Electronic Supplementary Material (ESI) for ChemComm.


# Supporting Information

# Gambogic acid suppresses the pentose phosphate pathway by covalently inhibiting 6PGD protein in cancer cells

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**Reagents and Materials**

Gambogic acid (GA) (purity≥98%) was purchased from Beijing Bethealth People Biomedical Technology (Beijing, China). N-acetyl-L-cysteine (NAC), Liproxstatin-1 (Lip-1), Z-VAD (OMe)-FMK (Z-VAD-FMK), and Physcion were obtained from MedChem Express (USA). Click chemistry reaction and LC-MS/MS reagents: Biotin-azide, TBTA, TCEP, Rhodmine-N3 and $CuSO_4$ were bought from Sigma (USA). TMT 10plex™ reagent, high capacity neutravidin agarose resin, and sequencing grade modified Trypsin were purchased from Thermo Fisher (USA). Specific primary antibodies against GPX4 and 6PGD were purchased from Abcam (Shanghai, China). GAPDH was obtained from Affinity Biosciences (USA).

**Cell culture**

A549 cell, HepG2 cell and HeLa cell were purchased from the Chinese Academy of Medical Sciences (Beijing, China). All cells were cultured in high glucose DMEM (Corning, USA) supplemented with 10% FBS (Corning USA), and 100 IU penicillin/streptomycin (Thermo Fisher) at 37 ℃ with 5% $CO_2$.

**Anticancer activity detection *in vitro***

Cells were seeded in 96-well culture plates at $8\times10^3$ cells per well for 24 h. After the desired treatment of GA (0, 0.5, 1, 2, 4, 8, 16, 32, 64 μM), cells were washed twice with PBS buffer to remove dead cells, and then the CCK-8 (Dojindo, Japan) assays were added to test the cell viability.

To further test the rescue effect of NAC, Lip-1 and Z-VAD-FMK on GA-induced cell death, 1 mM NAC, 100 nM Lip-1 or 40 μM Z-VAD-FMK was added to the system with GA and incubated at 37 °C for 24 h, and then the cell proliferation was detected as above described.

**Measurement of intracellular ROS**

Flow cytometry was used to measure intracellular reactive oxygen species (ROS). The A549 Cells ($3\times10^5$/well) were seeded into a 6-well culture plate and then incubated

with or without serial dilutions of GA for 6 h. 10 μM DCFH-DA probe (Beyotime Biotechnology) was added and incubated for 30 min. Finally, the fluorescence intensity was then measured with a flow cytometer (Beckman, USA).

**Apoptosis assay**

The experiment referred to the instructions of the apoptosis detection kit (Beyotime Biotechnology). A549 cells were treated with 8 μM GA for 4 h, DMSO used as the control, then harvested and stained with Annexin V-FITC/PI at room temperature for 10-20 min. The apoptotic cells were detected by flow cytometry.

**Western blotting assay**

Proteins were extracted from A549 cell lysis and separated by SDS-PAGE gel, following by electro-transferring to a PVDF membranes. Subsequently, samples were treated with primary antibodies and secondary antibody. Bands were visualized by enzyme-linked chemiluminescence assay using the Azure C400 system (USA), and analyzed by image J software.

**6PGD enzyme assay**

The enzymatic activity was measured by 6PGD activity detection kit (Solarbio, China). GA and phy were added to 0.5 mg/mL 6PGD (0.25 μL) in PBS, and the absorbance at 340 nm was measured to react enzyme activity. We used a microplate reader to scan every three minutes until 21 minutes.

**NADPH/$NADP^+$ ratio assay**

NADPH/$NADP^+$ were measured using the $NADP^+$/NADPH Assay (Beyotime Biotechnology). Briefly, A549 Cells ($1\times10^6$ cells/well) were put into a 6-well culture plate for 24 h, and collected after the GA treatment for 24 h. Then PBS was used to wash cells and follow the kit instructions. The total amount of NADPH and $NADP^+$ in the samples was determined by a microplate reader (EnVision2105, PerkinElmer,

USA), and then the content of NADPH was determined. According to the following formula, calculate the ratio of $NADP^+$/NADPH in the sample.

[NADPH /$NADP^+$] = [NADPH] / ([NADP total]-[NADPH])

**Measurement of R-5-P**

Intracellular R-5-P content was measured with R-5-P detection kit (Xian Biotechnology, China). A549 cells were seeded into 6-well culture plates and incubated with serial dilutions of GA for 4-6 hours and quantified referred to the kit instructions.

**Cellular Imaging**

Fluorescence imaging experiments were carried out to demonstrate the co-localization interactions of GA and potential cell target 6PGD. A549 cells were cultured in a 4-chamber glass bottom dish (Cellvis) and treated with 0.5 mL of DMEM containing 10% FBS in the absence or presence of GA (0 and 10 μM). After treatment for 4 h, the medium was replaced by 20 μM IAA-yne for 1 h. After that, cells were gently washed with 0.5 mL PBS. Subsequently, cells were fixed with cell fixative for 20 min and permeabilized in 0.2% Triton X-100 for 15 min. Cells were then subjected to click reactions as previously described for 2 h at room temperature, followed by incubation with anti-6PGD (1:1000) at 4 ℃ overnight and goat anti-rabbit IgG (1:1000) (Alexa Fluor 488) for 2 h. Finally, Hoechst (1:500 dilution) was used to treat cells for 30 min prior to image acquiring by a laser scanning confocal fluorescence microscopy (Leica TCS SP8 SR).

DCFH-DA probe was used to monitor the accumulation of ROS in A549 cells. Cells ($5\times10^5$ cells/well) were treated with 10 μM GA in the absence or presence of NAC (1 mM) for 4 h, and then incubated with 10 mM DCFH-DA and hoechst for 30 min. After treatment, cells were examined by the confocal fluorescence microscope. DMSO-treated cell was used as a control.

**The competitive in-gel fluorescence labeling of GA in A549**

A549 cells ($1\times10^6$ cells/well) were cultured in the 6-cm plates and grown to 90% confluence before they were incubated with different concentrations of GA (0, 20, 40, and 60 μM) for 2 h. Then PBS was used to wash cells for three times to discard the GA solution. Cells were harvested with cell Scraper, and sonicated on ice until clear after addition of cell lysates (200 μL RIPA containing 1% protease inhibitor). The protein lysis can be obtained by centrifugation at 20, 000 g for 10 min, and diluted to 2 mg/ml with PBS using a BCA kit. After treatment with different concentrations of GA, 200 μg cell lysis were chosen to label the cysteine-specific probe (IAA-yne) for 1 h in shaker (800 rpm, 37 °C). Next, each sample was added 13 μL click buffer, including 9 μL TBTA (10 mM in DMSO), 3 μL TCEP (50 mM in $ddH_2O$), 3μL $CuSO_4$ (50 mM in $ddH_2O$), and 1 μL TAMRA-azide (10 mM in DMSO), and incubated in a shaker at 37 °C for 2 h. 1 mL acetone was added into the mixtures to precipitate the labeled proteins at -80 °C for 30 minutes. The supernatant was discarded by centrifugation for 10 minutes at 20, 000g and evaporated the acetone. The samples were dissolved with 100 μL 1× loading buffer, denatured by metal bath heating at 90 °C for 5 min and finally separated in 12% SDS-PAGE gel. Azure Sapphire RGB NIR scanner (USA) was used to capture fluorescence gel images, and coomassie brilliant blue was used to visualize the total proteins.

**The streamlined cysteine activity-based protein profiling**

To search for activity-based interacting cellular protein targets of GA, we performed competitive mass spectrometry experiments. A new desthiobiotin iodoacetamide (DBIA, ChomiX Biotech Co, Nanjing, China) probe with an optimization enrichment than alkyne-iodacetamide was selected for the following steps. Here are three groups: 500 μM DBIA, 50 μM GA +500 μM DBIA and 100 μM GA+500 μM DBIA. GA was uesd for the competition experiment. The cell culture, GA treatment, and DBIA labeling procedures were similar to those previously described for in-gel fluorescent labeling of GA in A549. 100 μM DBIA was added into 100 μL GA-treatment cell lysis and the mixture was incubated in the shaker for 1 h at 37 ℃. After the probe labeling was

completed, 5 mM DTT was added to the reaction in the dark at room temperature for 30 min to quench the excess DBIA and reduce disulfide bonds, followed by 20 mM IAA alkylating reduced cysteine residues for 20 min in the dark at room temperature. Subsequently, to remove excess DBIA, the protein was precipitated using a chloroform/methanol/water system (1:4:3), and centrifuged at 20, 000 g at room temperature for 3 min. Additionally, 500 μL of methanol was used to wash the precipitate twice. For digestion, 200 μl 200 mM EPPS at pH 8.52 M and 2 μl of trypsin and LysC (1 μg /μL, Promega) were added to re-dissolve the proteins and digest for the whole night at 37 ℃ using a ThermoMixer at 800 rpm. For peptide labeling, digested peptides containing DBIA-conjugated cysteines were labeled by a TMT10plex™ Mass Tag reagent (Thermo scientific) according to the instructions. 0.3% hydroxylamine was used to quench the excess TMT reagent. Then, all TMT-labeled samples were combined and dried by a Speedvac to remove all acetonitrile. For peptide enrichment, 100 μL streptavidin magnetic beads (Thermo scientific) were added into TMT-labeled pooled sample dissolved by 1 mL PBS, rotating for 4 h at room temperature to enrich peptides. To remove nonspecific bindings, beads were washed following the procedure: three times of 1 ml PBS, 1 ml 0.1% SDS and 1 ml dd water. Next, 200 μL 0.1% FA in 50% acetonitrile was added for 30 min in the shaker at room temperature to elute peptides on the beads and transferred to a new tube after centrifugal. Then, repeat the elution steps and combined all cysteine-containing peptides together using a Speedvac to dry it. Finally, after desalting with 0.1% formic acid, DBIA-labeled peptides were eluted with 0.1% FA in 50% acetonitrile from C18 column, and samples were analyzed by orbitrap fusion lumos (Thermo scientific).

### *In situ* pull-down experiment

To identify the interacting cellular target protein of GA, we performed pull-down experiments, followed by immunoblotting. Cell culture, GA treatment and IAA-yne labeling procedures were performed as previously described in the in-gel fluorescence labeling of GA in A549. Samples were divided into three groups: DMSO, IAA-yne, and GA+IAA-yne. 100 μL cell lysis of each group was mixed with the 13 μL click

chemistry:9 μL TBTA (10 mM in DMSO), 3 μL TCEP (50 mM in $ddH_2O$), 3μL $CuSO_4$ (50 mM in $ddH_2O$), and 1 μL TAMRA-N3 (5 mM in DMSO), and incubated in a shaker at 37 °C for 2 h at room temperature, prior to precipitation by pre-chilled acetone (-20°C). Subsequently, labeled proteins were discarded by centrifugation and air-dried, before dissolving in PBS containing 1.2 % SDS (1 mL), and heating at 95 °C for 15 min. 1.2% SDS containing soluble proteins were diluted to 0.1% SDS with 4 mL 1 × PBS and 70 μL streptavidin beads were added to pull down the interacting cellular target proteins. After rotating incubation for 4 h at RT, these beads were collected through centrifuging at 1400 g for 3 min, and washed with 5 mL 1% SDS three times, 5 mL 0.1% SDS three times, and 5 mL 6 M Urea three times successively. After eluted and denatured from beads with 1 × loading buffer for 10 min at 90 ℃, the enriched proteins were separated by SDS-PAGE gel for immunoblotting.

**The whole-proteome experiment**

Firstly, A549 cells treated with DMSO or 5 μM GA were harvested and the cell lysis was collected as previously described. The concentration of the protein was determined by a BCA kit and diluted to 1 mg/mL. Secondly, proteins were reduced using 10 mM DTT for 15 min at room temperature. Subsequently, reduced cysteine residues were alkylated using 10 mM IAA for 30 min, and precipitated with chloroform/methanol as previously described. Next, the protein was re-dissolved in EPPS buffer, and digested with trypsin (1:100 enzyme/protein ratio) at 37 ℃ overnight. Samples were desalting with a commercial C18 column (Waters)for further use in the end.

**Binding site identification of GA on 6PGD**

To find the direct binding site of GA on 6PGD, we performed the following experiment. First, 20 μg of 6PGD was mixed with 2 mM GA and incubated for 2 h in 200 μL PBS system. Then, 5 mM DTT was added for 30 min, and then 20 mM IAA was added to the mixed solution for alkylation. To remove excess drug and reagents, a methanol/chloroform system was chosen to precipitate proteins. As in the previous step,

trypsin was then used to cleave the protease into peptides. After desalting the C18 column, 0.1% FA was used to dissolve peptides and prepared for the MS detection.

**GO and KEGG enrichment analysis**

Differential protein analysis was performed based on changes in abundance in the DMSO (control) and GA (treated) groups in the whole proteome experiment. Differential proteins were selected based on absolute fold change ≥ 1.5 and P-value (FDR) < 0.05. According to previous literature [1], heatmap and volcano map were produced, and biological process and kegg were selected to visualized functional profiles and pathway enrichment.

**Metabolomics**

A549 cells grown to 70~80% confluent were treated with DMSO or 5 μM GA for 12 h. About $1 \times 10^7$ cells were washed twice in PBS and harvested for each sample. Each treatment contains 5 biological replicates. The samples were resuspended in pre-chilled 80% methanol, then sonicated for 6 min and centrifuged at 5,000 rpm for 1 min at 4°C. The supernatant was freeze-dried and dissolved in 10% methanol, and then the solution were used for LC-MS/MS system analysis. Then the samples were melted on ice and whirled for 30 s. After the sonification for 6 min, they were centrifuged at 5,000 rpm, 4 °C for 1 min. The supernatant was freeze-dried and dissolved with 10% methanol. Finally, the solution was injected into the LC-MS/MS system for analysis using an ExionLC™ AD system (SCIEX) coupled with a QTRAP® 6500+ mass spectrometer (SCIEX) as previous studies[2, 3]. To identify and quantify the metabolites, MRM (Multiple Reaction Monitoring) was used to detect the samples based on novogene in-house database. For metabolite quantification, the Q3 were used. For metabolite identification, the Q1, Q3, RT (retention time), DP (declustering potential) and CE (collision energy) were used. The SCIEX OS Version 1.4 was used to process the data files generated by HPLC-MS/MS to integrate and correct the peak. The main parameters were set as follows: minimum peak height, 500; signal/noise ratio, 5;

gaussian smooth width, 1. Relative content of the corresponding substance was represented by the area of each peak.

**Statistical analysis**

Statistical analysis was performed by GraphPad Prism 8.0 software (GraphPad Prism, USA). Student's *t*-test, one-way ANOVA test and two-way ANOVA test were used for statistical analyses of the data. P values <0.05, <0.01, <0.001 or <0.0001 were considered statistically significant.

**References**


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2. C. A. Sellick, R. Hansen, G. M. Stephens, R. Goodacre and A. J. Dickson, *Nat. Protoc.,* 2011, **6**, 1241-1249.
3. M. Yuan, S. B. Breitkopf, X. Yang and J. M. Asara, *Nat. Protoc.,* 2012, **7**, 872-881.

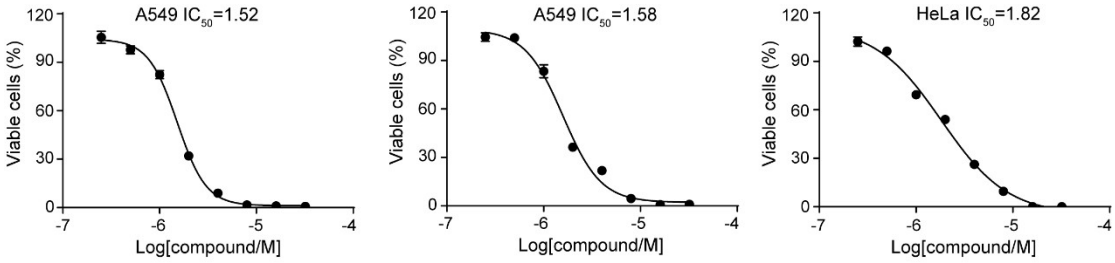


**Figure S1**. Detection of the killing effect of Gambogic acid on A549, HepG2 and HeLa cancer cell lines by CCK-8 assay.

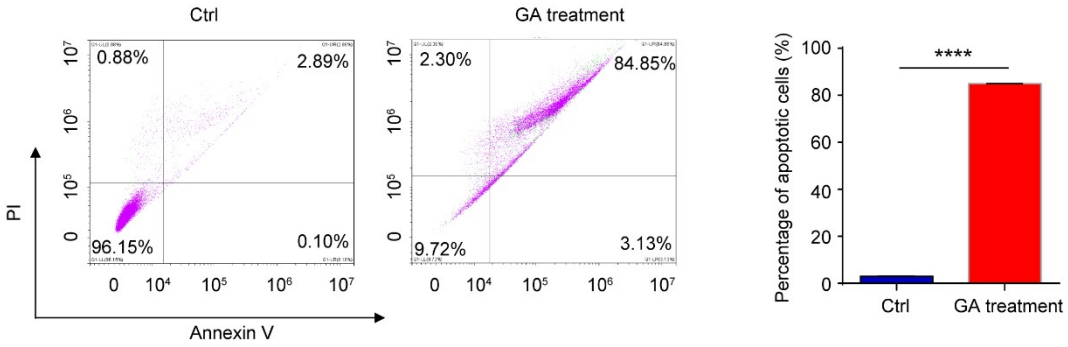


**Figure S2**. Detection of Gambogic acid-induced apoptosis in A549 by flow cytometry. Left, representative flow cytometric plots measuring apoptosis in Gambogic acid or DMSO treated A549 cells by annexin V and propidium iodide (PI) staining. Right, quantitation of the percentage of apoptotic cells. Data are mean ± s.e.m., *t*-test used. ****P<0.0001.

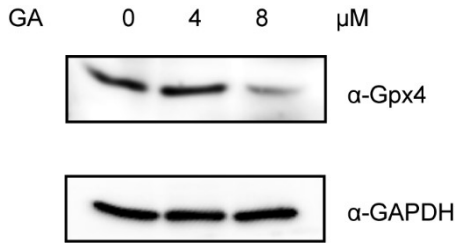


**Figure S3**. Gambogic acid treatment reduced the protein expression of GPX4 in A549 cells by western blotting.

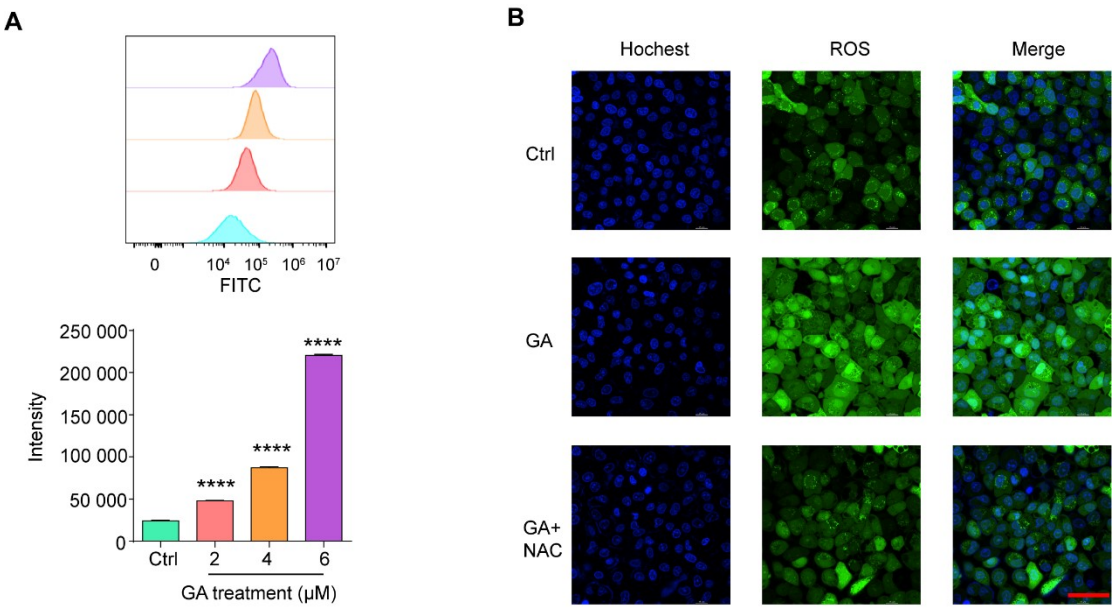


**Figure S4**. Gambogic acid induced the ROS level in A549 cells by flow cytometry (A) and immunofluorescence (B). Data are mean ± s.e.m., one-way ANOVA test used. ****P<0.0001. Bar = 50 μm.

**Table S1**. Significance analysis for Fig 1B.

| Tukey's multiple comparisons test | Significance | Summary |
|---|---|---|
| 0.25 μM | | |
| GA vs. GA+Z-VAD-FMK | No | ns |
| GA vs. GA+Liproxstatin-1 | No | ns |
| GA vs. GA+NAC | No | ns |
| 0.5 μM | | |
| GA vs. GA+Z-VAD-FMK | Yes | **** |
| GA vs. GA+Liproxstatin-1 | Yes | **** |
| GA vs. GA+NAC | Yes | **** |
| 1 μM | | |
| GA vs. GA+Z-VAD-FMK | Yes | **** |
| GA vs. GA+Liproxstatin-1 | Yes | **** |
| GA vs. GA+NAC | Yes | **** |
| 2 μM | | |
| GA vs. GA+Z-VAD-FMK | Yes | **** |
| GA vs. GA+Liproxstatin-1 | Yes | **** |
| GA vs. GA+NAC | Yes | **** |
| 3 μM | | |
| GA vs. GA+Z-VAD-FMK | Yes | **** |
| GA vs. GA+Liproxstatin-1 | Yes | * |
| GA vs. GA+NAC | Yes | **** |
| 4 μM | | |
| GA vs. GA+Z-VAD-FMK | Yes | **** |
| GA vs. GA+Liproxstatin-1 | No | ns |
| GA vs. GA+NAC | No | ns |
| 5 μM | | |
| GA vs. GA+Z-VAD-FMK | Yes | **** |
| GA vs. GA+Liproxstatin-1 | No | ns |
| GA vs. GA+NAC | No | ns |
| 6 μM | | |
| GA vs. GA+Z-VAD-FMK | Yes | *** |
| GA vs. GA+Liproxstatin-1 | No | ns |
| GA vs. GA+NAC | No | ns |

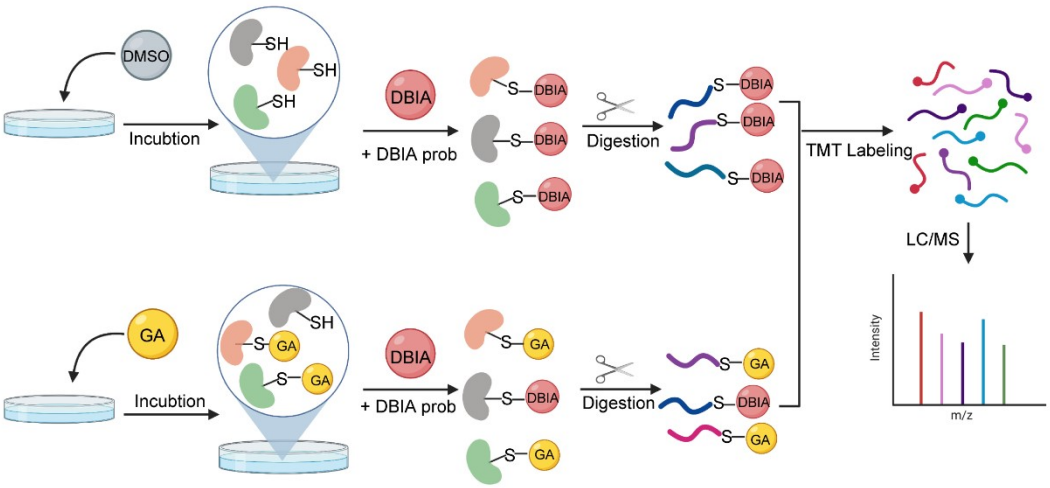


**Figure S5**. Flow chart of identifying the protein targets of gambogic acid in A549 cells by ABPP.

**Table S2**. The list of targets of GA identified by LC MS/MS (top 30)

| NO. | Protein Accessions | Master Protein Descriptions | Annotated Sequence | Cystines position | Ratio |
|---|---|---|---|---|---|
| 1 | P52209 | 6-phosphogluconate dehydrogenase | SAVENCQDSWR | 402 | 43.063 |
| 2 | Q99733 | Nucleosome assembly protein 1-like 4 | CAHIEAK | 77 | 34.82 |
| 3 | P30043 | Flavin reductase | CLTTDEYDGHSTYPSHQYQ | 188 | 26.011 |
| 4 | Q5TFE4 | 5'-nucleotidase domain-containing protein 1 | YLHSCPESVK | 197 | 24.944 |
| 5 | P52209 | 6-phosphogluconate dehydrogenase | VGTGEPCCDWVGDEGAGHFVK | 170, 171 | 21.813 |
| 6 | P00338 | L-lactate dehydrogenase A chain | YSPNCK | 131 | 21.378 |
| 7 | P07737 | Profilin-1 | CYEMASHLR | 128 | 20.167 |
| 8 | P49354 | type-1 subunit alpha | ALELCEILAK | 341 | 16.096 |
| 9 | P39023 | 60S ribosomal protein L3 | YCQVIR | 157 | 15.979 |
| 10 | P26641 | Elongation factor 1-gamma | WFLTCINQPQFR | 194 | 15.328 |
| 11 | P83731 | 60S ribosomal protein L24 | VELCSFSGYK | 6 | 14.367 |
| 12 | P50395 | Rab GDP dissociation inhibitor beta | TYDATTHFETTCDDIK | 414 | 13.38 |
| 13 | P63104 | 14-3-3 protein zeta/delta | DICNDVLSLLEK | 94 | 12.79 |
| 14 | Q99613 | Eukaryotic translation initiation factor 3 subunit C-like protein | CLEEFELLGK | 79 | 12.561 |
| 15 | P14618 | Pyruvate kinase PKM | CCSGAIIVLTK | 423, 424 | 12.392 |
| 16 | P07864 | L-lactate dehydrogenase | VIGSGCNLDSAR | 164 | 12.355 |
| 17 | Q9Y3U8 | 60S ribosomal protein L36 | EVCGFAPYER | 48 | 12.17 |
| 18 | Q9Y536 | Peptidyl-prolyl cis-trans isomerase A | GSCFHR | 52 | 12.092 |
| 19 | P15121 | Aldo-keto reductase family 1 member B1 | LIQYCQSK | 200 | 12.026 |
| 20 | O60218 | Aldo-keto reductase family 1 member B10 | LIQYCHSK | 200 | 11.81 |
| 21 | P27695 | DNA-(apurinic or apyrimidinic site) lyase | ICSWNVDGLR | 65 | 11.538 |
| 22 | Q92598 | Heat shock protein 105 kDa | LVEHFCAEFK | 245 | 11.268 |
| 23 | P04083 | Annexin A1 | CLTAIVK | 263 | 11.213 |
| 24 | P23396 | 40S ribosomal protein S3 | GCEVVVSGK | 134 | 10.927 |
| 25 | P37837 | Transaldolase | ALAGCDFLTISPK | 250 | 10.912 |
| 26 | P52895 | Aldo-keto reductase family 1 member C1 | KLLDFCK | 206 | 10.78 |
| 27 | Q01518 | Adenylyl cyclase-associated protein 1 | TDGCHAYLSK | 416 | 10.727 |
| 28 | P14618 | Pyruvate kinase PKM OS=Homo sapiens | AGKPVICATQMLESMIK | 326 | 10.351 |
| 29 | P62888 | 60S ribosomal protein L30 | VCTLAIIDPGDSDIIR | 92 | 10.216 |
| 30 | O95757 | Heat shock 70 kDa protein 4 | GCALQCAILSPAFK | 376 | 9.944 |

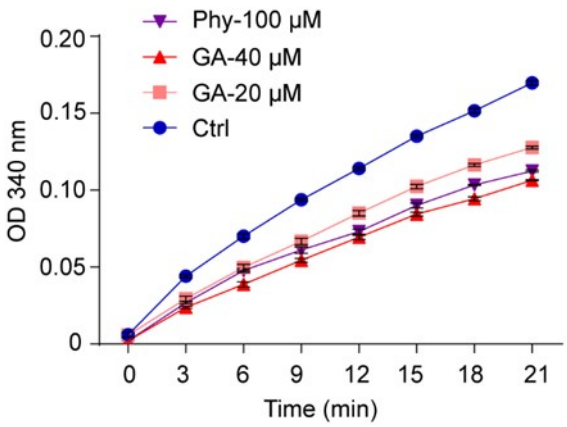


**Figure S6**. GA inhibits the enzymatic activity of 6PGD protein *in vitro*.

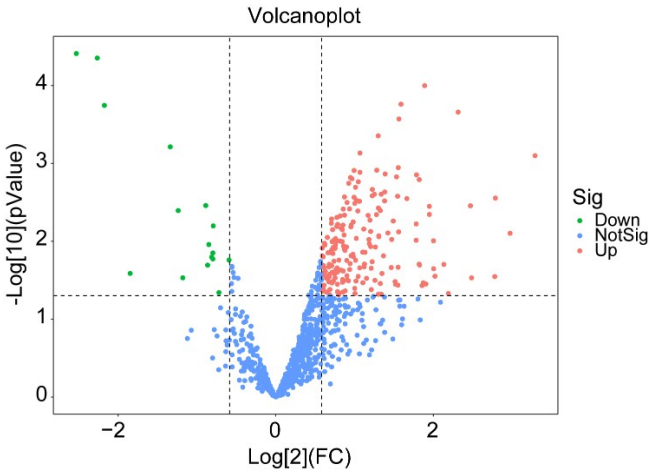


**Figure S7**. Volcano plot of up-regulated (red dots) or down-regulated peptides with oxidized methionine (green dots) in GA treatment vs control groups.