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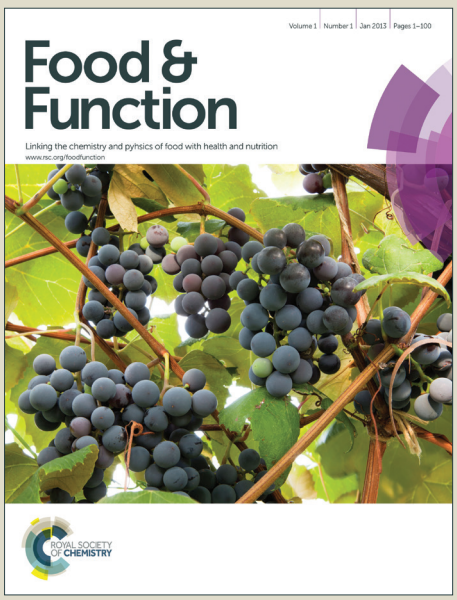

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Identification of hepatoprotective xanthones from the pericarps of *Garcinia mangostana*, guided with *tert*-butyl hydroperoxide induced oxidative injury in HL-7702 cells

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Abstract

Bioactivity-guided fractionation of an ethanol-soluble extract from the pericarps of *Garcinia mangostana*, using *tert*-butyl hydroperoxide (*t*-BHP) induced oxidative damage in human normal hepatocytes (HL-7702), led to the identification of 10 known xanthones. Among them, γ-mangostin (γ-Man) exhibited the most potent activity to attenuate *t*-BHP induced hepatocytes injury. γ-Man significantly ameliorated *t*-BHP induced reactive oxygen species accumulation, mitochondrial membrane depolarization and cell nuclei morphology change in HL-7702 cells. *t*-BHP decreased intracellular key enzymes levels, including glutamate oxaloacetate transaminase and glutamate pyruvate transaminase, which was totally reversed by γ-Man. Moreover, γ-Man significantly decreased the level of lipid peroxidation and increased the levels of superoxide dismutase and reduced glutathione, resulting in alleviative oxidative stress. The above results suggest γ-Man as a potential hepatoprotective agent in *t*-BHP induced oxidative injury, which may benefit the further application of *G. mangostana* as healthy food.

Keywords: *Garcinia mangostana*, xanthones, γ-mangostin, hepatoprotection, oxidative stress

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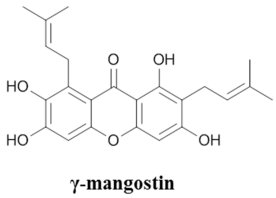
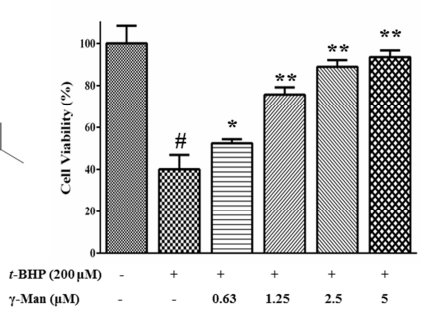
This study identified γ-mangostin from the pericarps of *Garcinia mangostana* as a potential hepatoprotective agent.



γ-mangostin



Introduction

Oxidative stress is occurred when excessive reactive oxygen species (ROS) production and/or cellular anti-oxidant defense system unbalanced,[1] which has inevitably influence on cell function and even lead to cell apoptosis or necrosis.[2] It is widely recognized excessive amount of ROS plays an important role in developing some chronic diseases.[3] Liver is likely injured at the condition of oxidative stress when drinking excessive amount of alcohol, attacking by hepatic virus or intaking some drugs.[4, 5] Therefore, agents ameliorating oxidative stress may bring great benefit to prevent and/or treat liver diseases.

Organic hydroperoxide, such as *tert*-butyl-hydroperoxide (*t*-BHP), is extensively used to induce oxidative stress *in vitro* and *in vivo*, and to make assessment of anti-oxidative capacity of extracts and compounds.[6] *t*-BHP is converted into free radicals by cytochrome P-450, which subsequently initiate lipid peroxidation, affect cell integrity, induce reactions with cellular molecules, damage DNA, and finally result in cytotoxicity.[7]

Garcinia mangostana L. (Clusiaceae) is a well-known tropical plant, widely distributed in Southeastern Asia, such as Thailand, Indonesia, Myanmarand and Sri Lanka.[8] The fruit, mangosteen, is referred to the ‘queen of fruits’ not only for its special tasty juice but also for its extraordinary medicinal values. The pericarps of *G. mangostana* was traditionally used for the treatment of diarrhea, inflammation, and ulcers in many countries.[9] Xanthones, the most characteristic constituents of *G.*

mangostana, show diverse bioactivities, including anti-oxidation,[8] anti-inflammation,[10] anti-cancer,[11] anti-virus,[12] anti-bacteria[13] and anti-allergy.[14] Due to its potential roles, many healthy botanical dietary supplements have been developed, such as 'Xango', 'Verve' and 'TriaXan'. In our systematic screening of hepatoprotective natural products, the ethanol extract from the pericarps of *G. mangostana* was found to significantly protect HL-7702 cells from *t*-BHP induced oxidative injury. Although many studies have disclosed xanthones from *G. mangostana* showed significant anti-oxidative activity on chemical assays or cell models,[15, 16] the hepatoprotective effect of these chemicals is merely investigated. In the present study, we carried out bioassay-guided isolation and identification of hepatoprotective principles of *G. mangostana* using *t*-BHP induced oxidative damage on normal human hepatocytes.

Results and discussion

Bioassay-guided isolation of xanthones from the pericarps of *G. mangostana*, protecting HL-7702 cells from *t*-BHP induced oxidative injury

In our systematic screening of hepatoprotective natural products, total extract (T.E) from the pericarps of *G. mangostana* was found to significantly protect HL-7702 cells from *t*-BHP-induced oxidative injury at concentrations of 1.25 and 2.50 μg mL^{-1} (Fig. 1A). Next, the T.E was further partitioned into petroleum ether fraction (P.E),

chloroform fraction (Chloro), ethyl acetate fraction (E.A), *n*-butanol fraction (*n*-Bu) and water fractions, respectively. Among them, P.E, Chloro and E.A fractions showed potent protective effect against *t*-BHP induced oxidative injury at concentrations of 1.25 and 2.50 μg mL^{-1} (Fig. 1A). Interestingly, pretreatment with 2.50 μg mL^{-1} Chloro fraction rescued more than 90% hepatocytes from oxidative damage. In the above range of concentrations, T.E and all fractions didn't show cytotoxicity on HL-7702 cells (Supplemental Table S1). Thus, the Chloro fraction was subsequently chosen for further isolation due to the most potent hepatoprotective effect with no cytotoxicity.

Additionally, anti-oxidative activities of T.E and fractions were examined on DPPH and ABTS radical scavenging assays, respectively. The results showed E.A and *n*-Bu fractions had the strongest activities, with the IC_{50} values comparable with that of Vitamin C (Table 1). The radical scavenging capacities of Chloro fraction on DPPH and ABTS assays were also significant, assembled to that of T.E (Table 1). The above data indicated Chloro, E.A and *n*-Bu fractions showed potent anti-oxidative property.

Further isolation and purification of the Chloro fraction resulted in identification of 10 known xanthones. After carefully analysis of mass spectrum, ^{1}H-NMR and ^{13}C-NMR, the isolates were elucidated as γ-mangostin (γ-Man) (**1**),[10] 1,3,6,7-tetrahydroxy-8-prenylxanthone (**2**),[17] gartanin (**3**),[18] garcinone E (**4**),[19] 8-deoxygartanin (**5**),[18] β-mangostin (**6**),[18] α-mangostin (7),[18] mangosharin (**8**),[20] 9-hydroxycalabaxanthone (**9**)[18] and 11-hydroxy-1-isomangostin (**10**).[21] The structure of γ-Man was shown in Fig. 1B, and others were shown in Supplemental Fig. S1.

Next, the hepatoprotective effect and anti-oxidative activity of all the isolates were

tested. Among them, γ-Man (**1**) showed the most potent hepatoprotective activity at concentrations of 0.63, 1.25, 2.50 and 5.00 μM (Table 2). Especially, treatment of 2.50 or 5.00 μM of γ-Man totally rescued HL-7702 cells from *t*-BHP-induce oxidative injury. Besides, 1,3,6,7-tetrahydroxy-8-prenylxanthone (**2**), gartinin (**3**), garcinone E (**4**) and 8-deoxygartanin (**5**) also showed significant protective effect at tested concentrations (Table 2). Further study found γ-Man protected *t*-BHP induced oxidative injury in a dose dependent manner (Fig. 1C). Additionally, all the xanthones had no cytotoxicity at concentration of 2.50 μM; and compounds **1**, **2**, **4** and **10** didn't show cytotoxicity even at concentration of 5.00 μM (Supplemental Table S2). Thus, γ-Man was identified as the most potent hepatoprotective xanthone in *G. mangostana* and selected in the following studies.

γ-Man also possessed significant anti-oxidative activity on DPPH and ABTS radical scavenging assays, with IC_{50} values at 6.84 and 11.28 μM, respectively (Table 3); and compounds **2**, **3** and **4** showed comparable anti-oxidative activity as that of γ-Man; while other xanthones almost had no effect (Table 3).

In the current study, bioassay guided strategy was taken advantage to identify several potential hepatoprotective xanthones from the pericarps of *G. mangostana*. All these isolates contained a xanthone core skeleton with different substituents, including hydroxyl, methoxyl and isoprenyl. The structure-activity relationship between these xanthones and their hepatoprotective effect was summarized as following: 1) The number of phenolic hydroxyl group in xanthones might play a key role in their hepatoprotective activity. The xanthones containing four phenolic hydroxyl groups,

such as compounds **1**-**4**, had significant hepatoprotective capacity, while other xanthones with two or three phenolic hydroxyl groups had less or no effect (Table 2). 2) The hepatoprotective ability of xanthones might also relate to the number of isoprenyl group. Xanthones with two isoprenyl groups, such as **1** and **3**, had more potent hepatoprotective activity than those with one (**2**) or three (**4**) isoprenyl groups (Table 2). 3) Additionally, the heaptoprotective ability of the xanthones might relate to the position of substituents. The methoxyl group on C-7 (such as compounds **6**, **7**, **9** and **10**) might attenuate its hepatoprotective effect. Interestingly, the same conclusion could be drawn for the radical scavenging capacity of xanthones (Table 3). Taken together, it was deduced that four phenolic hydroxyl groups, two isoprenyl groups and without methoxyl group on C-7 might be necessary for xanthones' radical scavenging ability and protection of oxidative injury in hepatocytes. Meanwhile, the cytotoxicity of xanthones on HL-7702 cells (Supplemental Table S2) was more significant when a methoxyl group or a hydrogen on C-7 than that of compounds with a hydroxyl group.

Surprisingly, the hepatoprotective effect of fractions from the pericarps of *G. mangostana* (Fig. 1A) was inconsistent with their radical scavenging activity (Table 1). The similar results have also been reported in other previous studies, which indicated the anti-oxidative capacity of herbal extracts or compounds were uncorrelated to their oxidative protection effects on cell or animal models. It was mainly due to that the anti-oxidative assays were not targeting relevant substrates and not reflecting gross characteristics of biological systems.[22, 23] On the other hand, the hepatoprotective effect and radical scavenging activity of the xanthones were totally

consistent (Tables 2 and 3). γ-Man was found as the most effective compound on radical scavenging assays and *t*-BHP induced oxidative injury model. To further analyze the content of γ-Man in each fraction, HPLC-UV experiments were performed. The data indicated the content of γ-Man in T.E, P.E, Chloro, E.A, *n*-Bu and water fraction was 5.59%, 1.03%, 8.12%, 5.08%, 0.05% and not detected, respectively (representative chromatogram shown in Supplemental Fig. S2). What puzzled us was that the E.A and *n*-Bu fractions were more effective on radical scavenging, in which the content of γ-Man was less than that in Chloro fraction. It might suggest other undiscovered compounds in the E.A and *n*-Bu fractions which contributed to the anti-oxidative activity. Remarkably, the content of γ-Man in each fraction was in agreement with their hepatoprotective effect, which demonstrated that γ-Man probably is the most potent effective compound on hepatoprotection among all the isolated compounds.

γ-Man significantly inhibited ROS accumulation in HL-7702 hepatocytes

2',7-dichlorofluorescein diacetate (DCFH-DA) is widely used to reflect cellular levels of hydrogen peroxide, hydroxyl radicals and peroxynitrite, which indicates ROS production. In the present study, we found *t*-BHP treatment robustly induced green fluorescence compared with that of control group, which suggested increased ROS accumulation (Fig. 2A). And pretreatment with γ-Man obviously decreased green fluorescence, indicating that γ-Man prevented *t*-BHP induced ROS production (Fig.

2A). Quantitative analysis also supported that treatment with *t*-BHP induced about 2.50 fold of fluorescent intensity compared with that of control group, which was attenuated by pretreatment with γ-Man in a dose dependent manner (Fig. 2B). Surprisingly, when treated 2.50 or 5.00 μM γ-Man, the level of intracellular ROS decreased to that of control group. Oxidative stress is commonly considered to correlate with many diseases, such as aging,[24] cardiovascular diseases,[25] diabetes,[26] cancers [27] and liver diseases.[4] Hence, γ-Man was found to effectively reduce *t*-BHP induced accumulation of ROS in HL-7702 cells, which might bring great benefit for prevention or treatment of these diseases.

γ-Man significantly prevented *t*-BHP-induced loss of mitochondrial membrane potential (MMP) and cell nuclei morphology change

Excessive accumulation of ROS induces depolarization of MMP, which is often accompanied with cell apoptosis or necrosis due to the open of mitochondrial permeability transition pore and mitochondrial swelling.[28] In the current study, the R123 (Rhodamine123) was used to investigate whether the hepatoprotective effect of γ-Man was through ameliorating *t*-BHP induced loss of MMP. As shown in Fig. 3A, the intracellular florescence was significantly enhanced when treated with *t*-BHP, which indicated that the MMP was obviously lost. However, pretreatment with γ-Man significantly attenuated the loss of MMP. Quantitative analysis of flow cytometry result showed that the loss of MMP in *t*-BHP treatment group was about 1.60 fold to

that of control group, which was decreased in a dose dependent manner when pretreated with γ-Man (Fig. 3B). The result indicated the hepatoprotective effect of γ-Man might be through decreasing *t*-BHP induced loss of MMP.

Intracellular accumulation of excessive ROS also has negative effect on the integrity of cell nuclei, which induces the occurrence of gene mutation.[29] In this study, the morphology of cell nuclei was also monitored for better explanation of the hepatoprotective effect of γ-Man. In Fig. 3C, the slight condensed and deformed cell nuclei were observed when treated with *t*-BHP, while the cell nuclei were maintained normally with the treatment of γ-Man.

γ-Man protected *t*-BHP induced intracellular key enzymes change and lipid peroxidation

Next the contents of alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutathione (GSH) and superoxide dismutase (SOD), as well as lipid peroxides product malondiadehyde (MDA) in HL-7702 cells were determined. As shown in Table 4, *t*-BHP treatment induced significant decrease of levels of ALT, AST, GSH and SOD, as well as increase of MDA content. Interestingly, pretreatment with 1.25 μM γ-Man significantly prevented *t*-BHP induced changes of the enzymes and MDA; and pretreatment of 5.00 μM γ-Man totally reversed the levels of enzymes and MDA to those of control group (Table 4). These results indicated that γ-Man might exert its hepatoprotective effect through attenuating key enzymes from being excessively

damaged by acute oxidative stress.

Experimental

Materials

Phosphate-buffered saline (PBS) powder, RPMI 1640 medium, penicillin-streptomycin (P/S), 0.25% (w/v) trypsin/1 mM EDTA and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY, USA). 2',7-dichlorofluorescein diacetate (DCFH-DA), Rhodamine 123, *t*-BHP, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2-Htetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO) , 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercial spectrophotometric kits for detection of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), superoxide dismutase (SOD), glutathione (GSH) and malondiadehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Vitamin C was purchased from Farco Chemical Supplies (Beijing, China).

Sample preparation

The fruits of *G. mangostana* were collected from Chiang Mai, Thailand, and identified by Prof. Jingui Shen (Shanghai Institute of Materia Medica). The dried pericarps of *G. mangostana* were smashed into fine powder and ultrasonically extracted by 95% ethanol for three times, each 2 h. The ethanol extract was

concentrated to yield total extract (T.E), which was further partitioned with petroleum ether, chloroform, ethyl acetate, and *n*-butanol successively, to obtain petroleum ether fraction (P.E), chloroform fraction (Chloro), ethyl acetate fraction (E.A), and *n*-butanol fraction (*n*-Bu), respectively. Subsequently, the Chloro fraction was further isolated and purified by column chromatography over silica gel, MCI and Sephadex LH-20, as well as preparative HPLC.

Chemical structure identification

The structures of the isolates were identified by ^{1}H-NMR, ^{13}C-NMR and mass spectra. The NMR spectra were recorded on a Bruker AM-400 NMR spectrometer (Bruker, Bremen, Germany). Chemical shifts were reported relative to tetramethylsilane (TMS) as an internal standard. ESI-MS spectra were measured on a Finnigan LCQ Deca instrument, and HR-ESI-MS spectra were performed on a Waters Micromass Q-TOF spectrometer.

DPPH radical scavenging assay

The DPPH radical scavenging capacity of samples was determined as described previously.[7] The samples (1.00-125.00 μg mL^{-1} and 1.00-500.00 μM for fractions and compounds, respectively, each 10.00 μL) were added to a volume of 190.00 μL methanol solution of DPPH radical (final concentration of DPPH was 40.00 μg mL^{-1}), followed by shaking vigorously and keeping at room temperature in dark for 30 min. Then the absorbance of the resulting solution was measured at 517 nm using a

microplate reader (SpectraMax M5, Molecular Devices, USA). The absorbance of DPPH methanol solution was tested as blank control. The radical scavenging capacity was calculated as following equation: DPPH Scavenging activity % = ($A_{Control}$ − $A_{Test}/A_{Control}$) × 100 %. Where $A_{Control}$ is the absorbance of 10.00 μL methanol and 190.00 μL fresh DPPH solution and A_{Test} is the absorbance of different concentrations of samples.

ABTS radical scavenging assay

The ABTS radical scavenging capacity of samples was determined as described previously.[7] Briefly, the samples (1.00-125.00 μg mL^{-1} and 1.00-500.00 μM for fractions and compounds, respectively, 10.00 μL each) were added to a volume of 190.00 μL solution of ABTS radical, followed by keeping at room temperature for 6 min. Then the absorbance of the resulting solution was measured at 734 nm by a microplate reader (SpectraMax M5, Molecular Devices, USA). The absorbance of ABTS methanol solution was tested as blank control. The radical scavenging capacity was calculated as following equation: ABTS scavenging activity % = ($A_{Control}$ − $A_{Test}/A_{Control}$) × 100 %. Where $A_{Control}$ is the absorbance of 10.00 μL Milli Q water and 190.00 μL fresh ABTS solution and A_{Test} is the absorbance of different concentrations of samples.

HPLC-UV analysis of γ-Man, total extract and fractions

HPLC analysis was performed on a Waters e2695 HPLC system composed of

photodiode array detector. Chromatograph was achieved with a gradient elution at a flow rate of 1.00 mL/min using reversed phase C18 silica gel as stationary phase (250 × 4.6 mm, 5 μm, Waters SunFire^{TM} column) which maintained at 35 ºC. The mobile phase was composed of 0.01% aqueous formic acid (A) and acetonitrile (B), and the gradient elution was as follows: 0-6 min, 40% A; 6-30 min, 40-16% A; 30-50 min, 16-5% A. The detection wavelength was set at 254 nm and the sample injection volume was 10.00 μL. Representative chromatogram was analyzed using a Waters Empower system and content of γ-Man (%) in each fraction was determined by external standard method. Fractions and γ-Man were dissolved by 60% methanol solution at concentration of 1.00 mg mL^{-1} and 50.00 μg mL^{-1}, respectively.

Cell culture

HL-7702 human normal liver cells were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in RPMI 1640 medium containing heat-inactivated 10% FBS, streptomycin (100 μg mL^{-1}) and penicillin (100 unit mL^{-1}) in a 37 ^{o}C incubator under a humidified atmosphere of 95% air and 5% CO_2. Adherent cells were detached by trypsin-EDTA and plated onto 96- or 6-well plate at 70–80% confluence for the following tests.

Cell viability assay

HL-7702 cells were seeded in 96-well plates at a density of 1×10^4 cells per well. When approximately 70-80% confluence, cells were treated with or without different

concentrations of samples for 12 h. Subsequently, the culture medium was discarded and cells were treated with or without 200.00 μM *t*-BHP solution for additional 6 h. Then cell viability was determined by incubation with RPMI 1640 containing MTT (1.00 mg mL^{-1}) for 4 h, followed by dissolving the formazan crystals with DMSO. The absorbance at 570 nm was measured by a microplate reader (SpectraMax M5, Molecular Devices, USA) and presented as relative cell viability. The results were analyzed based on at least three independent experiments.

Assessment of ROS production

Intracellular formation of ROS was assessed using the oxidation sensitive dye DCFH-DA, as described previously.[7] Briefly, HL-7702 cells were seeded in a 96-well black plate at a concentration of 1×10^4 cells per well for 12 h. Then cells were treated with or without different concentrations of γ-Man for additional 12 h, followed by treatment with or without 200.00 μM *t*-BHP for 3 h. Subsequently, cells were incubated with DCFH-DA (10.00 μM) at 37 ^{o}C in dark for 15 min. The florescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a spectrofluorometer (SpectraMax M5, Molecular Devices, USA). The intracellular ROS was also quantitated using an In Cell Analyzer 2000 (GE Healthcare Life Sciences, USA).

Measurement of mitochondrial membrane potential (MMP)

The MMP was monitored using the fluorescent dye R123. Briefly, cells were

pretreated with or without different concentrations of γ-Man, followed by *t*-BHP treatment for 2 h. Then cells were stained with R123 (10.00 μM) for 10 min. The fluorescence was observed and quantitated by flow cytometer (Becton Dickinson, USA).

Nuclei morphology analysis

Hochest 33342 staining was performed as previously reported.[30] In brief, cells were treated with or without different concentrations of γ-Man for 12 h and then incubated with *t*-BHP for additional 6 h. Then cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with Hochest 33342 (1.00 μg mL^{-1}) for 10 min. The morphology of cell nuclei was monitored using an In Cell Analyzer 2000 (GE Healthcare Life Sciences, USA).

Hepatotoxicity assessment

The intracellular levels of AST and ALT were determined using commercial detection kits according to the manufacturer's instructions as described previously.[31]

Determination of GSH, SOD and MDA levels

The levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) in HL-7702 liver cells were determined using commercial assay kits according to the manufacturer's protocols.[31] Protein concentration was determined by a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). All

values were normalized to total protein.

Statistical analysis

Values were expressed as mean ± SEM based on at least three independent experiments. Between-group comparison was evaluated by one-way ANOVA test using SPSS software 16.0 (Chicago, IL, USA). A *p* value less than 0.05 and 0.01 was considered significantly and very significantly different, respectively.

Conclusions

In conclusion, γ-Man was extraordinarily effective in protecting hepatocytes from *t*-BHP induced oxidative damage through decreasing the ROS accumulation and the loss of MMP, and reducing the depletion of anti-oxidant enzymes GSH and SOD. Based on the current data, it is believed that γ-Man holds great potential for being developed as healthy foods and therapeutic agents for treatment or prevention of oxidative stress related liver diseases. Bioassay guided strategy is an effective and direct way for identification of bioactive components from natural products. However, a weak point of this study is that some potential anti-oxidative principles, which were not identified, might be neglected. Future work need to focus on identification more xanthones from *G. mangostana*, which might supply powerful evidence for better research and development of this fruit as hepatoprotective agent.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

Financial support by Science and Technology Development Fund, Macao S.A.R (FDCT 120/2013/A3) and the Research Fund of University of Macau (MYRG2014-00020-ICMS-QRCM and MYRG2015-00153-ICMS-QRCM) are gratefully acknowledged. The authors thank Prof. Jingui Shen for identification of plant material.

382 **References:**

383

384 1. G. W. Thorpe, C. S. Fong, N. Alic, V. J. Higgins and I. W. Dawes, *Proc Natl*
385 *Acad Sci USA*, 2004, 101, 6564-6569.

386 2. H. U. Simon, A. Haj-Yehia and F. Levi-Schaffer, *Apoptosis*, 2000, 5, 415-418.

387 3. G. Waris and H. Ahsan, *J Carcinog*, 2006, 5, 14.

388 4. A. I. Cederbaum, Y. Lu and D. Wu, *Arch Toxicol*, 2009, 83, 519-548.

389 5. C. Loguercio and A. Federico, *Free Radic Biol Med*, 2003, 34, 1-10.

390 6. C. J. Wang, J. M. Wang, W. L. Lin, C. Y. Chu, F. P. Chou and T. H. Tseng,
391 *Food Chem Toxicol*, 2000, 38, 411-416.

392 7. Y. S. Kim, J. W. Hwang, S. H. Sung, Y. J. Jeon, J. H. Jeong, B. T. Jeon, S. H.
393 Moon and P. J. Park, *Food Chem*, 2015, 168, 572-579.

394 8. H. A. Jung, B. N. Su, W. J. Keller, R. G. Mehta and A. D. Kinghorn, *J Agric*
395 *Food Chem*, 2006, 54, 2077-2082.

396 9. Q. Y. Liu, Y. T. Wang and L. G. Lin, *Food Funct*, 2015, 6, 383-393.

397 10. L. G. Chen, L. L. Yang and C. C. Wang, *Food Chem Toxicol*, 2008, 46,
398 688-693.

399 11. P. Moongkarndi, N. Kosem, S. Kaslungka, O. Luanratana, N. Pongpan and N.
400 Neungton, *J Ethnopharmacol*, 2004, 90, 161-166.

401 12. S. X. Chen, M. Wan and B. N. Loh, *Planta Med*, 1996, 62, 381-382.

402 13. G. Gopalakrishnan, B. Banumathi and G. Suresh, *J Nat Prod*, 1997, 60,
403 519-524.

14. K. Nakatani, M. Atsumi, T. Arakawa, K. Oosawa, S. Shimura, N. Nakahata and Y. Ohizumi, *Biol Pharm Bull*, 2002, 25, 1137-1141.

15. L. Yu, M. Zhao, B. Yang, Q. Zhao and Y. Jiang, *Food Chem*, 2007, 104, 176-181.

16. N. Kosem, Y.-H. Han and P. Moongkarndi, *Science Asia*, 2007, 33, 283-292.

17. Q. Zhou, L. Chen, Q. Chen, H. Chen and J. Dong, *Zhong Yao Cai*, 2013, 36, 1444-1447.

18. A. R. Han, J. A. Kim, D. D. Lantvit, L. Kardono, BS, S. Riswan, H. Chai, E. J. Carcache de Blanco, N. R. Farnsworth, S. M. Swanson and A. D. Kinghorn, *J Nat Prod*, 2009, 72, 2028-2031.

19. S. I. Sakai, M. Katsura, H. Takayama, N. Aimi, N. Chokethaworn and M. Suttajit, *Chem Pharm Bull*, 1993, 41, 958-960.

20. G. Ee, S. Daud, Y. Taufiq-Yap, N. Ismail and M. Rahmani, *Nat Prod Res*, 2006, 20, 1067-1073.

21. G. L. Sia, G. J. Bennett, L. J. Harrison and K. Y. Sim, *Phytochemistry*, 1995, 38, 1521-1528.

22. M. Serafini and D. Del Rio, *Redox Rep*, 2004, 9, 145-152.

23. L. K. MacDonald Wicks, L. G. Wood and M. L. Garg, *J Sci Food Agric*, 2006, 86, 2046-2056.

24. B. S. Berlett and E. R. Stadtman, *J Biol Chem*, 1997, 272, 20313-20316.

25. T. Heitzer, T. Schlinzig, K. Krohn, T. Meinertz and T. Münzel, *Circulation*, 2001, 104, 2673-2678.

26. R. Da Ros, R. Assaloni and A. Ceriello, *Diabet Med*, 2004, 21, 1249-1252.

27. D. Trachootham, J. Alexandre and P. Huang, *Nat Rev Drug Discov*, 2009, 8, 579-591.

28. J. J. Lemasters, T. P. Theruvath, Z. Zhong and A. L. Nieminen, *Biochim Biophys Acta*, 2009, 1787, 1395-1401.

29. K. Ishikawa, K. Takenaga, M. Akimoto, N. Koshikawa, A. Yamaguchi, H. Imanishi, K. Nakada, Y. Honma and J. I. Hayashi, *Science*, 2008, 320, 661-664.

30. N. Plesnila, C. Zhu, C. Culmsee, M. Gröger, M. A. Moskowitz and K. Blomgren, *J Cereb Blood Flow Metab*, 2004, 24, 458-466.

31. H. Miyao, T. Arao, M. Udayama, J. Kinjo and T. Nohara, *Planta Med*, 1998, 64, 5-7.

Tables

Table 1. Anti-oxidative capacity of total extract and fractions from the pericarps of *G. mangostana* on DPPH and ABTS radical scavenging assays.

	DPPH	ABTS
	IC_{50} (μg mL^{-1}) [1]	
T.E	12.12 ± 0.58	6.82 ± 0.30
P.E	15.64 ± 0.43	16.95 ± 0.68
Chloro	13.70 ± 0.41	11.15 ± 0.12
E.A	3.51 ± 0.27	3.04 ± 0.16
n-Bu	3.86 ± 0.07	4.39 ± 0.37
Water	34.46 ± 0.48	45.86 ± 0.68
Vitamin C	2.51 ± 0.35	2.62 ± 0.09

[1]values are expressed as means ± SD (n=6).

444 **Table 2.** Hepatoprotective effects of xanthones from the pericarps of *G. mangostana* on *t*-BHP induced oxidative injury HL-7702 cells.

NO	Compounds	Cell viability (%) [1]			
		0.63 μM	1.25 μM	2.50 μM	5.00 μM
1	γ-mangostin	58.62 ± 4.72 **	86.01 ± 1.53 **	91.22 ± 5.83 **	95.26 ± 1.84 **
2	1,3,6,7-tetrahydroxy-8-prenylxanthone	48.13 ± 8.12 *	48.42 ± 3.36 *	41.15 ± 5.83	40.63 ± 1.85
3	gartanin	53.37 ± 0.93 **	61.12 ± 1.34 **	63.01 ± 1.12 **	---
4	garcinone E	32.34 ± 1.25	41.33 ± 3.35	49.83 ± 3.72 *	67.35 ± 1.67 **
5	8-deoxygartanin	46.83 ± 7.02 *	48.35 ± 4.03 *	41.56 ± 7.47	---
6	β-manostin	37.79 ± 7.30	39.82 ± 2.77	35.56 ± 4.63	---
7	α-mangostin	30.74 ± 1.05	30.92 ± 0.66	28.17 ± 0.84	---
8	mangosharin	36.23 ± 0.95	33.03 ± 2.07	31.34 ± 1.19	---
9	9-hydroxy-calabaxanthone	30.83 ± 1.56	28.37 ± 1.78	30.15 ± 1.47	---
10	11-hydroxy-1-isomangostin	37.62 ± 5.38	32.73 ± 2.25	37.34 ± 5.16	32.43 ± 3.67

445 [1] values are expressed as mean ± SD (n=6). Control group (100.00 ± 6.03) and model group (38.84 ± 5.17), $p < 0.01$ model group *vs* control
446 group.

447 * $p < 0.05$, ** $p < 0.01$, sample group *vs* model group.

448 **Table 3.** Anti-oxidative capacity of xanthones from the pericarps of *G. mangostana*

449 on DPPH and ABTS radical scavenging assays.

NO	compound	DPPH	ABTS
		IC_{50} (μM) [1]	
1	γ-mangostin	6.84 ± 0.08	11.28 ± 0.41
2	1,3,6,7-tetrahydroxy-8-prenylxanthone	9.83 ± 0.03	12.70 ± 0.40
3	gartanin	6.97 ± 0.13	14.62 ± 0.16
4	garcinone E	11.73 ± 0.58	21.16 ± 0.58
5	8-deoxygartanin	40.73 ± 0.17	111.33 ± 1.07
6	β-mangostin	NI	NI
7	α-mangostin	61.97 ± 0.23	133.30 ± 1.68
8	mangosharin	254.40 ± 1.02	308.00 ± 2.35
9	9-hydroxycalabaxanthone	NI	NI
10	11-hydroxy-1-isomangostin	NI	NI
	Vitamin C	0.01 ± 0.00	0.01 ± 0.00

450 [1] values are expressed as means ± SD (n=6). NI, No inhibitory effect was observed at

451 concentration up to 500.00 μM.

452 **Table 4.** γ-mangostin reversed *t*-BHP induced changes of intracellular key enzymes and MDA in HL-7702 cells.

Group	SOD (U g^{-1} protein)	MDA (U g^{-1} protein)	GSH (μmol g^{-1} protein)	AST/GOT (U g^{-1} protein)	ALT/GPT (U g^{-1} protein)
Control	71.11 ± 3.96	5.28 ± 0.39	179.82 ± 5.56	23.29 ± 3.05	10.46 ± 0.84
t-BHP (200.00 μM)	13.54 ± 1.34 $^{\#}$	10.87 ± 0.58 $^{\#}$	29.89 ± 6.38 $^{\#}$	17.12 ± 0.70 $^{\#}$	8.14 ± 0.22 $^{\#}$
γ-Man (0.63 μM) + *t*-BHP	34.18 ± 3.30 *	7.30 ± 0.50 *	64.23 ± 3.90 *	15.18 ± 3.59	7.97 ± 0.26
γ-Man (1.25 μM) + *t*-BHP	52.54 ± 7.60 *	7.52 ± 0.21 *	83.31 ± 2.86 *	25.47 ± 1.94 *	11.87 ± 0.35 *
γ-Man (2.50 μM) + *t*-BHP	52.38 ± 1.58 *	4.78 ± 0.18 *	140.59 ± 2.59 *	25.29 ± 1.63 *	12.64 ± 0.73 *
γ-Man (5.00 μM) + *t*-BHP	58.23 ± 1.75 *	5.48 ± 0.22 *	183.43 ± 3.19 *	25.26 ± 2.01 *	12.35 ± 0.79 *

453 All data were expressed as mean±SD (n= 6).

454 $^{\#}p < 0.05$, *t*-BHP group *vs*. control group; $^{*}p < 0.05$, sample group *vs*. *t*-BHP group.

Figure legends

Fig. 1. Bioassay guided isolation of xanthones from the pericarps of *G. mangostana*, protecting HL-7702 cells from *t*-BHP induced oxidative injury. (A) Protective effects of total extract and other fractions from the pericarps of *G. mangostana* on *t*-BHP induced oxidative injury. (B) Structure of γ-mangostin. (C) Protective effect of γ-Man on *t*-BHP induced oxidative injury HL-7702 cells. All values represent mean ± SEM (n=6). # $p < 0.05$, control group *vs* model group; * $p < 0.05$ and ** $p < 0.01$, sample group *vs* model group.

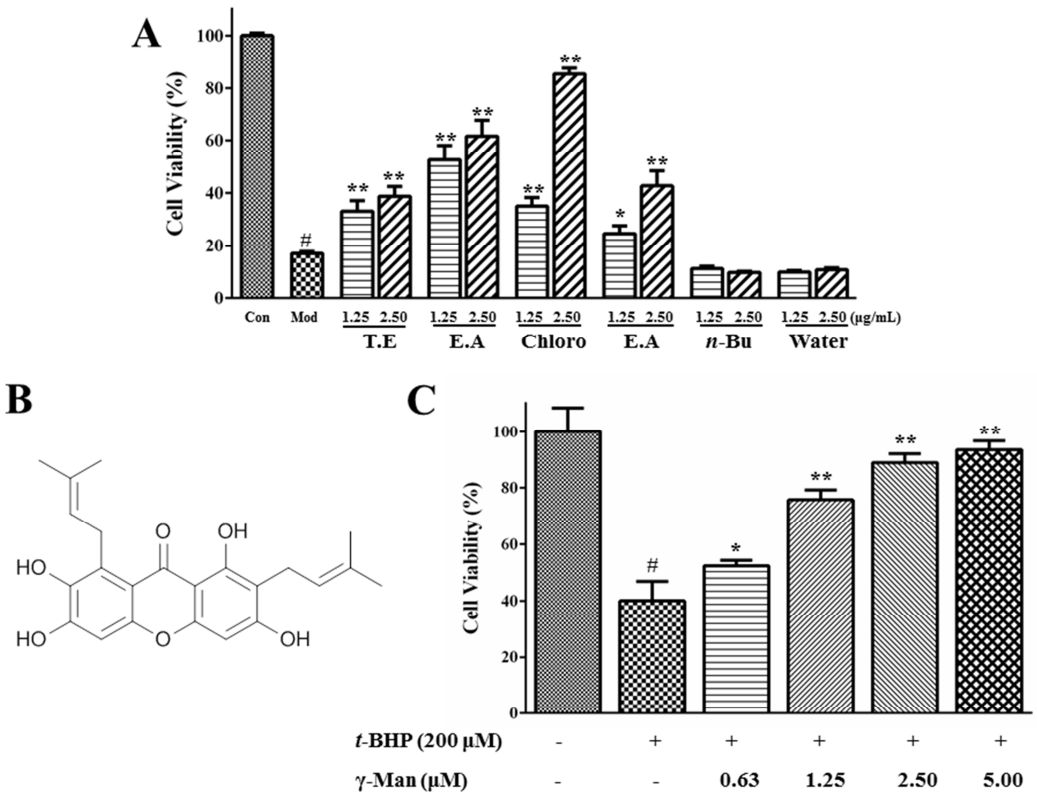


Fig. 2. γ-Man significantly inhibited the ROS accumulation in hepatocytes. (A) Representative photograph of intracellular florescence of ROS monitored by Incell Analyzer 2000 (1, control; 2, model; 3-6, γ-Man 0.63-5.00 μM). (B) Quantitative analysis of intracellular ROS content. All values represent mean ± SEM (n=6). # $p < 0.05$, control group *vs* model group; * $p < 0.05$ and ** $p < 0.01$, sample group *vs* model group.

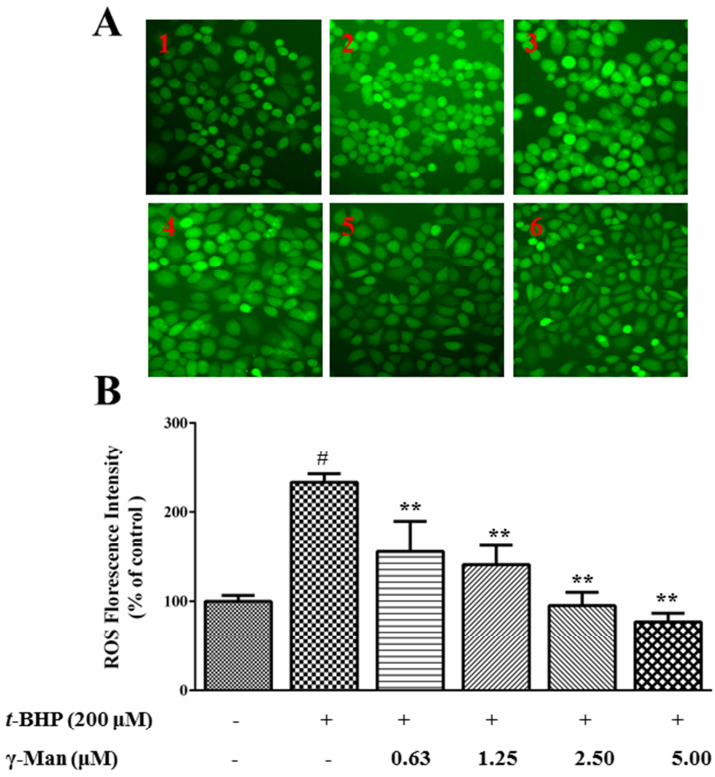
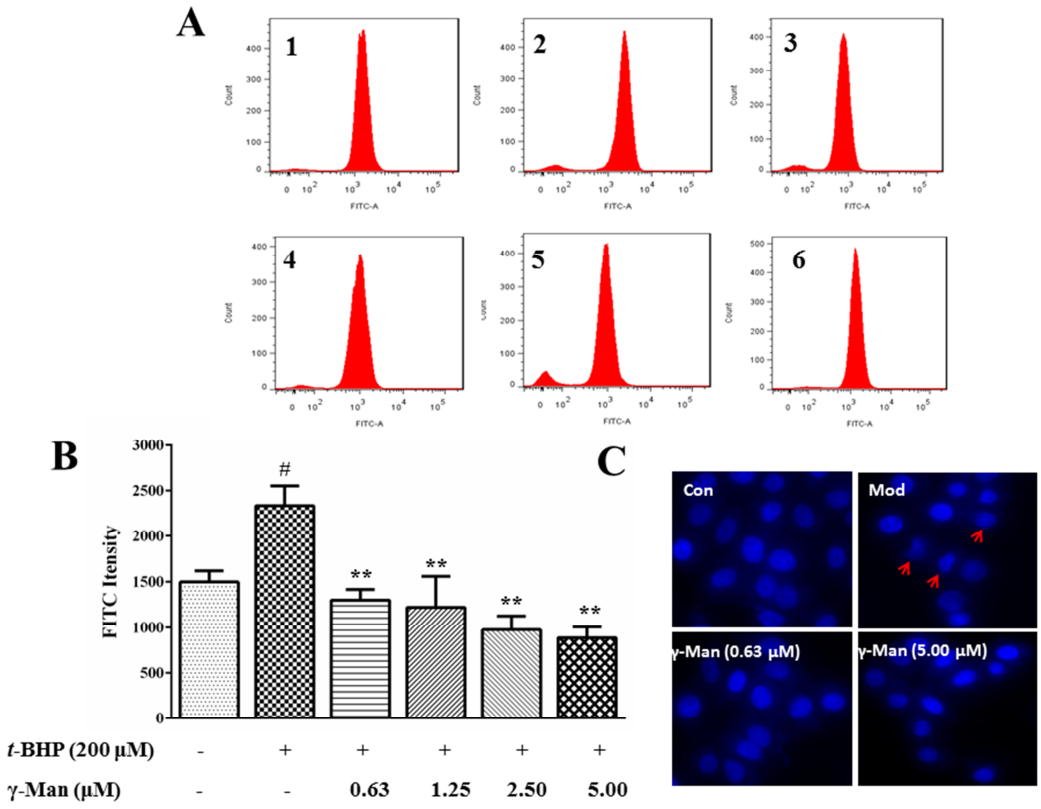


Fig. 3. γ-Man significantly decreased the loss of MMP induced by *t*-BHP and maintained the morphology of cell nuclei. (A) γ-Man protected *t*-BHP induced loss of MMP detected by flow cytometry (1, control; 2, model; 3-6, γ-Man 0.63-5.00 μM). (B) Quantitative analysis of FITC fluorescent intensity. (C) Nuclei morphology analysis. All values represent mean ± SEM (n=6). # $p < 0.05$, control group *vs* model group; * $p < 0.05$ and ** $p < 0.01$, sample group *vs* model group.



Supplemental materials

Table S1. Cytotoxicity of total extract and fractions from the pericarps of *G. mangostana* on HL-7702 cells.

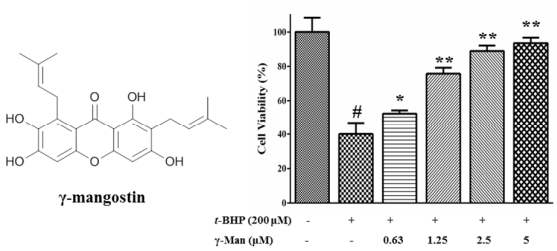
Table S2. Cytotoxicity of xanthones from the pericarps of *G. mangostana* on HL-7702 cells.

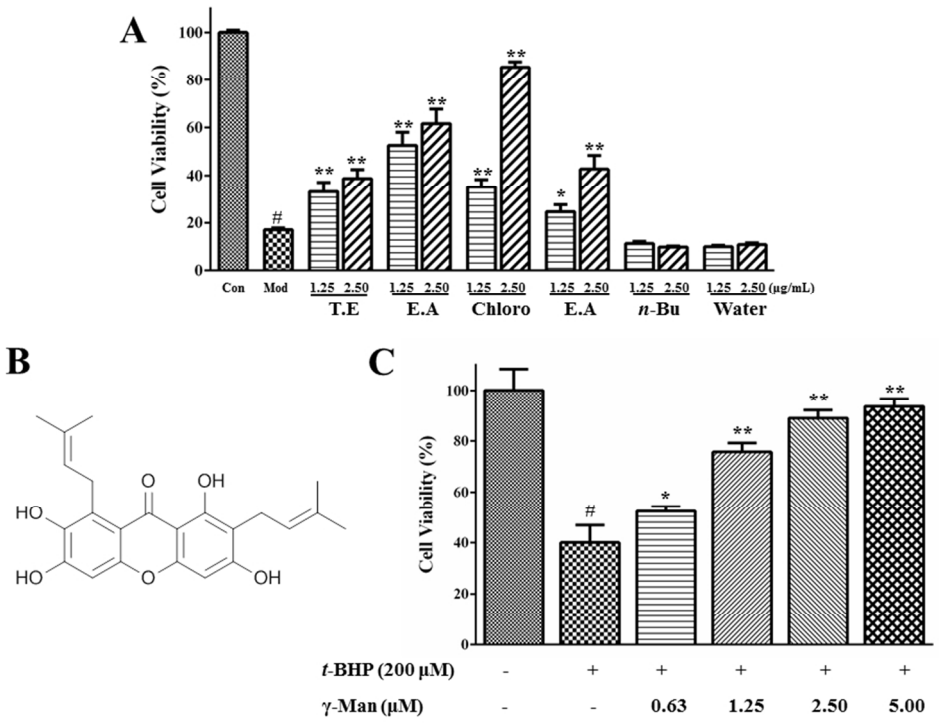
Fig. S1. Chemical structures of xanthones isolated from the pericarps of *G. mangostana*.

Fig. S2. Chromatogram of γ-magostin, T.E and fractions from the pericarps of *G. mangostana* (from top to bottom represent γ-magostin and α-magostin, 50.00 μg mL^{-1}; T.E, P.E, Chloro, E.A, *n*-Bu and water fraction, 1.00 mg mL^{-1}, respectively. Wavelength was set at 254 nm).

Table of contents

This study identified γ-mangostin from the pericarps of *Garcinia mangostana* as a potential hepatoprotective agent.





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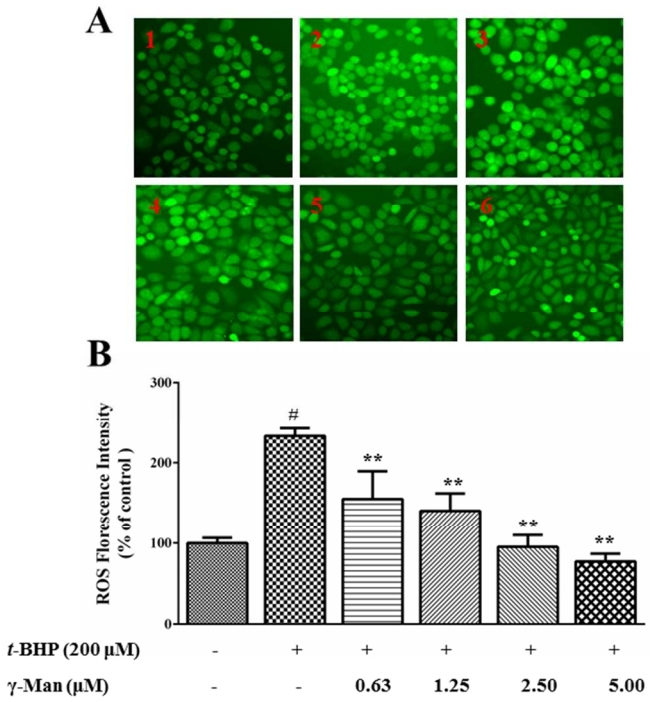

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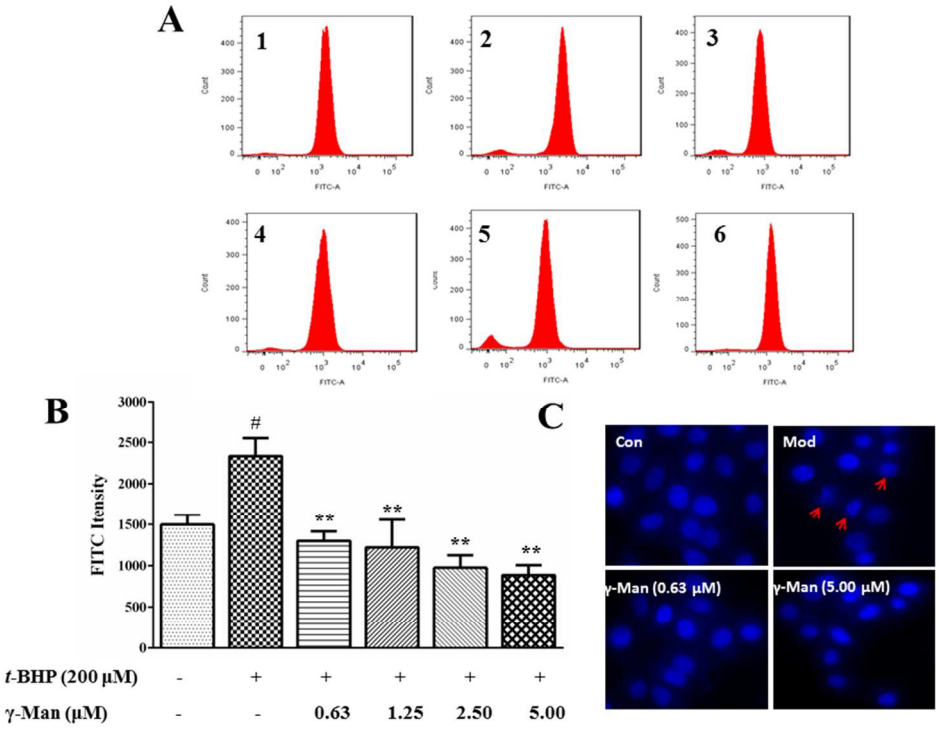

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