Electronic Supplementary Material (ESI) for ChemComm.

Supplemental Materials

Quantitative chemical proteomics for investigating the biomarkers of dioscin against liver fibrosis caused by CCl_4 in rats

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Supplementary materials and methods

Real-time PCR assay

Total RNA from cells and livers was extracted using RNAiso Plus reagent following the manufacturer's protocols. RNA (1 μg) was reverse-transcribed using a PrimeScript® RT reagent Kit in a TC-512 PCR system (TECHNE, UK), and single-stranded cDNA was quantified using real-time PCR with SYBR® PremixEx Taq™II (Tli RNaseH Plus) in an ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The primers used in the present work are listed in Supplemental Table 1. A no-template control was analyzed in parallel with each gene, and the GAPDH gene was used as the housekeeping gene in our study. The unknown template was calculated using a standard curve for quantitative analysis.

Western blotting assay

Total protein was extracted from cells and livers tissues using an appropriate cold lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and the protein concentration was determined using a BCA protein assay kit (Beyotime Biotechnology, China). Samples were subjected to SDS-PAGE (10%-15%) and transferred onto a PVDF membrane (Millipore, USA). Membranes were blocked and incubated overnight at 4°C with the primary antibodies listed in Supplemental Table 2. Membranes were incubated at room temperature with an appropriate secondary antibody, and proteins were detected using an enhanced chemiluminescence (ECL) method. Protein bands were imaged using a Bio-Spectrum Gel Imaging System (UVP, USA). Bands were normalized with GAPDH as an internal control.

Investigation of dioscin-induced proteins alterations using proteomics

Protein samples were extracted from rat livers of control, model and dioscin-treated groups, and were treated with liquid nitrogen and grinded into a uniformly powder. 1200 µl Alklysis buffer (7M urea, 2M thiourea, 4% (W/V) CHAPs and PMSF) (Sigma, USA) was used for protein extraction. Samples were sonicated on ice (80 W, 0.8 s duration, 10 times, 0.8 s intervals, repeated six times), nucleic acid fragmentation, then centrifuged at 12000 × g for 20 min at 4°C. The supernatant was transferred to a new centrifuge tube, and four volumes of acetone was added, then precipitated overnight at -20°C, and centrifuged at 12000 × g for 20 min at 4°C, then the precipitate were collected and stored at -80°C. After the protein quantification by BCA assay, the concentration of each sample protein was adjusted to 10 µg/ml. Protein labeling and 2D-DIGE analysis were performed as the described method [1]. The sample proteins were labeled with CyDye DIGE Fluor (minimal dye) Labeling Kit (GE Healthcare, CT, USA). A 400 pmol of the sample dry was added per 50 µg of sample. The investigation was performed in three biological duplicates. Equal amounts (25 µg) of proteins from the test samples were pooled together as the internal standards and labeled with Cy2. And 50 µg of protein from control, model and dioscin were labeled with Cy3 and Cy5. The labeling reactions were performed on ice in the dark for 30 min, and then quenched with 50-fold molar excess of free lysine to dye for 10 min on ice. Immobilized pH gradient (IPG) strip (24 cm, pH 4-7) (GE Healthcare, CT, USA) were rehydrated with a mixture of 120 µg Cy-labeled samples in immobile drystrip rewelling tray overnight at 20ºC. The first-dimension isoelectric focusing (IEF) was performed at 20ºC, using voltages and running times as follows: S1 stp 300 V 30 min; S2 stp 700 V 30 min; S3 stp 1500 V 30 min; S4 grd 9000 V 3 h; S5 stp 9000 V 4 h. After IEF, gel strips were incubated in equilibration solution

(50 mM Tris/HCl, pH 8.8, 6 M urea, 30% Glycerol, 2% SDS, 0.002% Bromophenol blue, 100 mM DTT) for 15 min, followed by 15 min incubation away from light with equilibration solution where the DTT was substituted by 250 mM iodoacetamide. Equilibrated IPG strips were transferred onto 12.5% uniform polyacrylamide gel and overlaid with 0.5% agarose solution. SDS-PAGE was carried out using EttanTM DALT six (GE, Healthcare), and performed at 2W/gel for 45 min, followed by 17 W/gel until the bromophenol blue dye front had run off the bottom of the gels. After second-dimension SDS-PAGE, gels were scanned at a resolution of 100 microns on a Typhoon 9400 variable mode imager (GE Healthcare). Cy2-, Cy3- and Cy5-dye images of each gel were acquired at excitation/emission values of 488/520, 523/580 and 633/670 nm, respectively. Image analysis was performed using the Image-Master 2D Platinum 7 (GE Healthcare), and analysis of variance (ANOVA) was applied to matched spots and the data were filtered to retain protein spots with $p \leq 0.05$ determined by 1-way ANOVA and a fold-change of ≥ 1.5. Preparative 2D PAGE gels were set up by loading 1200 μg of protein extract into 24 cm IPG strips, which were then focused and subjected to 2-DE electrophoresis as described above. And the gel was subjected to mass-compatible silver staining in order to track the spots to be excised for protein identification. Proteins spots with significant change were excised from preparative gels that matched with the 2D-DIGE image, and subjected to digestion with trypsogen (Promega). The peptides resulting from tryptic digestion were subsequently desalted for MALDI-TOF/TOF MS (Ultraflex III TOF/TOF, Bruker Dalton) analysis. In the survey full scan MS spectra (from 700 Da to 3200 Da), the UV wavelength was set at 355 nm, the repetition rate was set 200 Hz, and acceleration voltage of 20000V, the optimal resolution mass was 1500Da. The flexAnalyaia

software (Bruker Dalton) was used to filter baseline peak and identify signal peaks. And the protein identification was done via Bio Tools (Bruker Dalton) software against NCBI database.

References

1 B. T. Sun, L. H. Zheng, Y. L. Bao, C. L. Yu, Y. Wu, X. Y. Meng and Y. X. Li, Eur. J. Pharmacol., 2011, 654, 129–134.

Supplemental Table 1 The primer sequences used for real-time PCR assay

Gene	GenBank accession	Primers (5'-3')
α-SMA	NM_031004	Forward: AGCCAGTCGCCATCAGGAAC
		Reverse: GGGAGCATCATCACCAGCAA
COL1A1	NM_053304	Forward: GACATGTTCAGCTTTGTGGACCC
		Reverse: AGGGACCCTTAGGCCATTGTGTA
COL3A1	NM_032085	Forward: TTTGGCACAGCAGTCCAATGTA
		Reverse: GACAGATCCCGAGTCGCAGA
GAPDH	NM_017008.3	Forward: GGCACAGTCAAGGCTGAGAATG
		Reverse: ATGGTGGTGAAGACGCCAGTA

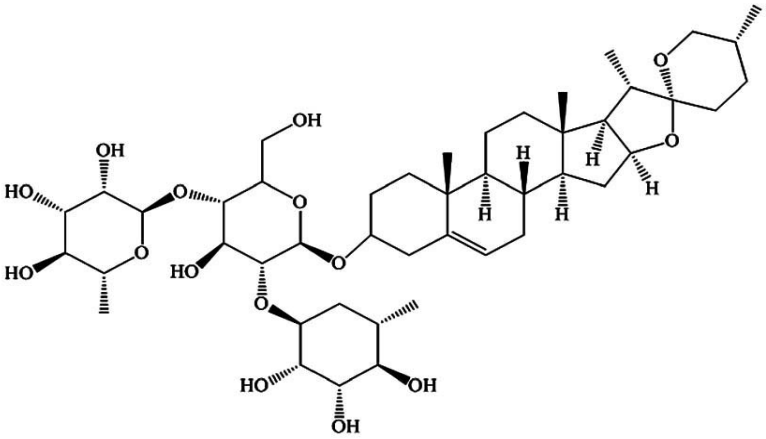
Supplemental Table 2 The differentially expressed proteins from rat livers identified by proteomics

Spot	Gene name	Protein name	Scores	Nominal mass (M_r)	pI value	Sequence Coverage
A10	EST3	Liver carboxylesterase 3	81	61961	5.63	8%
A11	PDIA3	Protein disulfide-isomerase A3	231	57044	5.88	18%
A13	PDIA3	Protein disulfide-isomerase A3	86	57044	5.88	18%
A19	SBP1	Selenium-binding protein 1	317	53069	6.1	36%
A22	ALDH2	Aldehyde dehydrogenase, mitochondrial	259	56966	6.63	11%
A23	AL9A1	4-trimethylaminobutyraldehyde dehydrogenase	194	54530	6.57	25%
A25	GSHB	Glutathione synthetase	376	52597	5.48	36%
A38	SUCB2	Succinyl-CoA ligase subunit beta, mitochondrial	110	47096	6.58	18%
A39	SUCB2	Succinyl-CoA ligase subunit beta, mitochondrial	321	47096	6.58	21%
A48	ARK72	Aflatoxin B1 aldehyde reductase member 2	164	41105	8.35	22%
A52	ALDR	Aldose reductase	158	36230	6.26	27%
A54	GPDA	Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic	312	38112	6.16	46%
A55	CK054	Ester hydrolase C11 or f54 homolog	96	35427	6.16	29%
A56	RGN	Regucalcin	321	33939	5.27	49%
A57	HMCS2	Hydroxymethylglutaryl-CoA synthase, mitochon - drial	248	57332	8.86	21%
A59	CSAD	Cysteine sulfinic acid decarboxylase	254	55841	6.84	19%
A68	ST1E1	Estrogen sulfotransferase, isoform 1	184	35828	5.78	35%
B05	HEMO	Hemopexin	183	52060	7.58	19%
B09	HEMO	Hemopexin	108	52060	7.58	22%
B16	KNT2	T-kininogen 2	148	48757	5.94	13%
B18	ALBU	Serum albumin	379	70682	6.09	39%
B24	KNT1	T-kininogen 1	236	48828	6.08	5%
B35	PDIA1	Protein disulfide-isomerase	234	57315	4.82	20%
B40	CALR	Calreticulin	231	48137	4.33	23%
B41	A1AT	Alpha-1-antiproteinase	102	46278	5.7	16%
B49	A1AT	Alpha-1-antiproteinase	248	46278	5.7	27%
B56	GSHB	Glutathione synthetase	264	52597	5.48	33%
B60	Not identified					

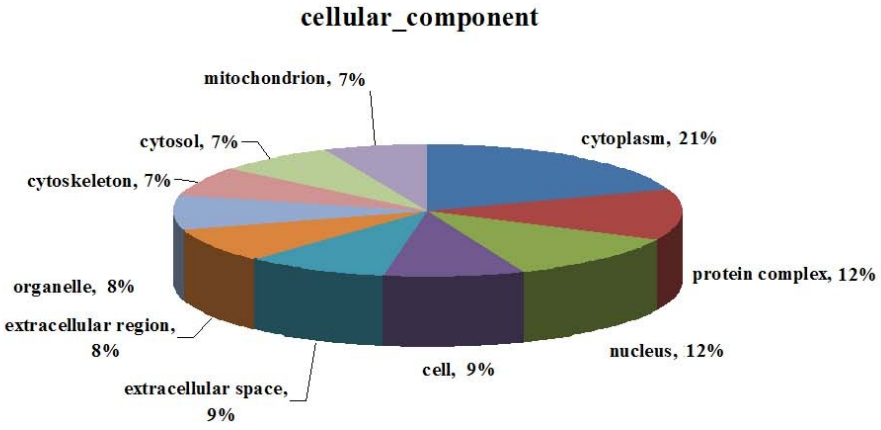
B61	K2C8	Keratin, type II cytoskeletal 8	441	53985	5.83	48%
B63	K2C8	Keratin, type II cytoskeletal 8	155	53985	5.83	18%
B64	Not identified					
B66	VIME	Vimentin	190	53757	5.06	38%
B69	RCN1	Reticulocalbin-1	186	38090	4.7	26%
B71	K1C18	Keratin, type I cytoskeletal 18	276	47732	5.07	35%
B72	HSP7C	Keratin, type I cytoskeletal 18	171	71055	5.37	13%
B74	K1C19	Heat shock cognate 71 kDa protein	190	44609	5.21	48%
B76	ACTB	Keratin, type I cytoskeletal 19	348	42052	5.29	26%
B77	K2C8	Actin, cytoplasmic 1	193	53985	5.83	22%
B90	HPT	Haptoglobin	145	39052	6.1	19%
B96	EFHD2	EF-hand domain-containing protein D2	413	26743	5.01	43%
B98	ANXA5	Annexin A5	478	35779	4.93	39%
B102	HSPB1	Heat shock protein beta-1	422	22936	6.12	51%
B104	HSPB1	Heat shock protein beta-1	145	22936	6.12	31%
B107	Not identified					
B109	APOA1	Apolipoprotein A-I	203	30100	5.52	47%
B111	STMN1	Stathmin	129	17278	5.76	30%
B112	MYL6	Myosin light polypeptide 6	154	17135	4.46	25%
B113	COTL1	Coactosin-like protein	172	16036	5.28	47%

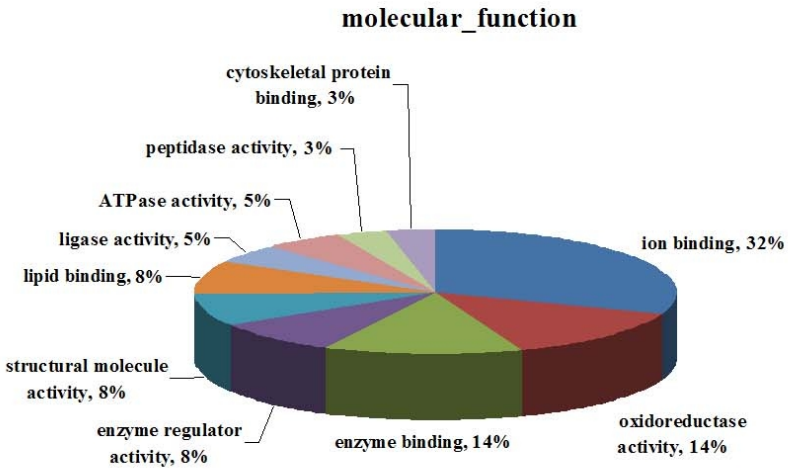
Supplemental Table 3 The information of the antibodies used in the present work

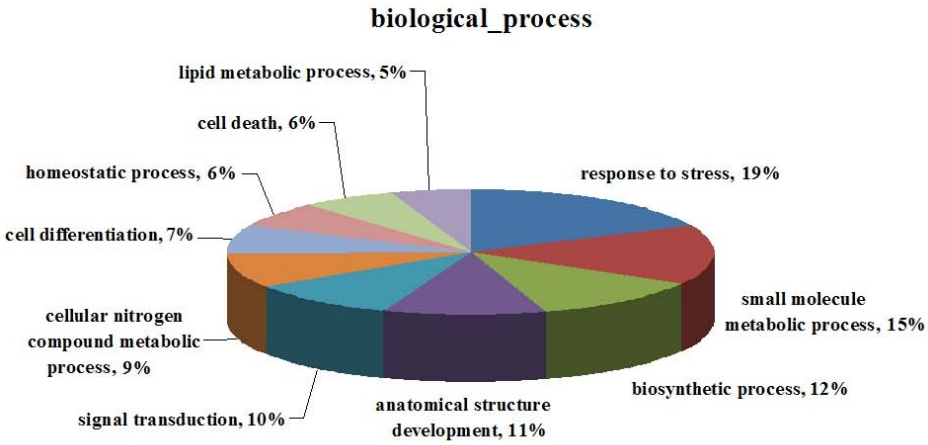
Antibody	Source	Dilutions	Company
PDIA3	rabbit	1: 1000	Proteintech Group, Chicago, USA
SELENBP1	rabbit	1: 1000	Bioworld Technology, USA
GSS	rabbit	1: 1000	Bioworld Technology, USA
RGN	rabbit	1: 1000	Proteintech Group, Chicago, USA
Hemopexin	rabbit	1: 1000	Bioworld Technology, USA
Keratin 8	rabbit	1: 1000	Proteintech Group, Chicago, USA
Vimentin	rabbit	1: 1000	Proteintech Group, Chicago, USA
Keratin 18	rabbit	1: 1000	Proteintech Group, Chicago, USA
ANXA5	rabbit	1: 1000	Proteintech Group, Chicago, USA
DPT	rabbit	1: 1000	Proteintech Group, Chicago, USA
GAPDH	rabbit	1: 1000	Proteintech Group, Chicago, USA



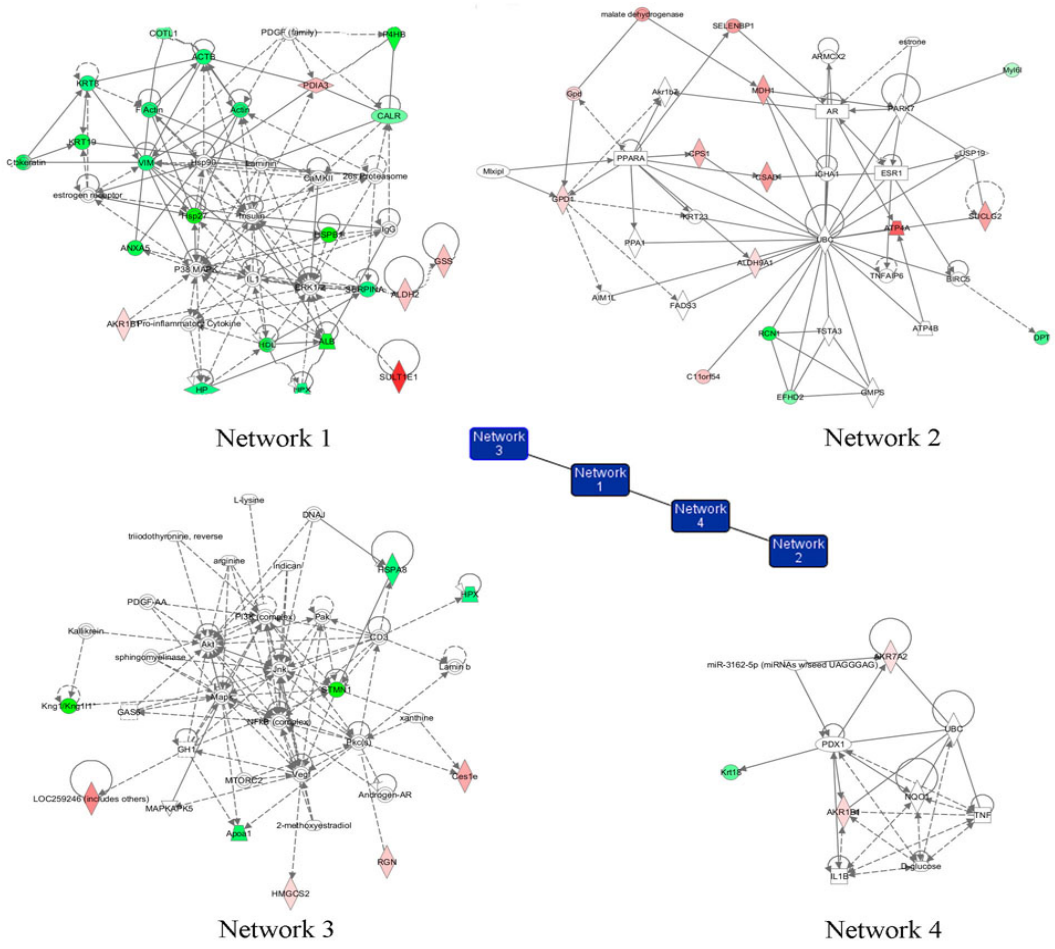
Supplemental Fig. 1 The chemical structure of dioscin.



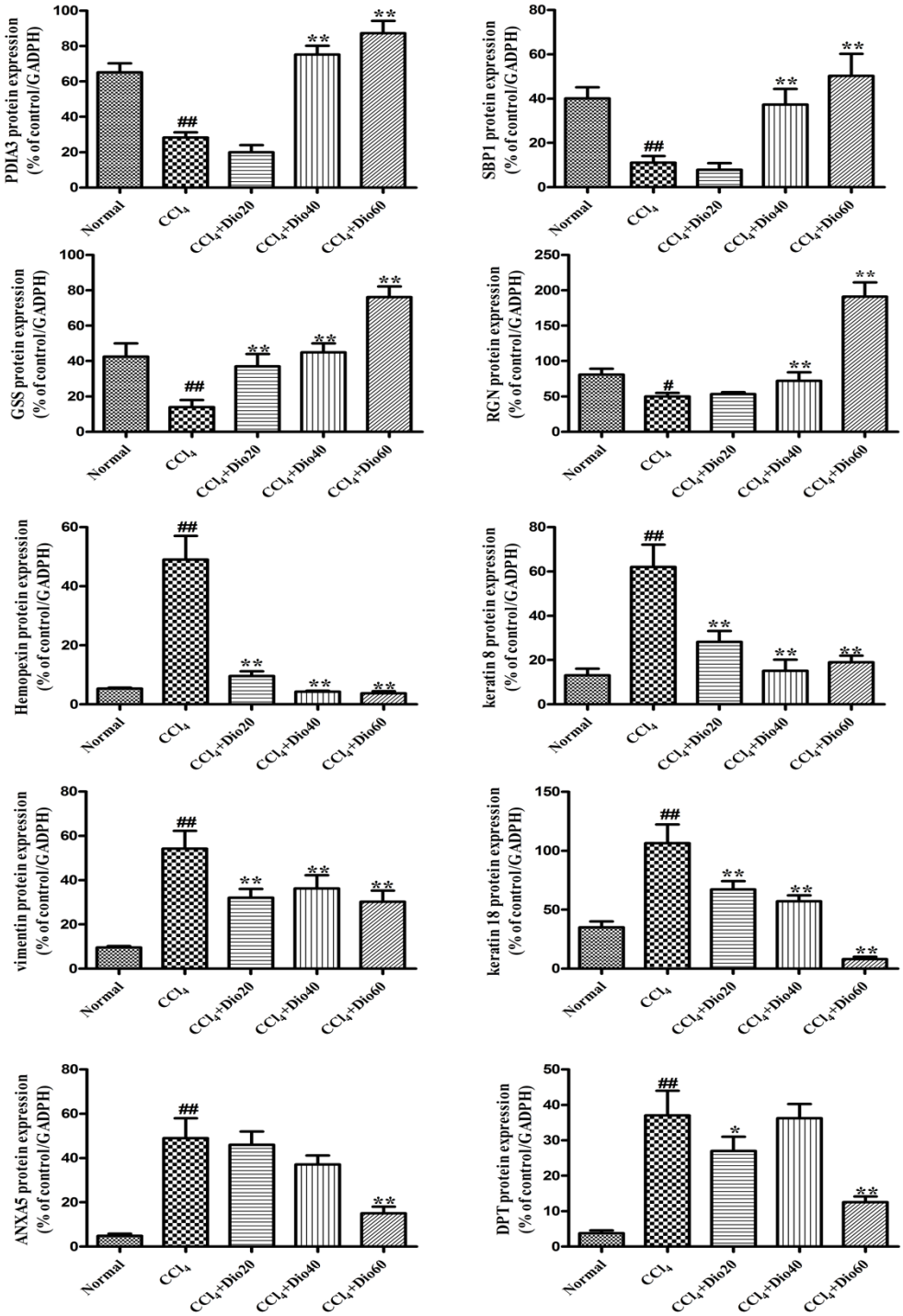




Supplemental Fig. 2 Classification of the differentially expressed proteins identified by proteomics according to the cellular component, molecular function and biological process by Gene Ontology (GO) analysis.



Supplemental Fig. 3 The regulation networks of the differentially expressed proteins caused by dioscin to treat liver fibrosis in rats based on IPA analysis. Each node represents one protein and the direct (represented by solid lines) and indirect (represented by dotted lines) association with other proteins. Proteins with red color represent up-regulation, and those with green color indicate down-regulation in the study. Moreover, the deeper color is, the larger difference will be.



Supplemental Fig. 4 Western blot analysis of PDIA3, SBP1, GSS, RGN, hemopexin, keratin 8, keratin 18, vimentin, ANXA5 and DPT in rat livers. Values are expressed as the mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ vs. CCl_4-treated group; $^{\#}p < 0.01$, $^{\#\#}p < 0.01$ vs. normal group.