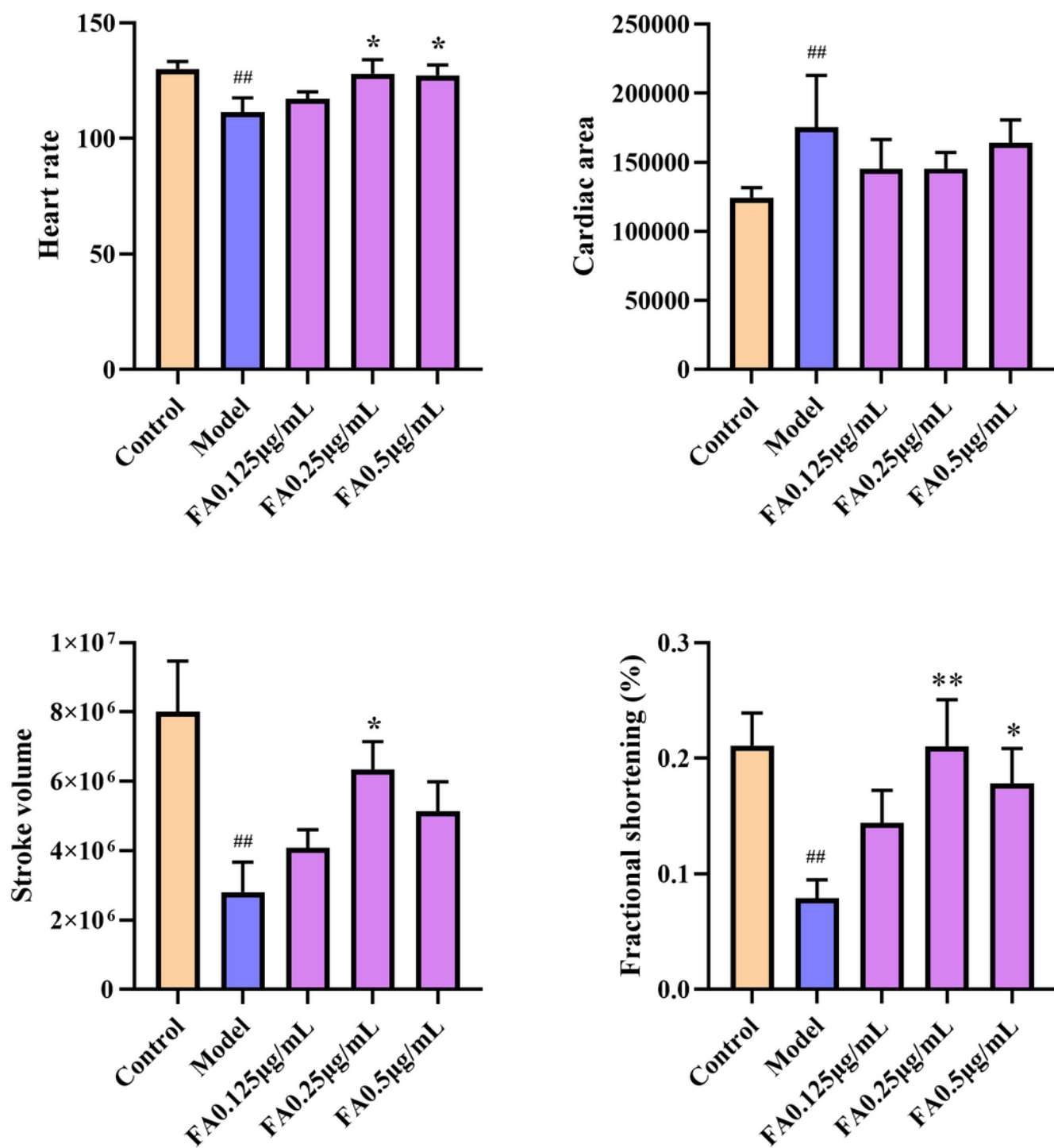


Figure 10

Protective effect of FA (18:4) on Dox-induced cardiotoxicity in zebrafish. The zebrafish at 24 hpf were exposed to different concentrations (0.125, 0.25, and 0.5 µg/mL) of the FA (18:4) for 6 h, followed by the addition of 30 µM Dox for 42h. Protective effect of FA (18:4) on the Dox-induced impairment of cardiac functions in the zebrafish(a-d). Heart rate (a), cardiac area (b), stroke volume (c), and fractional

shortening (%) (d). Data are presented as mean $\pm$ SD. $^{\#}P < 0.05$ and $^{##}P < 0.001$ vs. control group, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. model group.

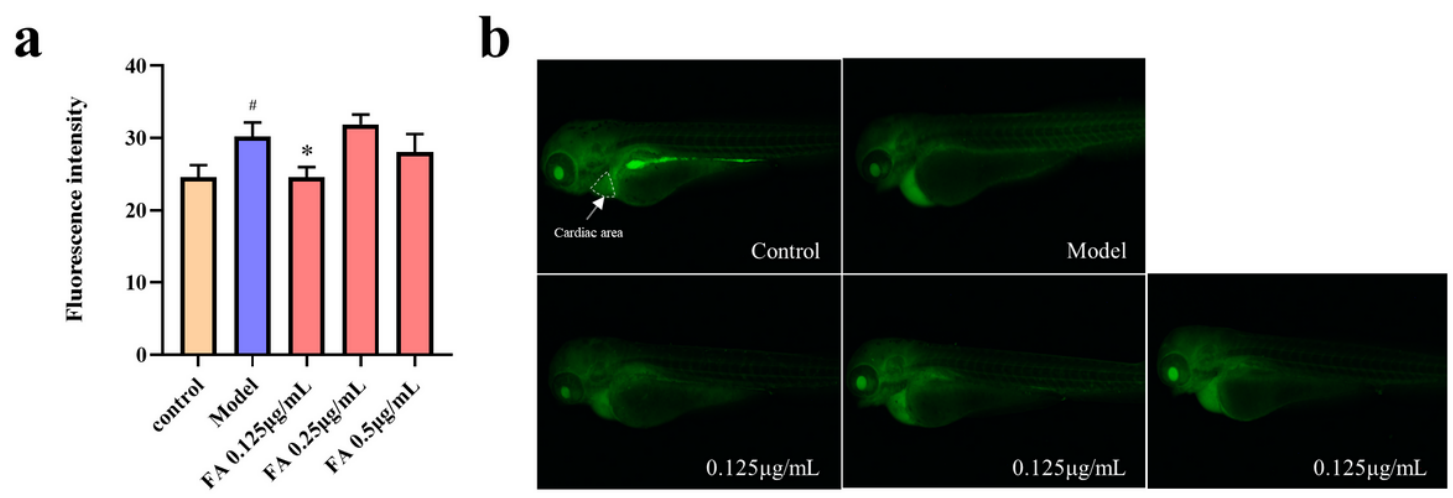


Figure 11

Protective effect of FA (18:4) on Dox-induced cardiotoxicity in zebrafish. The zebrafish at 24 hpf were treated with the FA (18:4) at different concentrations (0.125, 0.25, and 0.5 $\mu\text{g/mL}$) for 6 h, followed by the addition of 30 μM Dox for 42 h. ROS staining was performed, and the intensity of green fluorescence represented the amount of ROS produced. (a) Protective effect of the FA (18:4), on the Dox-induced impairment of cardiac function in the zebrafish. (b) Representative fluorescence microscopy images of the zebrafish. Data are presented as mean $\pm$ S.D. $^{\#}P < 0.05$ vs. control group, $^{*}P < 0.05$ vs. model group.