Supporting Information

For

Bouchardatine analogue alleviates NAFLD/NASH in high fat fed mice via blunting ATP synthase activity

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Table S1. The fomulation of the high fat diet and high fructose diet

Product #	D12492		D02022704	
	gm%	kcal%	gm%	kcal%
Protein	26.2	20	19	20
Carbonhydrate	26.3	20	67.3	70
Fat	34.9	60	4.3	10
Total kcal/gm		100		100
Ingredient	gm	kcal	gm%	kcal%
Casein, 80 Mesh	200	800	200	800
L-cystine	3	12	3	12
Com starch	0	0	90	360
Maltodextrin 10	125	500	0	0
Sucrose	68.8	275.2	0	0
Fructose	0	0	610	2440
Cellulose, BW200	50	0	50	0
Soybeam Oil	25	225	25	225
Lard*	245	2205	20	180
Mineral Mix, S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix, V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Blue Dye #1	0.05	0	0.05	0
Total	773.85	4057	1055.05	4057

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

*Typical analysis of cholesterol in lard = 0.95 mg/gram.

Cholesterol (mg)/4057 kcal = 232.8

Cholesterol (mg)/kg = 300.8

Table S2. Primers sequence information

Species	Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
Mice	IL-6	CTGGTGACAACCACGGCCTTCCCTA	ATGCTTAGGCATAACGCACTAGGTT
	TNF-α	CACAAGATGCTGGGACAGTGA	TCCTTGATGGTGGTGCATGA
	IFN-γ	TGCTGATGGGAGGAGATGTCT	TTTCTTTCAGGGACAGCCTGTT
	TGF-β	ATTCCTGGCGTTACCTTGG	AGCCCTGTATTCCGTTCTCT
	Collagen Ⅰ	TCCGGCTCCTGCTCCTCTTAG	AGGCCATTGTGTATGCAGCTGAC
	CD68	CCCACCTGCTTCTCTCATTC	CCATGTAGCTCAGGTAGACAAC
	Actin	GACCTCTATGCCAACACAGTGC	GTACTCCTGCTTGCTGATCCAC
Human	Collagen Ⅰ	TCCTGGTCCTGCTGGCAAAGAA	CACGCTGTCCAGCAATACCTTGA
	TGF-β	GAGCTGCTTATCCCAGATTCA	GGCAGTGGAGACGTCAGATT
	TNF-α	ACCCTCAACCTCTTCTGGCTCAAA	AATCCCAGGTTTCGAAGTGGTGGT
	IL-6	ATAGGACTGGAGATGTCTGAGG	GCTTGTGGAGAAGGAGTTCATAG
	Actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCTGTAACAACGCA

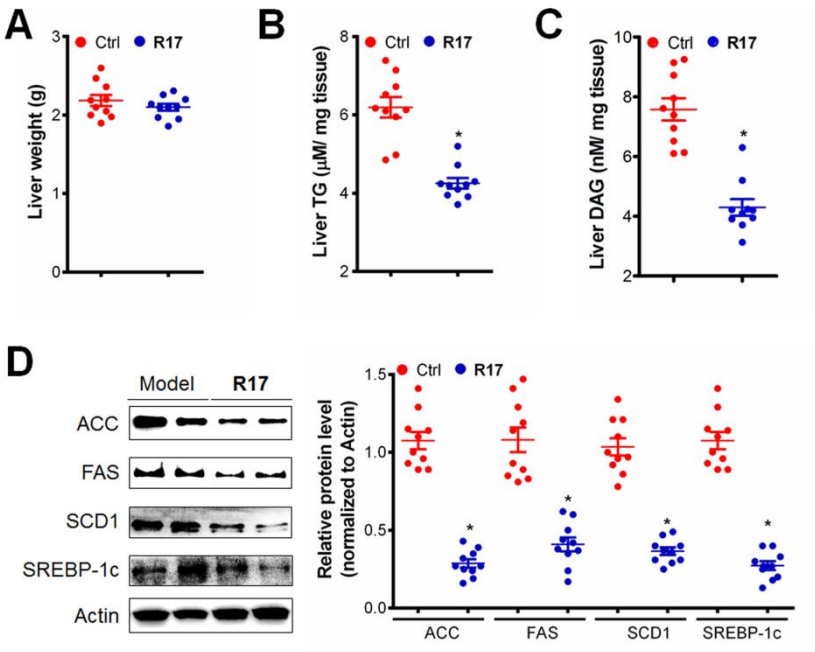

Figure S1. Effects of **R17** on TG level in liver of fast/refeding mice. Male adult C57BL/6J mice were fasted overnight and fed with fed a high fructose diet with or without the treatment of **R17** at the dose of 20 mg/kg by *i.p.* each other day for 10 days. Livers were isolated and weighted, the TG, DAG level and protein related to lipogenesis were determined. (A) Liver weight. (B) Liver TG level. (C) Liver DAG level. (D) Expression of proteins related to lipogenesis and quantification. Data are statistically analysed as means ± SEM, each circle indicated one mice (N = 10 mice/group). $^{*}p<0.05$, *vs*. Ctrl group mice.

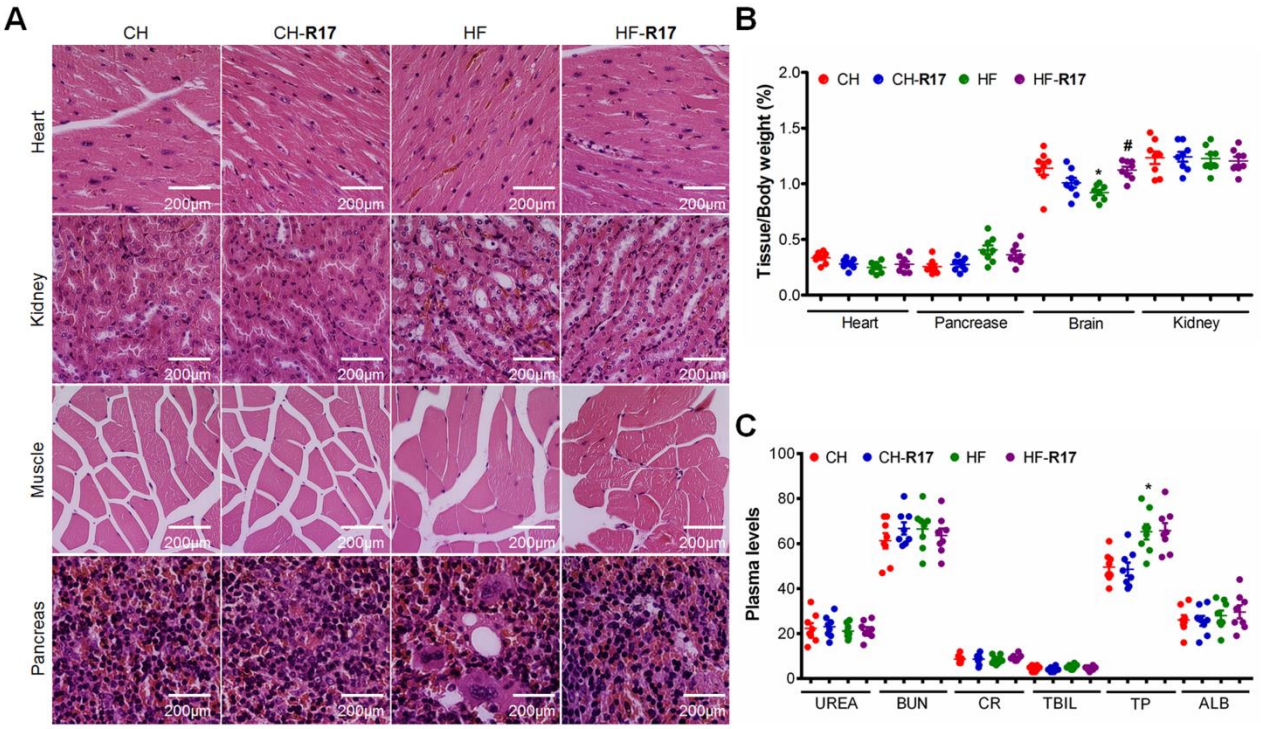

Figure S2. Evaluation of toxicity of **R17** in mice after 5 weeks treatment. Male adult C57BL/6J mice were fed with chow (CH) or high fat (HF) diet for consecutively 16 weeks, and **R17** was administrated at last 5 weeks at the dose of 20 mg/kg by *i.p.* each other day. After 5 weeks treatment, plasma and interested tissues were isolated and related parameters of plasma and tissues were determined. (A) Hematoxylin & eosin (× 200 magnification) staining of heart, kidney, muscle and pancreas. (B) Ratio of tissue/Body weight of heart, kidney, muscle and pancreas. (C) Determination the level of chemicals in plasma indicative of organ toxicity in mice. Data are statistically analysed as means ± SEM, each circle indicated one mice (N = 8 mice/group). $^{*}p < 0.05$, *vs.* CH control mice; $^{\#}p < 0.05$, *vs.* HF control mice.

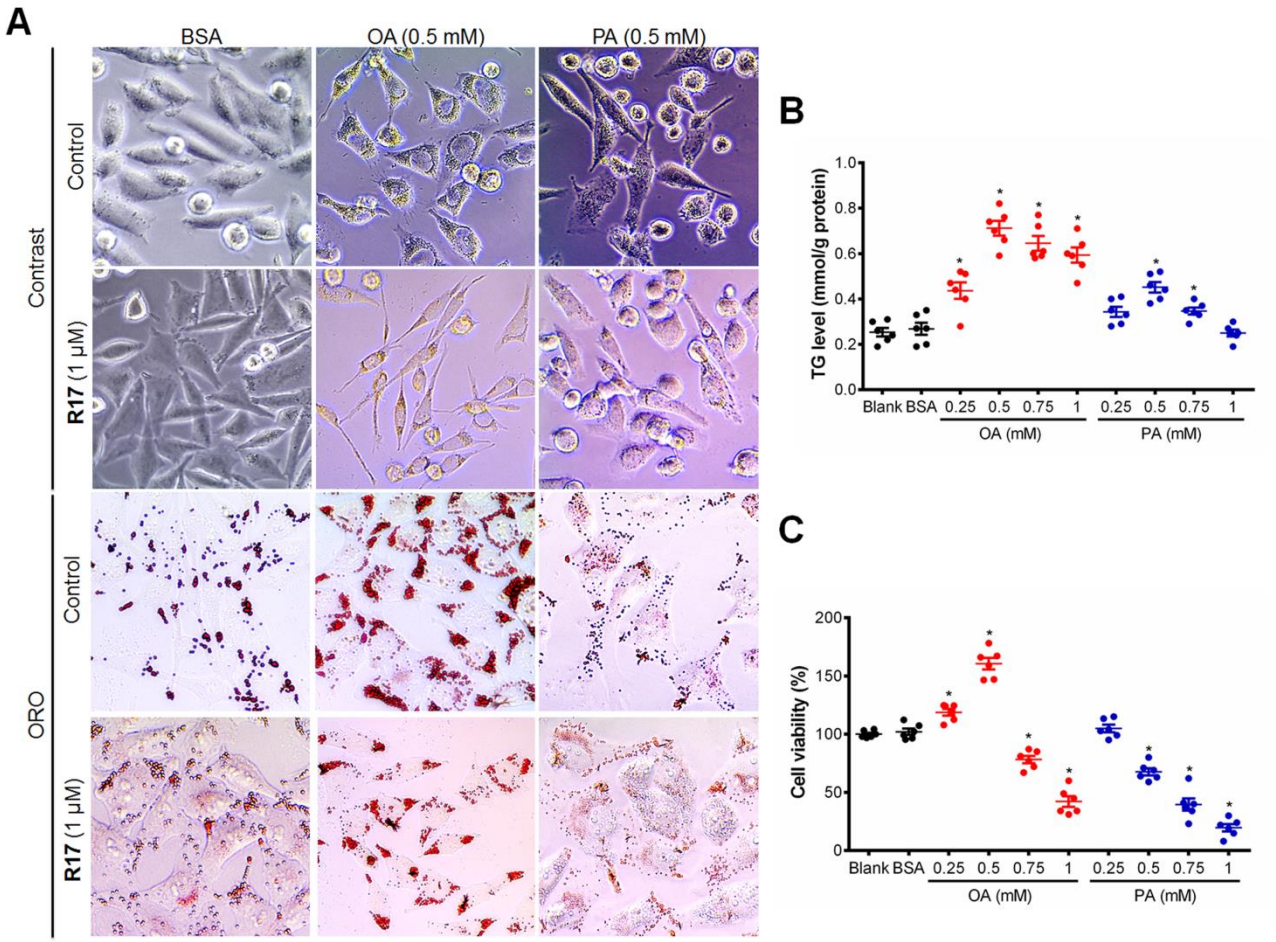

Figure S3. Change of TG levels in HuH7 hepatocytes after FAs induction. HuH7 cells were incubated with fatty-free BSA or OA (0.5 mM) and PA (0.5 mM) for 24 hr, TG level and cell viability were determined. (A) Images were captured under a contrasted eyesight or after Oil-Red O staining, × 200 magnification. (B) Cellular TG level. (C) Cell viability. Data are statistically analysed as means ± SEM, each circle indicated one independent experiment (N = 5 independent experiments). $^{*}p<0.05$, *vs*. BSA treated group.

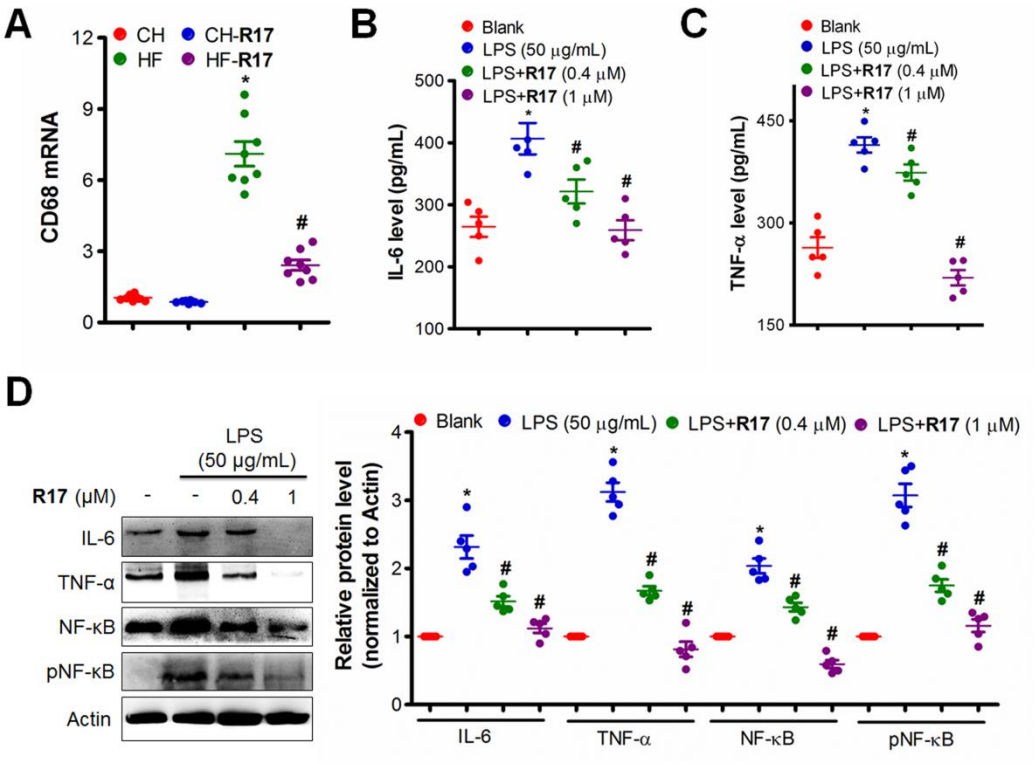

Figure S4. Effect of **R17** on the inflammation in LPS stimulated cells. (A) CD68 mRNA level in liver of mice. Data are statistically analysed as means ± SEM, each circle indicated one mice (N = 8 mice/group). $^{*}p < 0.05$, *vs.* CH control mice; $^{\#}p < 0.05$, *vs.* HF control mice. HepG-2 cells were exposed to LPS (50 μg/mL) with or without the treatment of **R17** (0.4, 1μM) for 24 hr, the inflammatory cytokines in culture medium and protein level of NF-κB pathway were determined by ELISA and immunoblotting assay, respectively. (B-C) Secretion levels of IL-6 and TNF-α; (D) Expression of IL-6, TNF-α and NF-κB pathway proteins, and quantification, relative folds were determined by comparative with the Blank group. Data are statistically analysed as means ± SEM, each circle indicated one independent experiment (N = 5 independent experiments). $^{*}p<0.05$, *vs*. Blank group; $^{\#}p<0.05$, *vs*. Ctrl group.

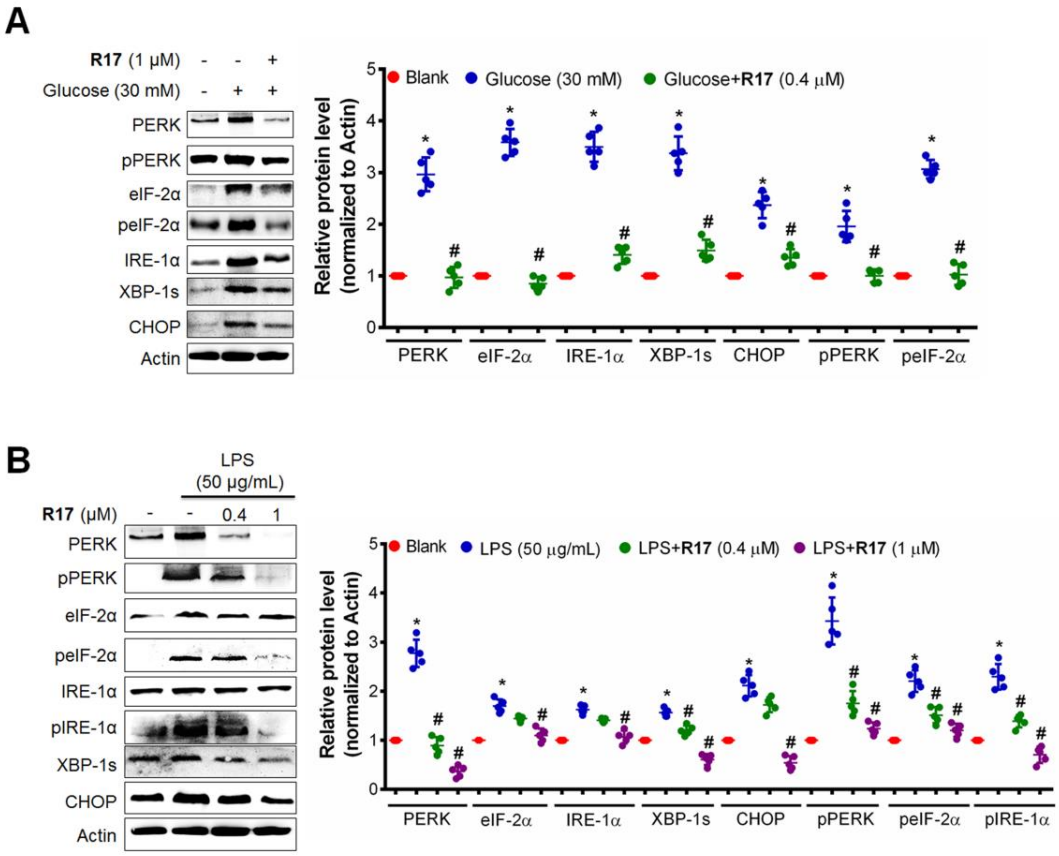

Figure S5. Effects of **R17** on ER stress in high glucose or LPS stimulated cells. (A) HuH7 cells were starved (cultured in a culture medium containing 5 mM) for 8 hr and then switched to a high glucose (30 mM) culture medium in the presence or absence of **R17** (1 μM) treatment for 24 hr. UPR pathway related proteins were determination by immunoblotting and quantification, relative folds were determined by comparative with the Blank group. (B) HepG-2 cells were exposed to LPS (50 μg/mL) stimulation with or without the treatment of **R17** (0.4, 1 μM) treatment for 24 hr. UPR pathway related proteins were determination by immunoblotting and quantification, relative folds were determined by comparative with the Blank group. Data are statistically analysed as means ± SEM, each circle indicated one independent experiment (N = 5 independent experiments). $^{*}p<0.05$, *vs*. Blank group; $^{\#}p<0.05$, *vs*. Ctrl group.

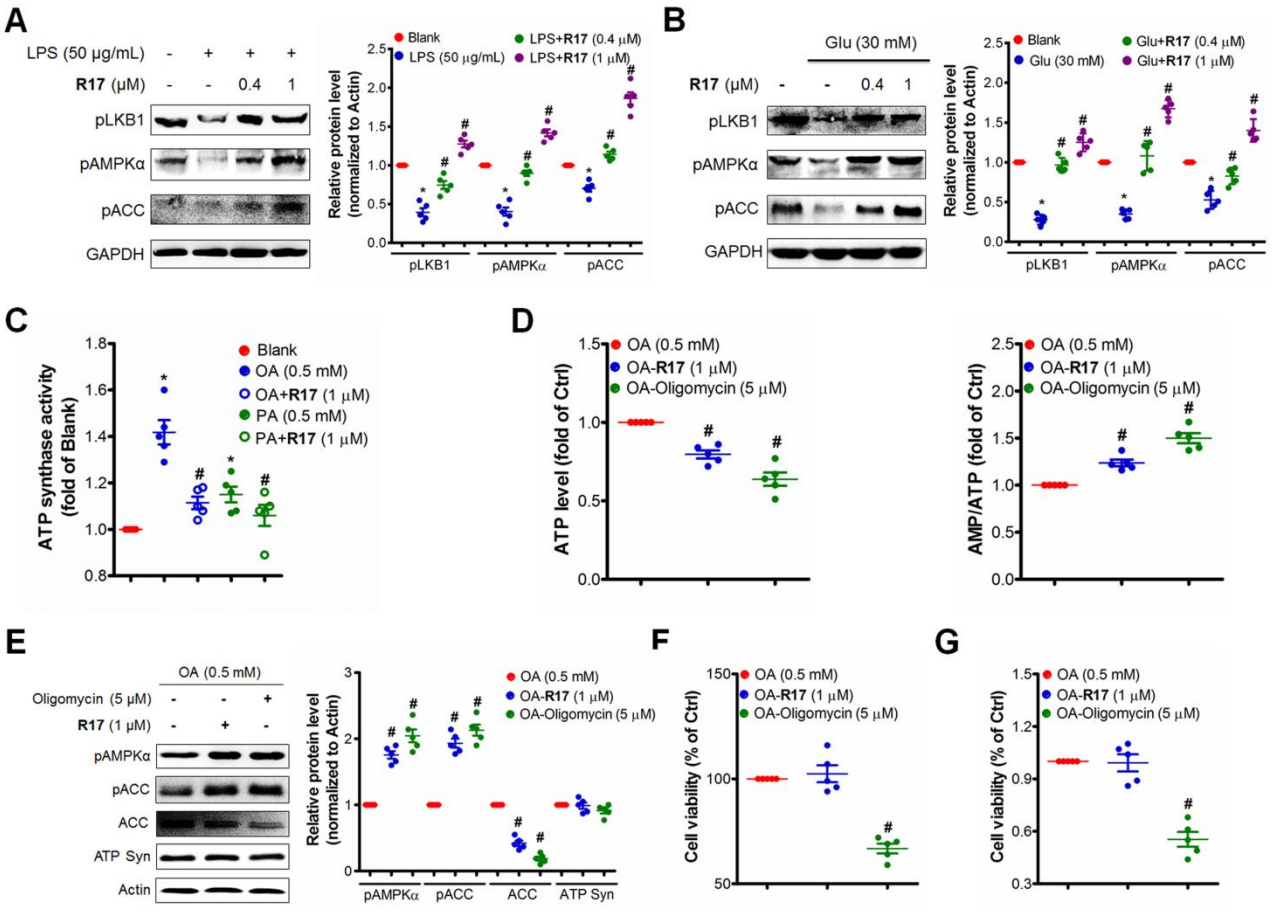

Figure S6. R17 inhibits lipotoxicity in hepatocytes *via* LKB1-AMPK axis activation. (A) Effect of **R17** on the LKB1-AMPK axis in the LPS stimulated HepG-2 cells. HepG-2 cells were exposed to LPS (50 μg/mL) stimulation for 24 hr with or without **R17** (1 μM) treatment, cells were collected and subjected to western blot assay and quantification, relative folds were determined by comparative with the Blank group. (B) Effect of **R17** on the LKB1-AMPK axis in the high glucose stimulated HuH7 cells as described in Methods. Protein levels were quantified and relative folds were determined by comparative with the Blank group. (C) Effect of **R17** (1 μM) on the ATP synthase activity in OA or PA (0.5 mM) stimulated-HuH7 cells. (D-G) Effects of ATP synthase inhibitor oligomycin on TG level and AMPK pathway in OA (0.5 mM)-stimulated HuH7 cells. Cells treated with oligomycin (10 μM) for 12 hr and collected for indicative assay. (D) ATP level and AMP/ATP ratio. (E) Proteins level of AMPK pathway, ATP synthase and quantification, relative folds were determined by comparative with the OA (Ctrl) group. (F) Cell viability. (G) Cell survival. Data are statistically analysed as means ± SEM, each circle indicated one independent experiment (N = 5

independent experiments). *p<0.05, *vs*. Blank group; #p<0.05, *vs*. Ctrl group.

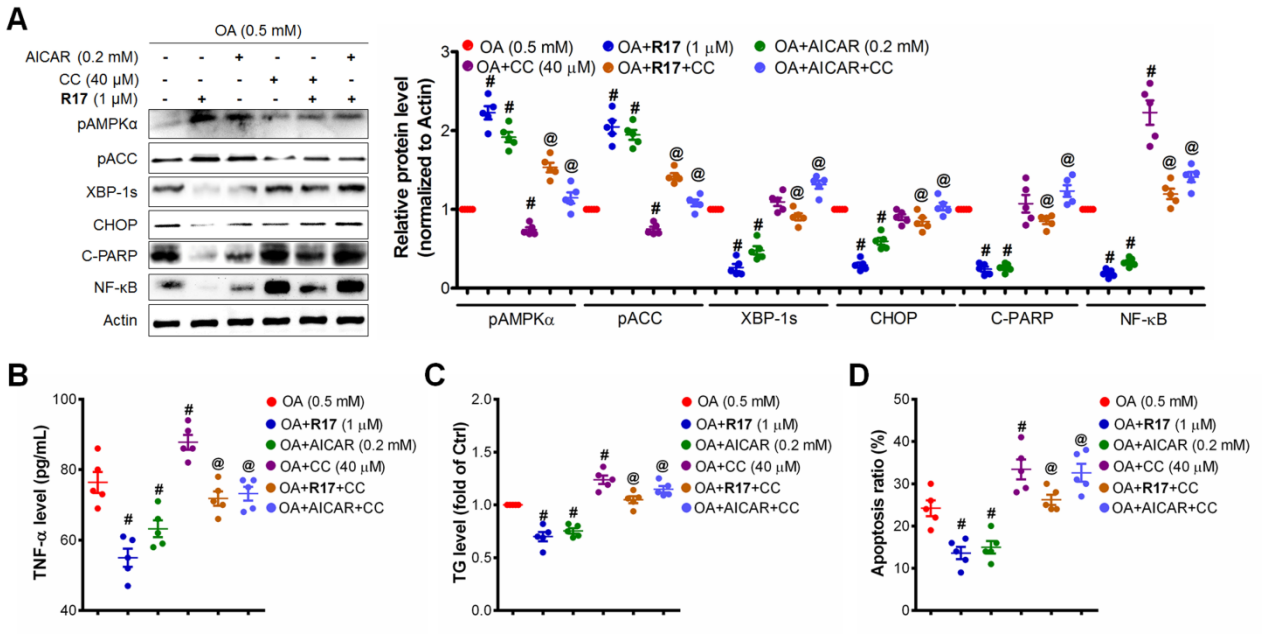

Figure S7. Compound C (CC) abolishes the hepatoprotective effects of **R17** in HuH7 cells. Cells were treated with AICAR (0.2 mM), **R17** (1 μM) alone or together with CC (40μM) in the presence of OA (0.5 mM) for 24 hr. (A) Expression of UPR pathway, inflammation and apoptosis association protein and quantification, relative folds were determined by comparative with the OA (Ctrl) group. (B) TNF-α secretion level. (C) TG level; (D) Cell apoptosis. Data are statistically analysed as means ± SEM, each circle indicated one independent experiment (N = 5 independent experiments). #p<0.05, *vs*. OA (Ctrl) group; @p<0.05, *vs*. **R17** or AICAR treated group.