Supplementary Information

Characterization of active antiplatelet chemical compositions of edible *Citrus limon* through ultra-performance liquid chromatography single quadrupole mass spectrometry-based chemometrics

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Fig. S2. Histogram of R2X and Q2 value based on secondary metabolome data of SC1-SC4. (A) PCA, (B) OPLS-DA.

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Fig. S5. Representative 2-DE gel maps of control and LIM-treated platelets. Total protein expressed in platelet treated with (A) methanol, (B) LIM. Differentially expressed spots were shown by the arrows.

Fig. S6. The result of the MALDI-TOF MS/MS analysis of the protein spot 7205. It was identified to be cAMP-dependent protein kinase type I-alpha regulatory subunit (NCBI accession number XP_002719538.1) by protein database search. (A) Peptide mass fingerprint of the tryptic digest of spot 7205. Peptide signals identified were marked with asterisk, (B) MS/MS profile of the peptide with a mass of 1294.425 Da and (C) 1450.505 Da. y-ions resulting from fragmentation of the peptides and amino acids they represent are indicated.

Fig. S7. Platelet aggregation pathway. The color-coded protein represented differentially signal transduction related proteins which changed after addition of limetin. Red color for up-regulated proteins and yellow color for down-regulated proteins.

Supplementary Methods

Preparation of rabbit platelet-rich plasma and washed platelet

Rabbit blood samples were collected in 3.2% sodium citrate with the ratio of 9:1 (blood: anticoagulant) from carotid artery after anesthetizing by 1.5% pentobarbital sodium (Adamas-beta, Adamas Reagent, Ltd., Shanghai, China). Platelet-rich plasma (PRP) was obtained by centrifugation of blood sample at 93 × *g* for 15 min at room temperature, and platelet-poor plasma (PPP) was obtained by further centrifugation from the remaining blood at 2259 × *g* for 10 min. The concentration of PRP was adjusted to 3×10^9 platelets/L by PPP.

To prepare washed platelet (WP), blood from the rabbit carotid artery was collected in plastic tubes with acid citrate dextrose (ACD) solution (1.32% trisodium citrate, 0.48% citric acid, 1.47% glucose) as anticoagulant (6:1, blood: anticoagulant, v/v). After centrifugation at 93 × *g* for 15 min at room temperature, PRP was isolated and further centrifuged at 2259 × *g* for 10 min. The bottom platelet pellet was washed twice by washing buffer [113 mM NaCl, 4 mM KCl, 24 mM $NaH_2PO_4 \cdot 2H_2O$, 24 mM $Na_2HPO_4 \cdot 12H_2O$, 0.2 mM EGTA, and 0.14 mM glucose (250 mL, pH 7.4)]. Platelets were suspended in PBS solution [0.8% NaCl, 0.02% KCl, 81 mM $Na_2HPO_4 \cdot 12H_2O$, 19 mM KH_2PO_4 (100 mL, pH 7.4)] to obtain a platelet count of 3×10^9 platelets/mL. This platelet suspension was used for proteomic analysis.

UPLC-IT-TOF-MS/MS identification

A Shimadzu LC-MS system (Shimadzu, Kyoto, Japan) consisting of a hybrid ion trap time-of-flight mass spectrometer (Shimadzu) equipped with an electrospray ionization (ESI) interface was connected to the LC system via a PEEK tube (0.13 mm i.d.) to perform high-resolution tandem mass spectrometry identification. The LC conditions were the same as described above. For the mass spectrometer, an accurate ion axis was calibrated using the sodium trifluoroacetate clusters as a reference. Nitrogen was used as the nebulizing and drying gas, whereas ultrahigh purity argon (Ar) was used as the cooling and collision gas for collision-induced dissociation (CID). The mass spectrometric parameters were set as below: ionization polarity, positive and negative; drying gas pressure, 100 MPa; curved desolvation line (CDL) voltage, constant level; interface voltage, 1.4 kV; nebulizing gas flow rate, 1.5 L/min; detector voltage, 1.40 kV; CDL temperature, 200°C; block heater temperature, 200°C; and IT vacuum, 1.9×10^{-2} Pa. Both positive and negative mass spectra were recorded in the full scan and automatic multiple stage fragmentation scan modes over a range of *m*/*z* 50 - 800 for all MS^1 and MS^2 spectra acquisition. The ion accumulation time was set at 100 ms and the collision energy of CID was set at 50%. Data acquisition and processing were performed with the LC-MS solution version 1.1 software package (Shimadzu). The accuracy of the assigned chemical formula was determined using a mass difference tolerance of ± 5 ppm, which was calculated by the deviation between the experimental mass and calculated mass.

Supplementary Results and Discussion

Platelet survival rate test and initial bioactivity study

The platelet death rate before and after incubation with different concentration of lemon ethanol extract were determined by lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Beyotime Biotechnology, Co., Ltd, Shanghai, China) was used, and the specific operation process was according to the introduction of the Kit. Platelet was incubated with PBS, blank control methanol as well as three different concentrations of LEE for 10 min, respectively. After platelets were treated, the 490 nm UV signals were detected using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., USA). In Fig. S1A, it was set as 100% survival rate for platelet incubated with PBS, solvent methanol had almost no effect on platelet activity, and the death rate of platelet was 0.15%. Therefore, methanol could be adapted to be a solvent for the subsequent activity test process. Fig. S1A showed the platelet survival rate after incubation with various concentrations of LEE sample solution (final concentration was 3.125, 6.25 and 12.5 mg/mL), and the death rate were 6.63%, 9.16% and 17.67% respectively. These results indicated that the compounds in the sample (under 6.25 mg/mL) did not influence platelet vitality much with relatively low platelet death rate (< 10%).

Table S1. List of differentially expressed proteins in the LIM treated platelet proteome identified by MALDI-TOF-MS/MS from the 2-DE profiling of rabbit platelets LIM compared to control.

Spot No.	Protein name	NCBI acc. no	MV/PI	Protein score	Sequence coverage	Fold changed	Function
Cytoskeleton structure related proteins							
8114	Annexin A5	G1TED6	36.0/4.86	177	16%	0.047	Bind to the cytoskeleton protein, such as the actin and the β subunit of integrin; inhibit phospholipase A2 (PLA2) in cytosolic, and inhibit the activity of PKC through its competitive combination of PKC with phospholipid.
1108	Septin-2	XP_008250392.1	41.7/6.26	292	20%	0.316	Required for normal organization of the actin cytoskeleton.
2019	Microtubule-associated protein RP/EB family member 1	XP_002710850.1	30.2/5.02	73	7%	0.184	Involved in the regulation of microtubule structures and chromosome stability.
4608	Cullin 2 isoform X1	gi|291409897	87.5/6.45	30	2%	0.106	Help of F-box or SOCS box proteins attaches to the substrate.
6309	T-complex protein 1 subunit zeta	O77622	58.4/6.46	350	21%	0.307	Interactions with the cytoskeletal proteins actin and tubulin, assist in the folding of several other proteins, including cyclin E, myosin, transducing.
6310	RecName: Full=Coronin-1B; AltName: Full=Coronin-like protein pp66; AltName: Full=Coroninse	Q9XS70.1	54.2/5.58	98	5%	3.332	Binds to both the cell membrane and the actin-related protein Arp2/3, plays a role in actin rearrangements during platelet activation.
1104	Annexin A5	XP_008266130.1	36.0/4.86	61	7%	0.324	Bind to the cytoskeleton protein; prevent formation of thrombin; inhibit the activity of PKC through its

							competitive combination of PKC with phospholipid and PLA2.
3514	F-actin-capping protein subunit alpha-2	Q09YN4.3	33.1/5/57	41	4%	3.696	Required for barbed end actin filament capping activity to limit growth of the actin filament at this end; regulates PKC signaling and calcium sensitivity in cardiac muscle.
4413	Alpha-actinin-1 isoform X1	XP_002719567.1	106.1/5.25	78	1%	0.307	A member of actin-cross-linking proteins family, associated into reorganized actin cable networks and may mediate FAK-dependent signals which impact the physical properties of the cytoskeleton.
4517	Heat shock cognate 71 kDa protein	XP_002708378.1	71.1/5.37	68	2%	0.227	May be involved in protecting the centrosome and intermediating filaments during heat shock.
7615	Talin-2	XP_017203855.1	271.3/5.41	60	0%	0.291	Links integrin to the actin cytoskeleton, play an important role in platelet adhesion.
Proteins related to material energy metabolism							
3217	ATP synthase subunit alpha, mitochondrial	XP_002713558.1	59.8/9.15	90	2%	5.560	Produces ATP from ADP in the presence of a proton gradient; involved in oxidative phosphorylation.
7408	Phosphoglucomutase-2 isoform X1	XP_002709315.1	69.2/6.06	67	3%	0.277	Catalyses the interconversion of G1P and G6P.
8007	Succinyl-CoA:3-ketoacid coenzyme A transferase 1	gi|291395294	56.5/8.62	142	3%	0.266	Activates acetoacetate by transferring the CoA group to produce acetoacetyl-CoA and succinate.
5112	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	XP_002713321.1	39.4/6.41	76	4%	4.761	Catalytic the decarboxylation of pyruvate.

Cell signaling related proteins

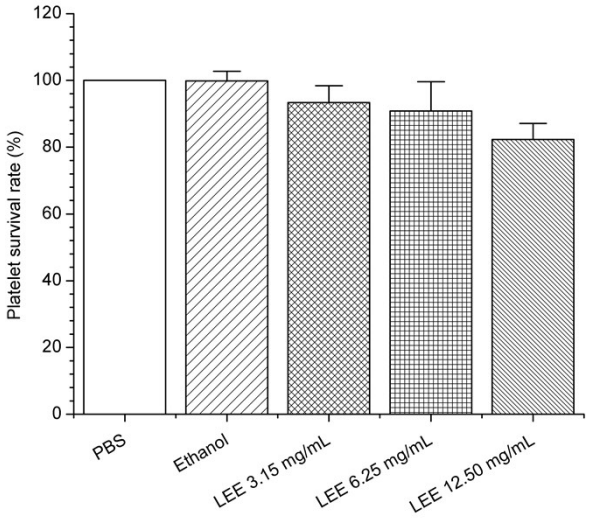
1012	Myosin regulatory light polypeptide 9	XP_002710827.1	19.9/4.80	53	11%	0.490	Can be phosphorylated by myosin light chain kinase in the presence of calcium and calmodulin and increases the actin-activated ATPase activities of myosins.
1209	Angio-associated migratory cell protein isoform X2	XP_002712519.3	47.5/4.25	40	2%	0.634	Interact with the TP alpha and TP beta isoforms of the human TXA_2 receptor.
2107	Prohibitin	gi|291405834	29.8/5.57	574	31%	1.975	Localized on the human platelet membrane and are involved in PAR1-mediated platelet aggregation, aIIbβ3 activation, and granular secretion.
3012	Prohibitin	G1SSJ7	29.9/5.57	334	25%	0.511	Localized on the human platelet membrane and are involved in PAR1-mediated platelet aggregation, aIIbβ3 activation, and granular secretion.
3016	Ras-related protein Rap-1b	gi | 291389525	21.0/6.65	99	7%	0.078	The most abundant Ras family member in platelets, invoved in platelet activation signal pathways.
3113	Guanine nucleotide-binding protein G(i) subunit alpha-2	gi | 655834605	40.9/5.35	293	20%	2.308	Invoved in platelet activation signal pathways.
4314	Guanylate cyclase soluble subunit beta-1	gi | 655856074	71.2/5.23	98	4%	1.577	Converts GTP into cGMP.
5113	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	G1SYI2	38.2/5.6	234	15%	0.554	Invoved in platelet activation signal pathways.
6311	Mitogen-activated protein kinase 3.	XP_008256167.1	44.9/6.43	38	6%	0.039	MAPK signaling.
8117	Small GTP-binding protein RhoA,	AAT68169.1	18.7/4.79	86	10%	6.394	Binds and activates rho kinase, which promotes

	partial						myosin light-chain phosphorylation by phosphorylating and inhibiting the myosin light-chain phosphatase.
8413	Phosphoinositide 3-kinase regulatory subunit 5 isoform X1	XP_002719015.1	98.5/6.15	46	2%	0.027	Phosphatidylinositol-3-kinase regulator activity, affecting the PI3K signaling pathway.
4313	Synaptotagmin-7	gi|655882206	71.2/9.63	33	2%	5.308	Calcium signaling.
7205	cAMP-dependent protein kinase type I-alpha regulatory subunit	XP_002719538.1	43.2/5.27	86	3%	0.324	A enzyme whose activity is dependent on cellular levels of cyclic AMP (cAMP); invoved in cAMP/PKA signaling pathway; phosphorylate the downstream target cell proteins.
Proteins are directly related to platelet functions							
4515	Thrombospondin-1	XP_002717845.1	133.7/4.78	201	4%	0.257	Interacts with fibrinogen, three kinds of integrins, CD36, GPIb-IX-V complexes, and collagen *et al*, promote the activation and aggregation of platelets.
5410	Coagulation factor XIII A chain	XP_008260735.1	29.5/9.03	41	8%	3.086	Leads to a covalent cross-linking of fibrin fibrils at the end of the clotting cascade; involved in platelet spreading, the later stages of platelet aggregation, clot retraction.
Proteins associated with redox balance and/or oxidative stress							
6016	Peroxiredoxin-6	G1T8Z0	25.3/5.74	84	10%	5.263	Associated with the H_2O_2 signaling pathway and protect against oxidative damage by scavenging ROS.
Unclassified							

1009/2014	Chain A, Atomic Structure Of The Actin: dnase I Complex	1ATN_A	41.7/5.04	92	4%	0.239	DNase I binds to the cytoskeletal protein actin; the DNase-actin complex might be a storage form of DNase I that prevents damage of the genetic information.
6015	Chloride intracellular channel protein	G1ST51	26.9/5.54	104	16%	0.266	The main transport channel of chloride ions and plays an important role in various physiological processes such as signal transduction, and ion homeostasis.
0401	14-3-3 protein epsilon isoform X1	XP_002718936.1	29.3/4.64	54	4%	0.263	Act as molecular adaptors to regulate a variety of biological processes, such as cell signaling, cell cycle progression, intracellular traffic, cytoskeletal reorganization, and transcription; interact with GPIb-IX through a binding site similar to that of 14-3-3ξ.
1020	Tyrosine 3 - monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide (predicted)	ACK44253.1	28.4/4.80	55	3%	0.157	Act as molecular adaptors to regulate a variety of biological processes, such as cell signaling, cell cycle progression, intracellular traffic, cytoskeletal reorganization, and transcription; interact with GPIb-IX through a binding site similar to that of 14-3-3ξ.
3314	60 kDa heat shock protein, mitochondrial	XP_002712414.1	61.2/5.70	291	7%	0.235	A highly conserved intracellular protein that is required for protein folding and transport; maintaining the stability of cell structure when cells are stimulated.
4206	Latexin	XP_002716378.1	25.8/5.86	36	5%	3.192	Known as mammalian carboxypeptidase inhibitor, increased during storage and has been implicated as a mediator of the hematopoietic stem cell compartment.

4214	Chain A, Atomic structure of the actin: DNASE I complex	1ATN	41.7/5.04	73	4%	4.454	DNase I binds to the cytoskeletal protein actin; the DNase-actin complex might be a storage form of DNase I that prevents damage of the genetic information.
4607	Ubiquitin-like modifier-activating enzyme 1	Q29504.1	118.7/5.51	75	1%	0.234	The E1 ubiquitin-activating enzyme, represents an important regulator of cellular protein homeostasis.
5513	Ubiquitin-like modifier-activating enzyme 1	Q29504.1	118.7/5.51	330	5%	5.190	The E1 ubiquitin-activating enzyme, represents an important regulator of cellular protein homeostasis.
6218	Uroporphyrinogen decarboxylase isoform X1	XP_002715187.1	41.0/5.95	31	2%	0.293	Oxidation of the substrate uroporphyrinogen (a reduced porphyrin) to uroporphyrin.
7110	Ubiquitin thioesterase OTU1	XP_002717592.1	38.4/5.25	42	4%	0.152	Hydrolase that can remove conjugated ubiquitin from proteins and participates in endoplasmic reticulum-associated degradation (ERAD) for misfolded lumenal proteins.
7407	Cytosolic non-specific dipeptidase isoform X1	XP_002713697.1	53.3/5.54	90	3%	0.267	Hydrolyzes a variety of dipeptides including L-carnosine but has a strong preference for Cys-Gly.

(A)



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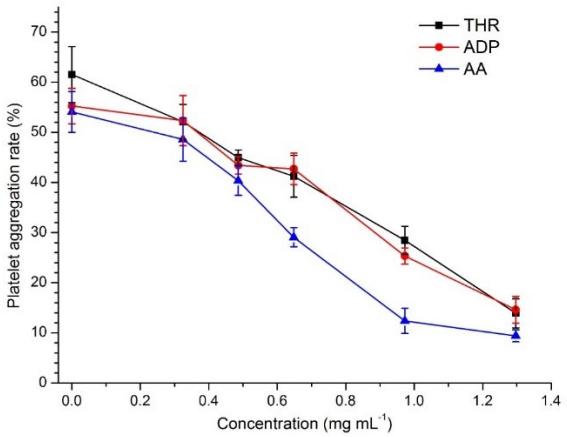
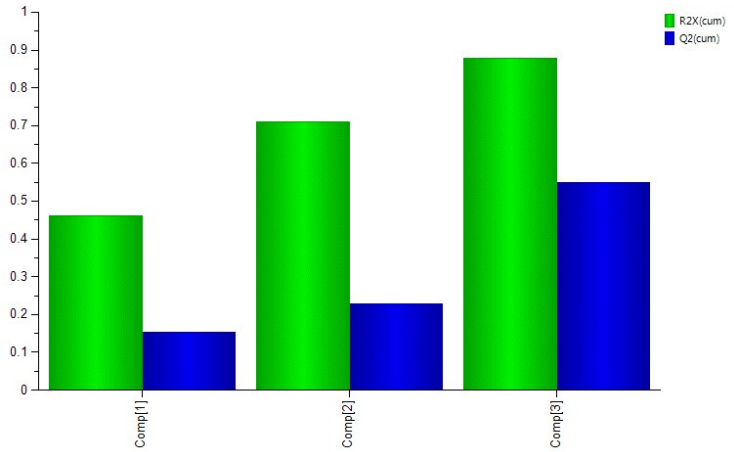


Fig. S1. Effect of LEE on (A) platelet vitality and (B) THR, ADP, AA induced platelet aggregation *in vitro.* Survival rate was evaluated by LDH Cytotoxicity Assay Kit. Data were expressed as the mean ± standard error of the mean (SEM) (n = 6).

(A)



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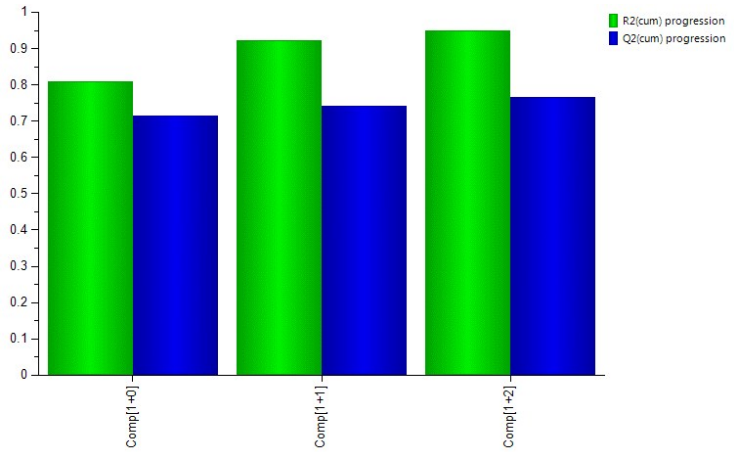
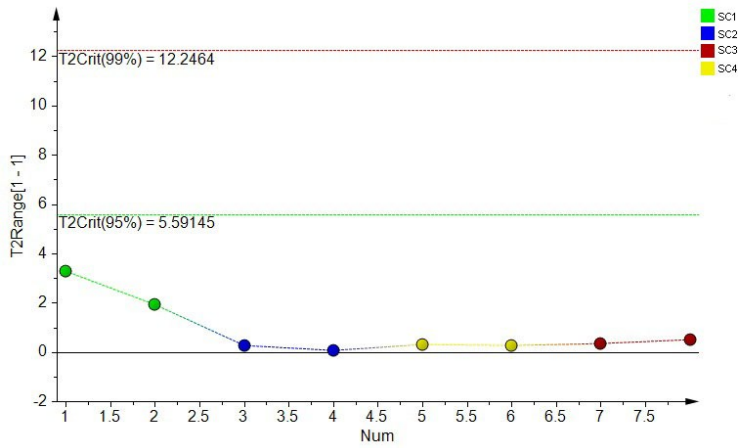


Fig. S2. Statistical parameters of PCA and OPLS-DA based on secondary metabolome data of SC1-SC4. (**A**) Histogram of R2X and Q2 value in PCA, (**B**) Histogram of R2X and Q2 value in OPLS-DA.

(A)



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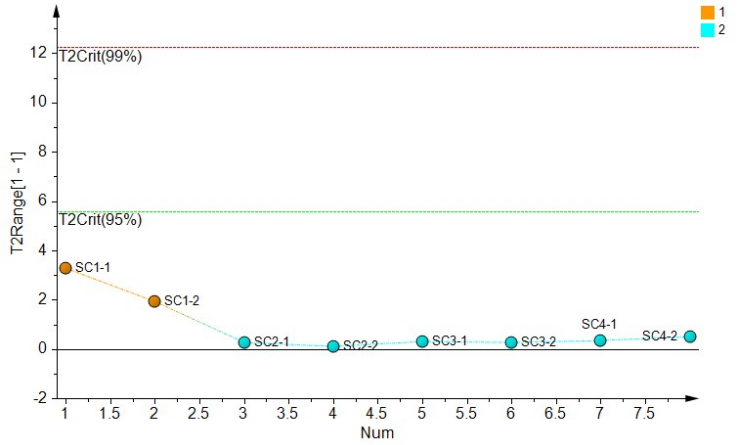
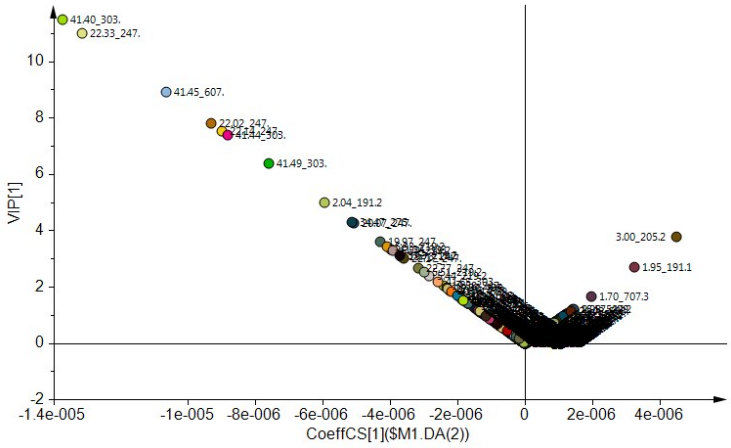


Fig. S3. Corresponding Hotelling's T2Range plot based on secondary metabolome data of SC1-SC4. (**A**) PCA, (**B**) OPLS-DA.



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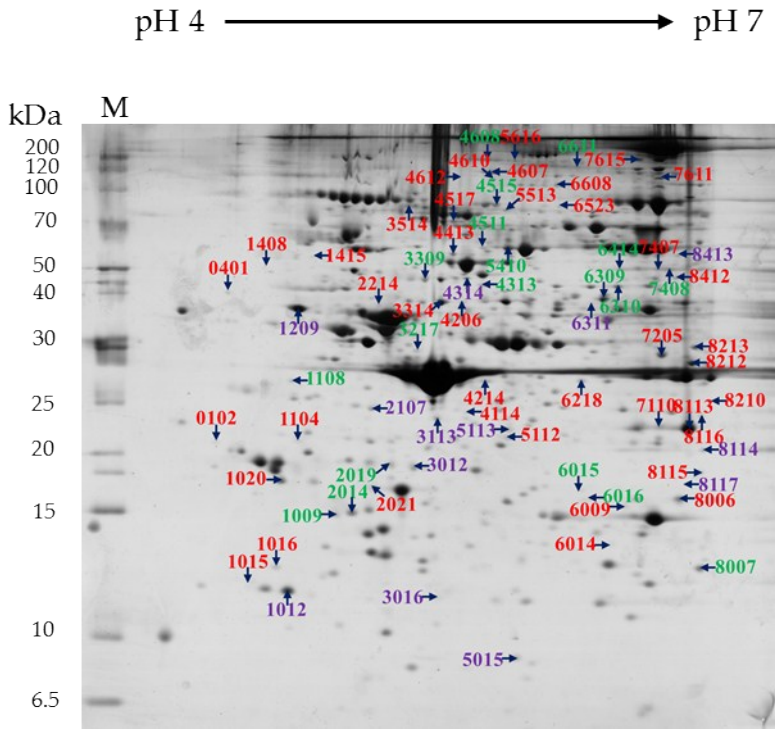
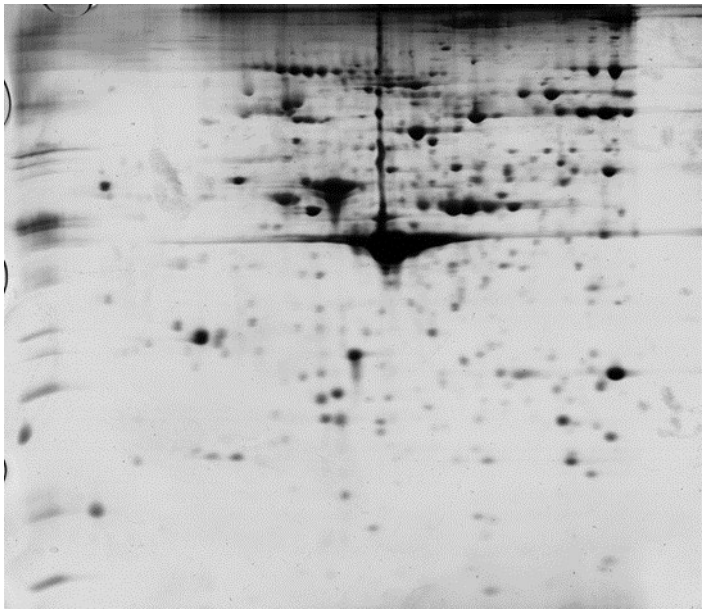
(A)



(B)



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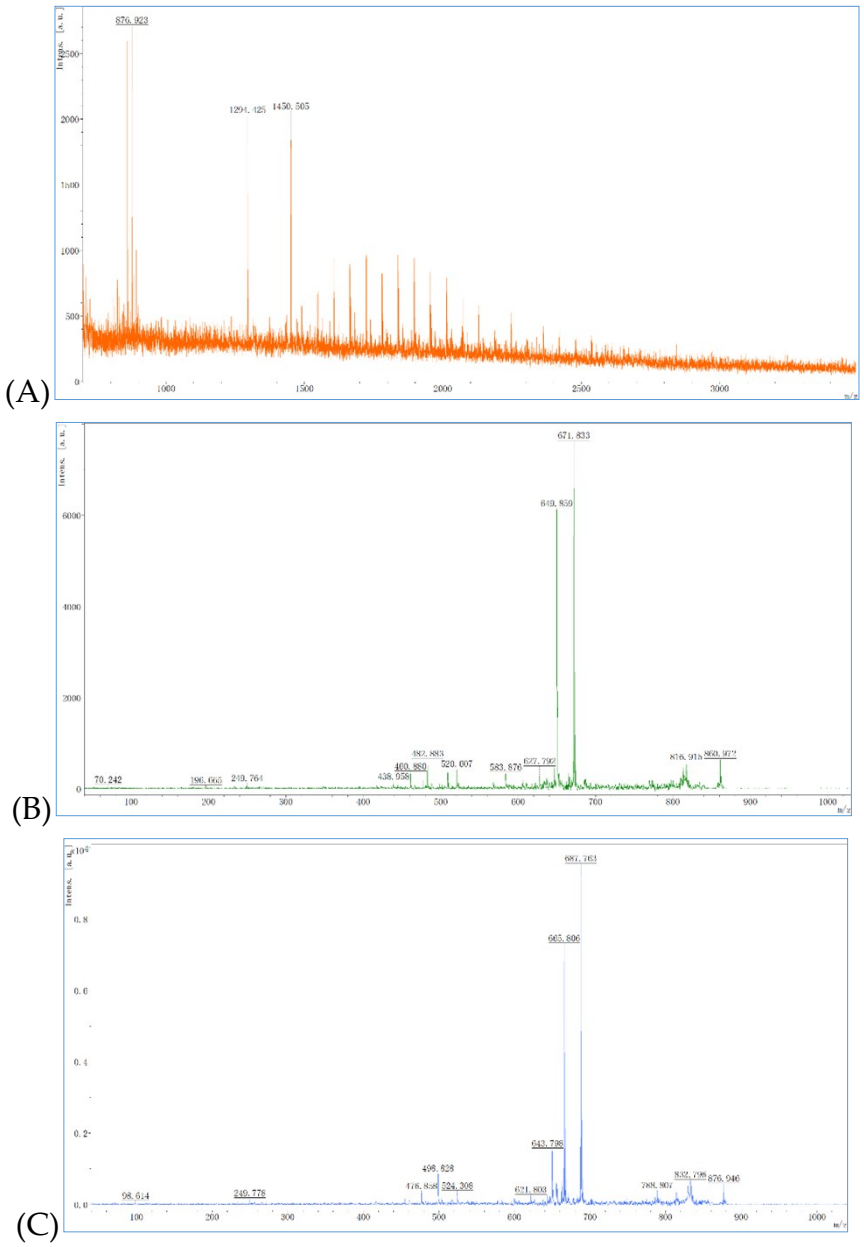

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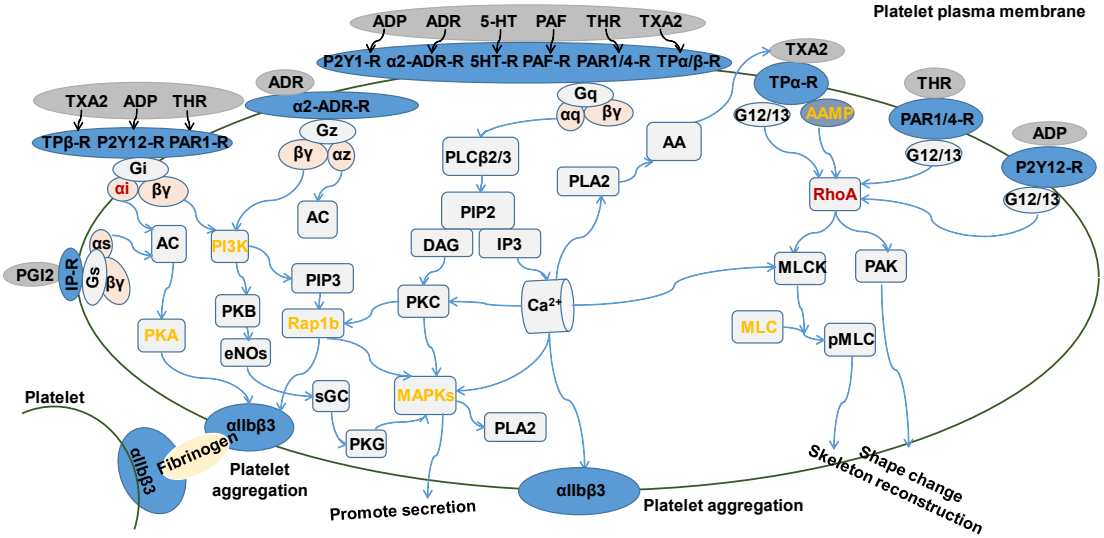

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