國立臺灣大學生命科學院生化科技學系

博士論文

Department of Biochemical Science and Technology

College of Life Science

National Taiwan University

Doctoral Dissertation

Monascus purpureus NTU 568 發酵產物對酒精性肝損傷及肝臟纖維化之保健功效評估

Protective effect of *Monascus purpureus* NTU 568-fermented products against alcoholic liver disease and hepatic fibrosis

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中華民國 106 年 4 月

April, 2017

doi:10.6342/NTU201700799

謝誌

承蒙恩師 潘子明教授悉心指導，得以讓學生順利畢業，學生在第一次入學期間，因學校英文門檻及家庭因素不得不休學數年進行工作，於 2015 年放棄原本學籍再次考試入學，恩師依然幫忙學生順利入學，並且願意再次指導學生，在許多論文及實驗上的問題，也都承蒙恩師耐心指正學生。學生在歷經兩次的入學，長達八年的在學時間，承蒙恩師指導及鼓勵學生才得以取得此學位，浩瀚師恩，永銘於心，特於卷首致衷心謝意。

論文口試期間承蒙國立台灣大學周正俊教授、食工所廖啟成所長、輔仁大學蔡宗佑主任、國立台東大學李俊霖主任、輔英科技大學王志傑研發長、萬芳醫院何秉穎博士、國立中興大學蔣慎思老師、晨暉生物科技許雅雯及陳建利博士，對輪文初稿詳加審閱，並提供許多寶貴的建議，使得本論文更臻完整，特致謝忱。

在研究期間，承蒙學長姐志輝、宗偉、姿杏、建利、秉穎、力川、慎思等，大力的幫忙指導協助，謝謝同窗保宏及小花對於討論實驗的協助，在此特別謝謝雅雯及雨青學姊，指導化學分析及實驗設計討論。當初的學弟妹人數眾多，無法一一介紹，在此特別感謝各位的包容及協助。再次入學後，感謝學弟家源、凱鈞、培祐、柏安、學妹暐婷、德華、偲涵、慧潔及聖方，對於實驗及口試的協助，特別謝謝學妹孟純，幫忙我準備口試及實驗的協助，讓我可以全心專注於實驗，加快畢業的時間。對於實驗室的同學、學長姊及學弟妹，由於在學期間斷斷續續橫跨八年，在此雖無法一一提及，特致謝忱。僅將此微薄的研究成果呈獻給我的家人，及曾經幫助我的朋友們，感謝辦公室的同仁宜霆及景中，協助處理公事及各種雜事，使得我能順利畢業取得學位。

智夫 謹致於

2017 春 四川

doi:10.6342/NTU201700799

縮寫表

ADH (alcohol dehydrogenase) 酒精去氫酶

ALT (alanine aminotransferase) 丙胺酸轉胺酶

AST (aspartate aminotransferase) 天門冬胺酸轉胺酶

CML (carboxymethyl-lysine) 羧甲基離胺酸

DBP (diastolic blood pressure) 舒張壓

DEXA (dual energy x-ray absorptiometry) 雙能量 X 射線吸收儀

ECM (extracellular matrixc) 細胞外基質

FFA (free fatty acid) 游離脂肪酸

GABA (γ-amino butyric acid) γ-胺基丁酸

GCL (glutamate-cysteine ligase) 谷胺酸半胱胺酸連接酶

HDL-C (high-density lipoprotein cholesterol) 高密度脂蛋白膽固醇

HR-LPL (heparin-releasable lipoprotein lipase) 肝素釋放脂蛋白脂解酶

HSCs (hepatic stellate cell) 活化肝星狀細胞

LDL-C (low-density lipoprotein cholesterol) 低密度脂蛋白膽固醇

LPO (Fe^{2+}-dependent lipid peroxidation) Fe^{2+} 依賴型脂質過氧化

Nrf-2 (nuclear factor-erythroid 2 related factor-2) 核因子-紅血球之 2 相關因子-2

NO (nitric oxide) 一氧化氮

PKC (protein kinase C) 蛋白激酶 C

PPAR-γ (peroxisome proliferator-activated receptor-γ) 過氧化物酶體增殖物活化受體 γ

RAGE (receptor for advanced glycation endproducts) 糖化最終產物受器

RMD (*Monascus*-fermented red mold dioscorea) 紅麴山藥

RMR (*Monascus*-fermented red mold rice) 紅麴米

RMRE (ethanol extract of red mold rice) 紅麴米酒精萃取物

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ROS (reactive oxygen species) 活性氧化物質

SBP (systolic blood pressure) 收縮壓

SREBP (sterol regulatory element-binding protein) 固醇調節元件結合蛋白

TC (total cholesterol) 總膽固醇

TG (triglyceride) 三酸甘油酯

VEGF (vascular endothelial growth factor) 血管內皮生長因子

doi:10.6342/NTU201700799

中文摘要

Monascus purpureus NTU 568 之發酵代謝產物具有廣泛的生物活性，包括抑制腫瘤、調節高三酸甘油酯、抗氧化及抗發炎等活性。然而，酒精性肝損傷的病理機制並未清楚。因此在本研究中，利用慢性酒精性肝損傷的小鼠作為實驗模式，以評估 *M. purpureus* NTU 568 發酵紅麴米 (*Monascus*-fermented red mold rice, RMR) 及紅麴山藥 (*Monascus*-fermented red mold dioscorea, RMD) 保護肝臟的功效。此實驗模式中，分別餵食酒精誘導肝損傷的小鼠紅麴米及紅麴山藥 307.5 mg/kg (一倍劑量)、615.0 mg/kg (兩倍劑量) 及 1537.5 mg/kg (五倍劑量) 持續餵食五周。餵食紅麴米及紅麴山藥組均顯著降低血清中天門冬胺酸轉胺酶 (aspartate aminotransferase, AST)、丙胺酸轉胺酶 (alanine aminotransferase, ALT)、瘦體素 (lepatin) 及肝臟中三酸甘油酯 (triglyceride, TG)、總膽固醇 (total cholesterol, TC) 及游離脂肪酸 (free fatty acid, FFA) 的累積，並且可提高血清中脂聯素 (adiponectin) 與肝臟中酒精去氫酶 (alcohol dehydrogenase, ADH) 含量。紅麴米及紅麴山藥也可進一步的提升肝臟抗氧化功能、減低肝細胞傷害 (脂肪肝) 並且降低肝臟的促發炎細胞激素含量。重複性的慢性或急性肝臟損傷會造成肝臟一再修復，並且導致肝纖維化疾病產生。而此疾病與活化肝星狀細胞 (hepatic stellate cell, HSCs) 增生及細胞外基質 (extracellular matrixc, ECM) 的累積具有相當大的關連性。以紅麴發酵產物中分離出之 ankaflavin (AK) 及 monascin (MS) 進行體外細胞實驗，不但有效提升 HepG2 細胞中過氧化小體增生劑活化接受體 γ (peroxisome proliferator-activated receptor-γ) 含量並降低酒精引起的固醇調節元件結合蛋白 (sterol regulatory element-binding proteins-1) 和 TG 上升，同時也可以抑制肝臟的纖維化。實驗發現利用 AK 及 MS (15 及 30 μM)，可有效抑制 HSC-T6 細胞的 Akt/nuclear factor (NF)-κB 及 p38 mitogen-activated protein kinase (MAPK) 分子路徑。同時於 AK (30 μM) 及 MS (30 μM) 濃度下可有效抑制細胞生長並導致細胞凋亡，與控制組相比細胞存活率分別為： 80.2 ± 5.4% 及

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62.8 ± 8.2%; P < 0.05。導致細胞週期停滯於 G1 期 (G1 期 AK 與 MS 百分比分別為： 76.1 ± 2.9% 及 79.9 ± 1.8%，控制組為： 65.9 ± 4.9% (P < 0.05)。由以上實驗發現紅麴發酵產物可以有效降低氧化壓力、發炎反應、脂肪肝及抑制肝臟星狀細胞的增生而達到保護酒精性肝疾病的功效。

關鍵詞：酒精性肝疾病、肝臟保護、*Monascus purpureus*、ankaflavin、monascin

doi:10.6342/NTU201700799

Abstract

Monascus purpureus NTU 568 fermented products are reported to exhibit a wide variety of biological effects, including antitumor, antihypertriglyceridemia, antioxidation, and anti-inflammatory activities. However, its role in the pathogenesis of alcoholic liver disease remains obscure. In this study, the hepatoprotective effects of *Monascus*-fermented red mold rice (RMR) and *Monascus*-fermented red mold dioscorea (RMD) were evaluated *in vivo* using chronic alcohol-induced mice as an experimental model. The alcohol-induced mice were orally treated with RMR or RMD at 307.5 mg/kg (1-fold), 615.0 mg/kg (2-fold), and 1537.5 mg/kg (5-fold) for 5 weeks, whereas controls received vehicle only. Treatment with RMR or RMD significantly attenuated the increased level of serum transaminases (AST, aspartate aminotransferase and ALT, alanine aminotransferase), leptin and hepatic triglyceride (TG), total cholesterol (TC), free fatty acid (FFA) accumulation, and increased serum adiponectin, hepatic alcohol dehydrogenase (ADH) levels. Furthermore, RMR and RMD elevates hepatic antioxidant ability that reduced hepatic cell damage (steatosis) and decreased tissue inflammatory cytokine levels. Hepatic fibrosis represents a wound-healing process in the liver and a response to acute or chronic hepatic injuries observed in diseases. The increased proliferation of activated hepatic stellate cells (HSCs) is associated with hepatic fibrosis and excessive extracellular matrix (ECM)-protein production. In this study, we isolated the compounds, ankaflavin (AK) and monascin (MS) from *M. purpureus*-fermented metabolites that were not only significantly induced peroxisome proliferator-activated receptor-γ expression and concomitant suppression of ethanol-induced elevation of sterol regulatory element-binding proteins-1 and TG in HepG2 cells but also inhibited hepatic fibrosis *in vitro*. We examined the inhibitory effects of the AK and MS (15 and 30 μM), on the Akt/nuclear factor (NF)-

doi:10.6342/NTU201700799

κB and p38 mitogen-activated protein kinase (MAPK) signaling pathways in HSC-T6 (activated hepatic stellate cell line). AK and MS (30 μM, 30 μM) induced apoptosis and significantly inhibited cell growth (cell viabilities: 80.2 ± 5.4% and 62.8 ± 8.2%, respectively, versus control cells; $P < 0.05$). Apoptosis and G1 phase arrest (G1 phase percentages: 76.1 ± 2.9% and 79.9 ± 1.8% respectively, versus control cells 65.9 ± 4.9%; $P < 0.05$) correlated with increased p53 and p21 levels and caspase 3 activity, and decreased cyclin D1 and Bcl-2-family protein levels ($P < 0.05$, all cases). These findings suggest that *Monascus*-fermented products may represent a novel, protective strategy against alcoholic liver disease by attenuating oxidative stress, inflammatory response, steatosis and inhibit heaptic stellate cell proliferation.

Keywords: alcoholic liver disease; hepatoprotective; *Monascus purpureus*; ankaflavin; monascin

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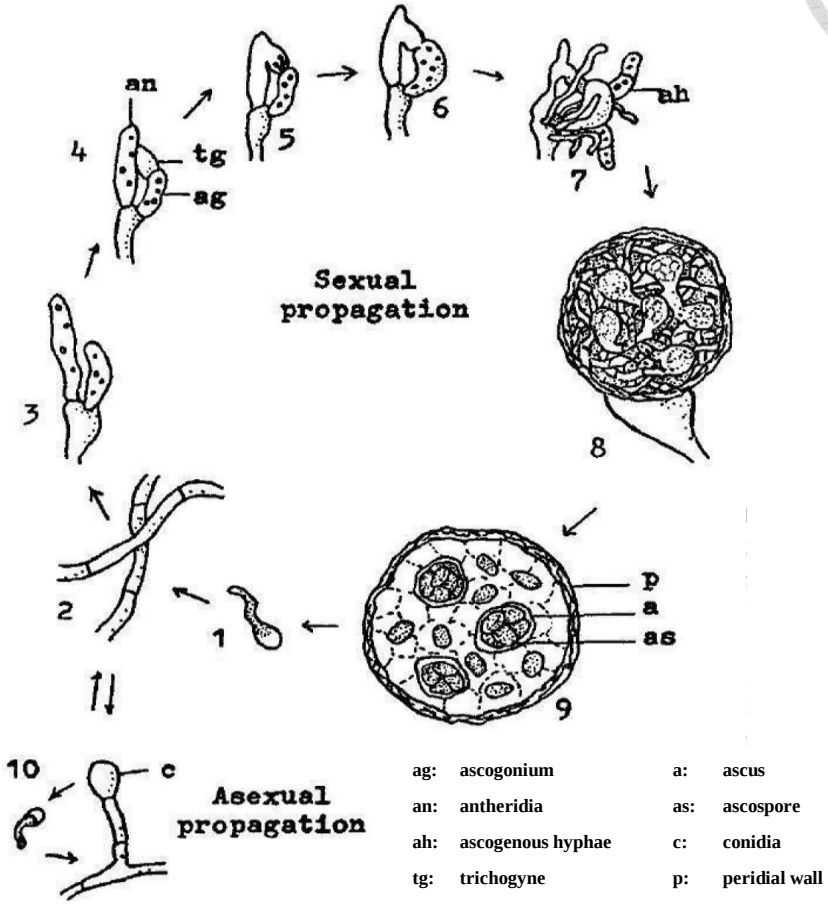
第壹章 文獻回顧

一、 紅麴介紹及製備

紅麴菌應用於飲食、工業、醫藥上已有千年歷史，自北宋朝初期便有相關文獻記載。紅麴菌屬 (*Monascus*) 的研究是由 1884 年法國學者 van Tieghem 分離菌種時開始進行命名。歐洲也以中國紅米 (red Chinese rice) 著稱 (陳及莊，2003)，而紅麴在日本稱為 beni koji 或 anka koji。分類學家將紅麴菌歸類為真菌界 (Fumgi)、子囊菌門 (*Plectomycetes*)、子囊菌綱 (*Ascomycetes*)、不整子囊菌目 (*Plectascales*)。其特徵為菌絲呈無色、褐色或紅色，具有橫隔 (septa)，在其末端會產生一個大型的有性厚壁子囊 (Ascocarp)。其廣泛存在於穀類、河川沉澱物及土壤中，為雌雄同體，並可藉由子囊果進行有性生殖或是由分生胞子進行無性生殖 (圖 1-1)。由於紅麴菌的無性繁殖是採由菌絲頂端往下切割形成鏈狀分生孢子的特異方式，故獨立成為單一屬 (genus) 的紅麴菌科 (*Monascaceae*)、紅麴菌屬，與同一目之麴菌 (*Aspergillus*) 與青黴菌 (*Penicillium*) 有所區別 (林，1994)。目前在文獻上被命名的紅麴菌約有 20 個不同的種名，常見的種名為 *M. pilosus*、*M. purpureus*、*M. ruber*、*M. floridanus*、*M. pallens* 及 *M. sanguineus*。

中國元朝以後紅麴普遍使用，當時的飲膳太醫忽思慧在飲膳正要中記錄紅麴的製法為「選擇土壤為暗紅色的地方，挖一深坑，上下周圍鋪以篾席，將粳米倒入其中，上壓以重石，使其發酵，而變為紅色。經三至四年後，米粒外皮呈紫色，內心亦為紅色，若內心有白點，表示尚未熟透，品質較差」。於明代李時珍所著的本草綱目 (1590 年) 中也記載紅麴主治消食活血，健脾燥胃。明末宋應星在其所著天工開物 (1637 年) 的「丹麴」一節中，除指出製紅麴要選用精白在來米外，其中記載：「其初時雪白色，經一二日成至黑色。黑轉褐，褐轉代赭，赭轉紅，紅極複轉微黃。目擊風中變幻，名曰生黃麴，則其價與入物之力，皆倍于凡麴也」，其中紀載了紅麴培養中色素的產生過程，並且指出黃色素的產生能使紅麴具有更高價值。紅麴是將蒸煮過的米飯接種紅麴菌進行發酵而成的食品。其發酵生長變異大，因生長條件不同造成二次代謝產物有所改變，紅麴菌生長速度緩慢，易受雜菌污染，因此製備過程需注意多個要點：米種的選擇 (以低黏性的米為主，此類米澱粉含量高、營養充足、水分吸收較容易)、優良的菌種以及適當的溫度等。

doi:10.6342/NTU201700799



(蘇，2001)。

圖 1-1 紅麴菌株生長生活史。

(說明) 1-2：子囊孢子形成營養菌絲；3-7：生殖器官形成與造囊菌絲發育；8-9：成熟子囊果；10：分生孢子無性生殖。

Fig. 1-1 The life cycle of *Monascus*.

Description: 1 and 2: Ascospore germinates to form vegetative hyphae; 3 to 7: The formation of reproductive organs and the development of ascogenous hyphae; 8 and 9: Mature cleistothecium; 10: The asexual reproduction of one-celled conidia.

doi:10.6342/NTU201700799

紅麴之製法如下：斜面培養→接種→翻拌→頭水→次水→完水→後熟→乾燥→成品。固態發酵生產紅麴的方式較費人力、時間、佔空間、易污染等，但是生產之產物可以直接利用，所需設備成本較低，可省略分離純化等步驟，且可得到較高產率之二次代謝產物，因此仍為目前製備紅麴的主要方法。

二、 紅麴於食品上的應用

紅麴色素在國內做為食品著色劑已許久，目前已知有八種化學結構被確定出來 (圖 1-2)，可分為紅色素 (monascorubramine 及 rubropunctamine)、橘色素 (monascorubrin 及 rubropunctatin) 及黃色素 (AK、MS、yellow II 及 xanthomonascin A) 三類 (Wild *et al.*, 2002)。許多中外學者的研究報告也指出紅麴色素的安全性極高 (蘇及黃，1981)，因此紅麴色素被認為是安全的食品添加物。在肉製品的加工過程中，添加紅麴萃出物的香腸，在 4^{o}C 真空保存 3 個月，其色素穩定性達 92-98%，且在食品官能品評時有較佳的風味及口感，認為可取代傳統肉製品之亞硝酸鹽或人工色素。而陳等學者 (1997) 亦得到類似的結果，經液體紅糟的浸漬，能賦予豬肉人工色素相當的紅色。已上市的紅麴傳統食品分類如下：(1) 紅麴葡萄酒、(2) 紅麴清酒、(3) 米醋、(4) 高鹽分食品：如醬油、味噌及紅豆腐乳、(5) 肉製品、(6) 麵食、(7) 低膽固醇雞蛋及雞肉、(8) 其他食品：如紅糟製成的食品、紅麴餅乾。(9) 紅麴機能性食品：如紅麴膠囊、紅麴飲料。

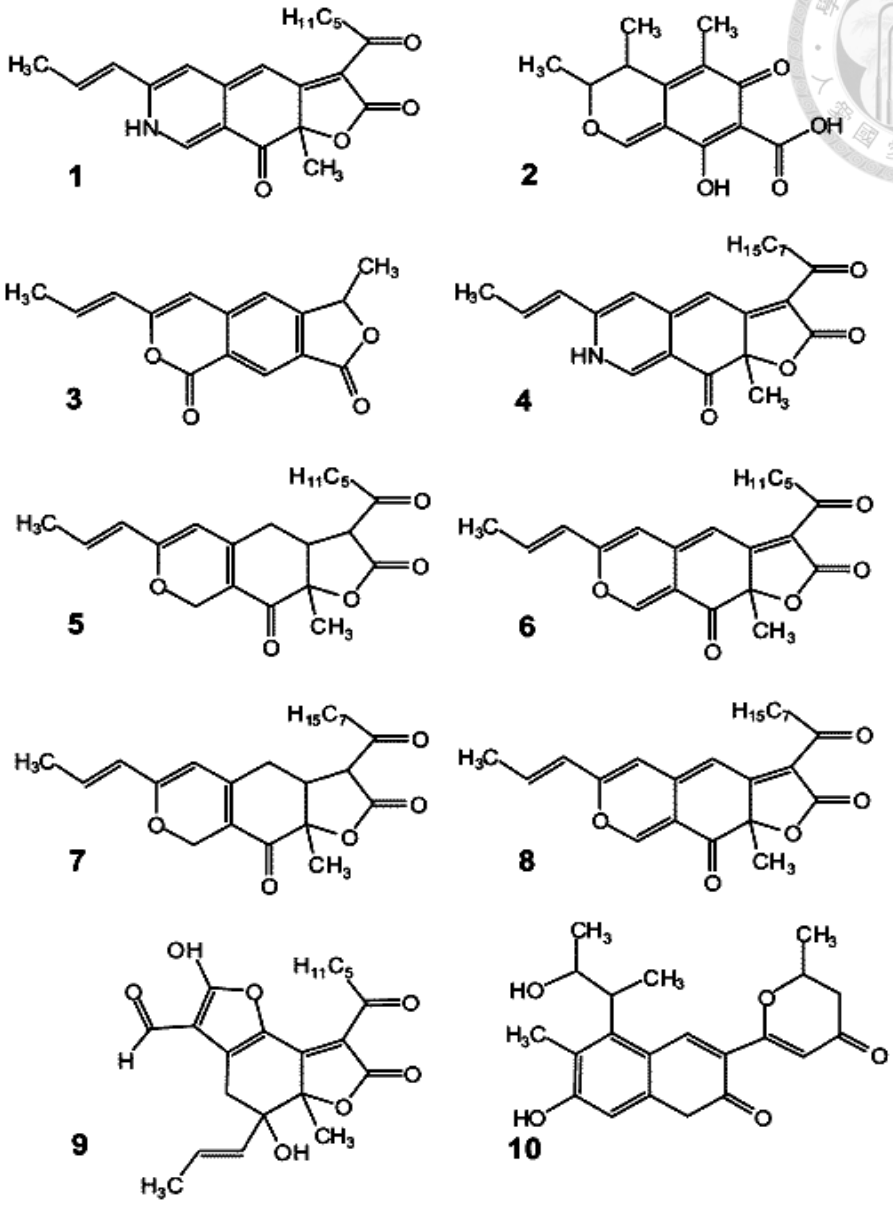
三、 紅麴之保健功效研究

紅麴的應用在過去多以色素生產與傳統食品開發為主，但近年來已有多數之研究指出紅麴的保健功效，包含降血脂、降血壓、抑制腫瘤生成、護肝功能、抗疲勞功效、降血糖、不易形成體脂肪、預防骨質疏鬆、改善酒精性肝損傷與預防阿茲海默症等。其各功效與活性成分之詳細說明如下：

(一) 紅麴菌發酵產物之降膽固醇功效

紅麴 (*Monascus*) 應用於食品在我國已有千年以上歷史，其二級代謝產物 monacolin K (MK) 和 γ-胺基丁酸 (γ-amino butyric acid，GABA) 經證實分別具有降膽固醇和降血壓功效，能有效預防心腦血管疾病 (Su *et al.*, 2003)。以敘利亞倉鼠 (Syrian Hamster) 做為高血脂之實驗動物模式，餵食紅麴變異株 *M.*

doi:10.6342/NTU201700799



(Wild *et al*., 2002)

圖 1-2 紅麴代謝產物-citrinin、monascodilone 及色素結構。

Fig. 1-2 Structures of monascodilone (**3**), citrinin **(2),** and *Monascus* pigments: rubropunctamin (**1**); monascorubramin (**4**); monascin (**5**); rubropunctatin (**6**); ankaflavin (**7**); monascorubrin (**8**); xanthomonasin A (**9**); and monankarin A (**10**).

doi:10.6342/NTU201700799

purpureus NTU 568 所生產紅麴米，進行連續四週的高膽固醇飲食與紅麴米共同餵食，四週以後以二氧化碳犧牲法犧牲，採集肝臟及血液檢測其總膽固醇 (total cholesterol, TC)、三酸甘油酯 (triglyceride, TG)、低密度脂蛋白膽固醇 (low-density lipoprotein cholesterol, LDL-C) 以及視為好的膽固醇之高密度脂蛋白膽固醇 (high-density lipoprotein cholesterol, HDL-C)，結果發現 TC 下降 31.2%、TG 下降 30.1%、LDL-C 下降 36.0%；HDL-C 上升 11.5%。顯示紅麴米確實具有降低不好的膽固醇、提升好的膽固醇之功效。為了解紅麴所含 citrinin 是否對肝臟造成傷害，也測定肝指數天門冬胺酸轉胺酶 (aspartate aminotransferase, AST) 及丙胺酸轉胺酶 (alanine aminotransferase, ALT)，結果兩者均無變化。肝切片顯示未受傷害 (Lee *et al.*, 2006b)。探討紅麴山藥降血脂及抗動脈粥狀硬化能力是否比傳統紅麴米較佳。紅麴米及紅麴山藥對倉鼠之餵食劑量為 96 mg/kg/day (1 倍劑量)，該劑量相當於成人每日攝食 1 g 之紅麴發酵產物。經紅麴米及紅麴山藥餵食高脂飲食倉鼠八週後，發現 0.5 倍劑量之紅麴山藥除可避免血清高密度脂蛋白減少外，更可降低血清中之 TC、TG 及 LDL-C 含量，其抑制活性分別為 13.78%、38.74% 及 43.11%，紅麴山藥調節血脂之能力明顯優於紅麴米，該結果係因紅麴山藥 (17.5 mg/g) 較紅麴米 (8.62 mg/g) 含有較多量之 MK、MS 及 AK 所致。除此之外，餵食 1 倍劑量之紅麴山藥更可有效提升肝臟抗氧化酵素 superoxide dismutase (SOD) 及 catalase 活性，並增加血液 total antioxidant status (TAS)，且紅麴發酵過程中，可生產大量抗氧化物質 (包括 dimerumic acid、tannin 及 phenol 等)，顯示紅麴山藥可有效預防高脂飲食所造成之氧化壓力及預防脂質堆積於血管壁上造成動脈粥狀硬化 (Lee *et al.*, 2007a)。

(二) 紅麴菌發酵產物之降血壓功效

高血壓為國人近年來十大死亡原因之一，是心臟冠狀動脈疾病與腦血管病變最重要的危險因素。因此血壓的控制對於中老年人之健康是一重要的預防工作。血壓是血流衝擊血管壁引起的一種壓力，當心臟收縮時血管內壓力較高，此時所測得的血壓稱為收縮壓 (systolic blood pressure, SBP)，心臟舒張時壓力較低，此時所得的血壓稱為舒張壓 (diastolic blood pressure, DBP)。紅麴發酵產物含有降血壓功效物質 GABA，為抑制性神經傳導物質，可引起血管的擴張。使用自發性高血壓大鼠為模式老鼠，以非侵入式血壓機測量大鼠的尾脈搏，分別餵食紅麴米

doi:10.6342/NTU201700799

與紅麴山藥，評估紅麴米與紅麴山藥在短效性 (24 h) 與長效性 (8 週) 降血壓效果。結果無論是在短效性或是長效性餵食紅麴山藥效果皆比紅麴米較佳。在短效性紅麴山藥餵食一倍劑量 (150 mg/kg) 可降低收縮壓 13 mmHg 與舒張壓 19 mmHg，且持續至八小時後仍有效果；餵食八週後紅麴山藥顯著降低收縮壓 27 mmHg 與舒張壓 22 mmHg ($P < 0.05$)，無論是短期 24 小時或長期八週餵食紅麴山藥均有顯著降低其血壓功效，在生化值檢測方面，紅麴山藥不會造成肝、腎代謝負擔，並未顯著造成 AST 及 ALT 濃度上升，且也未造成肌肉、心律影響及體內電解質不平衡 (Wu *et al*., 2009)。

(三) 紅麴菌發酵產物之降血糖功效

玉田英明 (1988) 發現：兔子在進食添加 0.2-0.3% 紅麴發酵產物的飼料後半小時內血糖降低 23-33%，而在一小時之後的血糖量仍比對照組下降了 19-29%，不過其有效成分尚待進一步分析鑑定。降血糖研究發現，以 150 mg/kg 紅麴發酵產物進行大鼠禁食後口服試驗，口服後 90 分鐘之血中葡萄糖值和對照組相比，血糖量下降 19.4%，胰島素量增加 60.2%，C-peptide 增加 63.6%，顯示紅麴萃取物可透過增加胰島素分泌量達到血糖調節功效 (Chang *et al*., 2006; Chen and Liu, 2006)。Statin 類物質可以刺激胰臟細胞分泌胰島素、改善胰島素抗性達到血糖調節效果 (Ishikawa *et al*., 2006)；statins 類化合物也可以促進脂肪細胞分泌細胞激素-adiponectin，增加胰島素敏感度，改善第二型糖尿病患症狀 (Takagi *et al*., 2008)。

(四) 紅麴菌發酵產物之抗疲勞功效

以紅麴菌株 *M. purpureus* NTU 568 所生產紅麴米餵食 16 周齡雄 Wistar 老鼠 28 天後進行游泳試驗，結果高劑量 (5 g/kg 體重) 組與低劑量 (1 g/kg 體重) 組之游泳時間各增加 65.90% 及 33.59%。血乳酸是醣在無氧條件下酵解的產物，醣酵解是短時間激烈運動的主要能源，血乳酸與負荷強度關係密切，另外，血液中尿素氮濃度的改變，導致大腦中神經傳導物質濃度的改變，為造成中樞疲勞的機制，故血乳酸及血尿素氮是判斷運動強度或疲勞程度的重要指標，也可代表運動後疲勞程度與恢復的情形。在游泳前之乳酸值控制組、低劑量組及高劑量組分別為 29.52 ± 1.44 mg/dL、27.72 ± 0.99 mg/dL 及 27.63 ± 1.17 mg/dL，控制

doi:10.6342/NTU201700799

組與高、低劑量組間並無顯著性的差異 (P ＞ 0.05)；游泳後之乳酸各組分別為 45.00 ± 0.90 mg/dL、31.41 ± 1.80 mg/dL 及 28.89 ± 1.62 mg/dL。由乳酸值的上升比率 (控制組 52.44%、低劑量組 13.31%、高劑量組 4.56%) 得知實驗組確實具有減緩及降低因運動後所產生之乳酸的能力。在游泳前之尿態氮值控制組、低劑量組及高劑量組分別為 16.37 ± 1.02 mg/dL、17.26 ± 0.81 mg/dL 及 17.74 ± 0.91 mg/dL，控制組與高、低劑量組間並無顯著性差異 (P ＞ 0.05)；游泳後各組之尿態氮分別為 21.87±0.75 mg/dL、20.33±0.83 mg/dL 及 20.53±1.09 mg/dL。由尿態氮的上升比率 (控制組 33.6%、低劑量組 17.8%、高劑量組 15.7%) 得知實驗組確實具有減緩及降低因運動後所產生之尿態氮的能力 (Wang *et al*., 2006)。

(五) 紅麴發酵產物抑制阿茲海默症之類澱粉樣蛋白沉積與改善記憶學習功效

1. 細胞模式

將 amyloid β-peptide 1-40 (Aβ40) 進行聚合反應後與鼠腎上腺髓質嗜鉻細胞瘤 (PC-12) 細胞共同培養使其產生 Aβ40 累積，再將紅麴米萃取物添加至細胞株培養液觀察 Aβ40 的累積量與神經毒性。在神經細胞毒性方面，*M. purpureus* NTU 568 發酵之紅麴米酒精萃取物 (ethanol extract of red mold rice, RMRE) 中的 MK 可藉由抑制下游產物 geranylgeranyl pyrophosphate (GGpp) 的生成，使 small G-protein 不被活化而抑制 Aβ40 所誘發的發炎反應，RE 568 亦提供有效的抗氧化能力以防止 Aβ40 所引發的氧化壓力。此外，RMRE 所呈現的效果會更勝於單純的 lovastatin 藥物處理，這可能是紅麴米中的 MK 及其他抗氧化物質與抗發炎成分共同呈現的協同效果。此外，RMRE 在 10 μg/mL 與 25 μg/mL 濃度下能夠將 PC-12 細胞存活率由經 Aβ40 處理的 62.1%，分別提升到 88.3% 與 99.2%，並有效抑一氧化氮 (nitric oxide, NO) 與活性氧化物質 (reactive oxygen species, ROS) 等氧化壓力與發炎反應，證實紅麴酒精萃取物有保護細胞不受 Aβ40 神經毒性影響 (Lee *et al*., 2008)。

2. 動物模式

以大鼠腦部輸注 Aβ40 作為阿茲海默症的動物模式，利用莫氏水迷宮與被動迴避學習研究 *M. purpureus* NTU 568 發酵產物對於記憶與學習能力的改善效果，並觀察大鼠犧牲後 Aβ40 在海馬迴 (hippocampus) 與大腦皮質層的累積狀況。而經歷過 28 天腦內注射 Aβ40 大鼠，無論餵食高劑量 (755 mg/kg/day) 或

doi:10.6342/NTU201700799

低劑量 (151 mg/kg/day) 之紅麴發酵產物於莫氏水迷宮與被動迴避學習作業表現上皆能有效改善 Aβ40 誘發的記憶與學習能力缺損，同時能回復 Aβ40 在大鼠大腦皮質層與海馬迴引發的乙醯膽鹼酶 (acetylchlinesterase) 活性，增加 SOD 活性。最後大鼠大腦皮質層與海馬迴切片染色顯示紅麴酒精萃取物能有效降低 Aβ40 在大腦皮質層與海馬迴的累積 (Lee *et al*., 2007c)。

(六) 紅麴萃取物降低體脂肪功效

紅麴米水萃取物、乙醇萃取物與紅麴山藥乙醇萃取物皆具有抑制前脂肪細胞增生之效果，於高劑量 (200 μg/mL) 作用 48 h 後抑制率分別為 22.2%、31.8%、69.1%；在抑制分化方面，紅麴米水萃取物與紅麴山藥水萃取物於高劑量 (200 μg/mL) 抑制率為 42.2% 與 60.3%，紅麴米乙醇萃取物於 100 μg/mL 抑制率為 53.0%，紅麴山藥乙醇萃取物於 50 μg/mL 抑制率為 54.9%，乙醇萃取物於高劑量下會降低細胞貼附性而影響細胞分化。紅麴米及紅麴山藥水萃取物皆可提高 lipolysis 效率，其中以紅麴米之效果較佳，提高效率高達 49%。紅麴山藥乙醇萃取物能抑制脂肪細胞肝素釋放脂蛋白脂解酶 (heparin-releasable lipoprotein lipase, HR-LPL) 活性，抑制率為 23.1%。動物實驗結果顯示，於高油脂飲食中添加 0.4% 及 2.0% (w/w) 紅麴米能顯著減緩體重上升，與對照組相較下降 21.5% 及 30.5%。亦可抑制腎臟及副睪周圍脂肪細胞增大，改善脂肪組織堆積情形。紅麴米可顯著降低體重，其可能因素包括提高腎臟及副睪周圍脂肪組織 lipolysis 效率及減少攝食量。紅麴發酵產物之降低體脂肪功效主要來自於抑制脂肪細胞增大，但其仍具有抑制前脂肪細胞增生及分化之影響，可避免過多的脂肪細胞生成。紅麴發酵產物能夠改善高油脂飲食引起之體脂肪堆積、血脂紊亂及高血胰島素症狀，有潛力發展為不易形成體脂肪之保健食品 (Lee *et al*., 2013b)。

(七) 紅麴代謝產物抑制腫瘤生長及轉移功效

紅麴酒精萃取物 (RMRE) 在小鼠荷瘤 (Lewis lung carcinoma) 細胞模式試驗中，植入小鼠肺癌細胞後開始餵食 RMRE，於第四週與腫瘤對照組比較，餵食 100 或 200 mg/kg RMRE 組，其腫瘤大小相對於腫瘤對照組分別減少 23.6% 與 50.8%。RMRE 中的活性物質 MK 除了抑制腫瘤生長外，並且與另一活性成份 AK 的參與構成加乘效應 (synergistic effect)，而提升腫瘤細胞凋亡的誘發比率。

doi:10.6342/NTU201700799

對於腫瘤誘導的血管新生 (tumor-associated angiogenesis) 作用，MK 抑制血管內皮生長因子 (vascular endothelial growth factor, VEGF) 所誘發的腫瘤侵襲作用 (invasion) 與新血管生成，達到抑制腫瘤肺轉移的發生。顯示紅麴酒精萃出物可有效抑制腫瘤生長及轉移的發生率 (Ho and Pan, 2009)。

(八) 紅麴代謝產物改善糖尿病功效

紅麴山藥對血糖之改善效果較紅麴米及紅麴薏仁為佳，此結果可能是因紅麴山藥含較多量 MS 及 AK 所致 (Lee *et al*., 2006a)。另外，紅麴山藥也可保護 streptozotocin 對胰臟之傷害，進而改善糖尿病大鼠之 insulin 分泌 (Shi and Pan, 2010)。而 MS 也被報告指出具抗氧化之效果以達預防糖尿病之能力 (Shi *et al*., 2012)。MS 及 AK 為過氧化小體增生劑活化接受體 (proliferator activated receptor-γ, PPAR-γ) 促效劑 (agonist) 而能活化 PPAR-γ，也可透過活化核因子-紅血球之 2 相關因子-2 (nuclear factor-erythroid 2 related factor-2, Nrf-2) 改善 methylglyoxal (MG) 所誘導之糖尿病鼠 (Lee *et al*., 2012; Hsu *et al*., 2013a; Hsu *et al*., 2013d)。MS 亦可透過活化 PPAR-γ 及 Nrf-2，進而改善 tumor necrosis factor (TNF-α) 所造成 C2C12 肌肉細胞之胰島素抗性 (Lee *et al*., 2011)。MS 更可透過活化 Nrf-2 來減緩 THP-1 單核球細胞的過度糖化最終產物受器 (receptor for advanced glycation enfprodicts, RAGE) 訊號活化所造成的氧化壓力及發炎細胞激素產生 (Lee *et al*., 2013b)。MS 也可減緩 MG 所引起 Balb/c 小鼠胰臟及 RINm5F 胰腺細胞之 PPAR-γ 磷酸化，且抑制氧化壓力及 Nrf-2 之活化，主要透過蛋白激酶 C (protein kinase C, PKC) 路徑之調節 (Hsu *et al*., 2013b)。此外，MS 及 MK 可改善糖化最終產物 (advanced glycation endproducts, AGEs) 誘導糖尿病 Balb/c 小鼠之胰臟功能 (Hsu *et al*., 2013c)。

(九) 紅麴預防骨質疏鬆症功效

動物在進行卵巢切除手術前後利用微型電腦斷層儀 (microcomputed tomography, micro-CT) 及雙能量 X 射線吸收儀 (dual energy x-ray absorptiometry, DEXA) 測定骨質狀況，結果顯示手術前各組骨質無明顯的差異，手術切除卵巢三個月後即誘導出骨質疏鬆症，經誘導出骨質疏鬆的大鼠在餵食樣品四週後，由 micro-CT 影像定量結果可知，餵食紅麴山藥組的骨質狀況相對於

doi:10.6342/NTU201700799

切除卵巢組有改善且最為顯著。由骨鈣素濃度測定結果可知，山藥與紅麴山藥餵食對骨質合成皆有改善效果。右股骨進行 micro-CT 影像分析結果可觀察出山藥與紅麴山藥餵食對骨質皆有正向的改善 (Chiang *et al*., 2011)。

(十) 紅麴代謝產物改善酒精性脂肪肝功效

由於酒精攝取會造成氧化壓力及促進脂質於肝臟中生合成，進而造成肝損傷。紅麴代謝產物已被發現可清除自由基並減緩氧化壓力，並具改善體脂肪功能，研究也發現其對酒精所造成脂肪肝及肝損傷具改善效應 (Cheng and Pan, 2011)。紅麴發酵代謝產物 dimerumic acid (DMA) 可增加 Nrf2 和谷胺酸半胱胺酸連接酶 (glutamate-cysteine ligase, GCL) 之活性，以抑制羧甲基離胺酸 (carboxymethyl-lysine, CML) 引起肝星狀細胞產生之氧化壓力，進而預防肝細胞纖維化 (Lee *et al*., 2013a)。

(十一) 紅麴發酵產物之抗氧化功效

紅麴的抗氧化能力於 1999 年被 Aniya 等人提出，Aniya 等人進一步純化紅麴抽出物得知其具抗氧化能力的成分為 dimerumic acid。Dimerumic acid 在第一次被報導時指出其為一天然的 siderophore，與 Fe^{3+} 有高度親和力，具有抗氧化的功效。在低濃度時即具有較佳清除 α,α-diphenyl-β-picrylhydrazyl (DPPH) 自由基的能力，並減低活性氧 (active oxygen species)，如超氧陰離子 (superoxide anion, $\cdot O_2^-$) 及氫氧自由基 (hydroxyl radical, ·OH) (Aniya *et al*., 2000)。Dimerumic acid 具有對抗四氯化碳在肝臟中所產生的自由基。Taira 等人於 2002 年提出 dimerumic acid 的抗氧化機制，當含量在 20-200 μM 時可抑制老鼠肝臟微粒體的 NADPH 與 Fe^{2+} 依賴型脂質過氧化 (Fe^{2+}-dependent lipid peroxidation, LPO)。Dimerumic acid 可清除 ·OH、$\cdot O_2^-$、ferryl-Mb、peroxyl radicasl 與對 LPO 的抑制作用，其會提供一個電子給氧化物使本身氧化為 nitroxide radical (Taira *et al*., 2002)。紅麴代謝產物及色素之結構如圖 1-2 所示。

四、紅麴發酵產物

(一) 膽固醇合成抑制劑—monacolins

MK 的別名有 lovastatin、mevinolin、mevacor、MK803、mevinacor 及

doi:10.6342/NTU201700799

mevlor 等，分子式為 $C_{24}H_{36}O_5$，分子量為 404.55，為白色結晶。日本東京農工大學的 Endo 教授在 1979 年首先自紅麴菌 *M. ruber* 培養液中分離出來 (Endo, 1979)。1980 年 Alberts 等人亦從 *Aspergillus terreus* 培養液中發現具有同樣化學結構的物質，並將其命名為 mevinolin (或 lovastatin) (Alberts *et al*., 1980)。1985 年 Endo 教授的研究團隊又發現除 MK 之外，紅麴菌亦可產生 monacolin J、L 等和 MK 結構相似的化學物質，相關化合物之結構式如圖 1-3 所示 (Li *et al*., 2004)。3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) 為膽固醇合成的速率限制酵素。由於 MK 的結構與 HMG-CoA 類似，因此會與 HMG-CoA reductase 競爭而抑制此酵素，使 mevalonic acid 無法生成，而間接無法合成膽固醇，進而達到降低膽固醇的效果。

使細胞內膽固醇的含量下降，會促使 LDL receptor 增加，提高 LDL 的代謝，也進而降低動脈硬化程度。亦有報導指出此種 statin 類藥物，亦具有抗發炎、降低心臟病、中風及糖尿病等之發病機率 (陳，1999)。

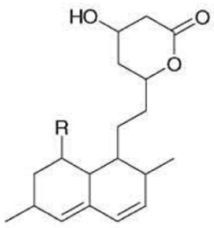
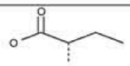
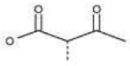
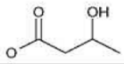
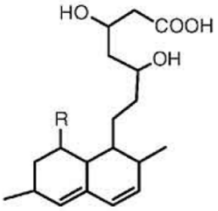
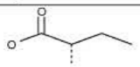
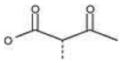
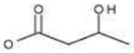
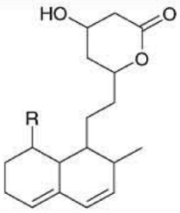
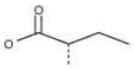
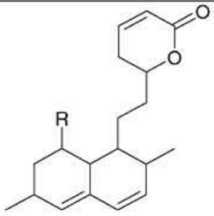
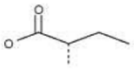
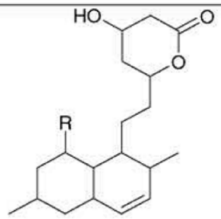
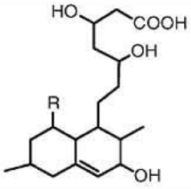
(二) 紅麴色素及 azaphilone

紅麴色素的研究很廣，目前已知有八種化學結構被確定出來，可分為三類：紅色素有 monascorubramine 及 rubropunctamine、橘色素有 monascorubrin 及 rubropunctatin、黃色素有 AK、MS、xanthomonascin A 及 xanthomonascin B，其中紅色素、橘色素及黃色素的 AK 和 MS 屬於 azaphilone 的結構。Azaphilones 是真菌特有的二次代謝物，是指一群含有含酮六圓環以及含氧六圓環的化合物 (pyrone-quinone structures)，並且具有一個四級的對掌中心 (chiral center)。

(三) 抗氧化物-dimerumic acid (DMA)、deferricoprogen (DFC)

紅麴的抗氧化能力於 1999 年被 Aniya 等人提出，紅麴抽出物具清除 α,α-diphenyl-β-picrylhydrazyl (DPPH) 自由基及抗油脂過氧化的能力，並在老鼠實驗中證實可預防肝臟的損傷。Aniya 等人進一步純化紅麴抽出物得知其抗氧化能力的成分為 DMA (Aniya *et al*., 2000)。DMA 及 DFC 的 DPPH 清除能力其 IC_{50} 值分別為 16.4 及 15.1 μg/mL，與 vitamin E 之清除能力相當，其結構式如圖 1-4 所示 (許，2011)。DMA 能夠抑制由 H_2O_2 以氧化壓力模式所誘導的人類大腸癌細胞 (SW620 cell) 侵襲，藉著抑制 AP-1 傳達 MMP-7 基因轉錄，經由

doi:10.6342/NTU201700799

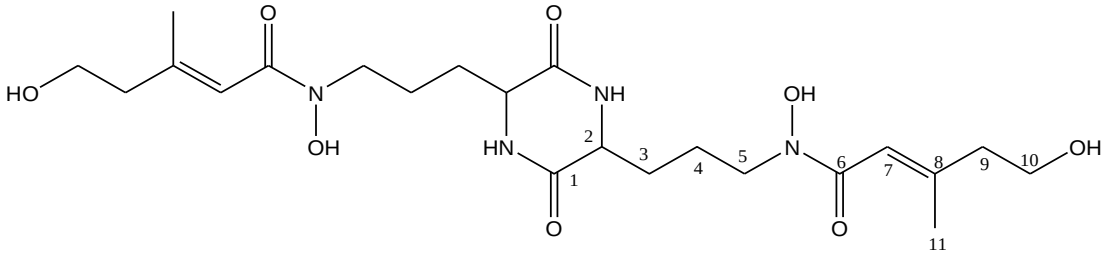
Structure	Name	R	MW
	Monacolin K (MK)		404
	Monacolin J (MJ)	OH	320
	Monacolin L (ML)	H	304
	Monacolin X (MX)		418
	Monacolin M (MM)		406
	MK acid form (MKA)		422
	MJ acid form (MJA)	OH	338
	ML acid form (MLA)	H	322
	MX acid form (MXA)		436
	MM acid form (MMA)		424
	Compactin (P1)		390
	Dehydromonaclin K (DMK)		386
	Dehydromonaclin L (DML)	H	306
	3 α -hydroxy-3,5-dehydromonaclin L (HDML)	H	340

(Li *et al*., 2004)

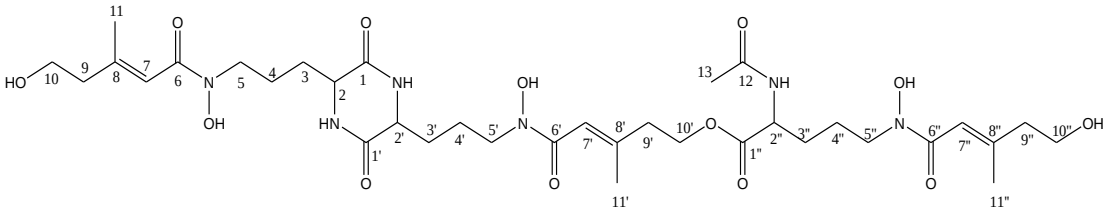
圖 1-3. 紅麴米的 monacolins 結構。

Fig. 1-3. Structural data of monacolins in fermented red mold rice.

doi:10.6342/NTU201700799



Dimerumic acid



Deferricoprogen

(許，2011)

圖 1-4. Dimerumic acid 及 deferricoprogen 之結構。

Fig. 1-4. Structure of dimerumic acid and defferricoprogen.

doi:10.6342/NTU201700799

JNK/c-Jun 及 ERK/c-Fos 的訊號傳遞，DMA 會降低水楊酸 (salicylic acid, SA) 所誘導的肝細胞脂質氧化，在肝細胞中可扮演抗氧化物的角色，藉著抑制氧化壓力的能力而降低其所造成的肝細胞毒性 (Yamashiro *et al*., 2008; Ho *et al*., 2011)。DFC 的 DPPH 自由基清除率相當於維生素 E，也被證實可抑制因橘黴素 (citrinin) 所誘導的人類腎細胞 (HEK-293 cell) 凋亡 (Hsu *et al*., 2012)。

(四) 胺基酸類化合物

γ-胺基丁酸 (γ-amino butyric acid, GABA) 是中樞神經系統主要抑制性的神經傳導物質。飼料中添加 0.2-0.3% 紅麴培養物可使患有先天性高血壓老鼠的血壓由 200 mmHg 以上降至 180 mmHg 以下 (陳，1999)。以 12-O-tetradecanoylphorbol-13-acetate (TPA) 活化 Raji 細胞的 Epstein-Barr virus early antigen (EBV-EA) 實驗，(+)-monascumic acid 和 (-)-monascumic acid 可抑制 TPA 的促癌效果 (Akihisa *et al*., 2005)。

(五) 黴菌毒素- Citrinin

紅麴產生的 citrinin，為一種典型的黴菌毒素，最早是由 *Penicillium citrinum* 培養液中發現。Blanc 等人 (1995) 指出，*M. purpureus* 和 *M. ruber* 會產生一種抗菌物質—citrinin，該物質對腎臟有毒害作用，無論固態培養或液態培養物中都可能發現這種物質。但也發現有些紅麴米產品中未檢測到 citrinin。此一研究引起了各國有關單位的高度重視。但許多學者認為紅麴產品是一種混合物，不能因為其中存在 citrinin 就否定紅麴的正常作用，紅麴中可能存在著某些物質可抵銷 citrinin 對人體的毒害作用。也有人認為雖然紅麴產品中有 citrinin 存在，但其在紅麴中的劑量甚低，在一定的範圍內，人體食用仍是安全的 (許及傅，1998)。Hsu 等 (2012) 發現紅麴代謝產 defferricoprogen 可幾乎去除 citrinin 之毒性 (Hsu *et al*., 2012)。

(六) 紅麴二次代謝產物 monascin 與 ankaflavin 之生理功效

1. 降低體脂肪之功效

以 3T3-L1 前脂肪細胞模式，探討 monascin 與 ankaflavin 對脂肪增生之影響。實驗結果顯示，monascin 與 ankaflavin 可分別降低三酸甘油酯

doi:10.6342/NTU201700799

(triglyceride, TC) 達 37.1% 及 41.1%。此外，monascin 與 ankaflavin 可抑制 peroxisome proliferator-activated receptor γ (PPARγ)、CCAAT/enhancer binding protein β (C/EBPβ)、C/EBPδ 及 C/EBPR mRNA 表現，進而抑制脂肪前驅細胞分化，並且可提升成熟脂肪細胞行脂解作用達原先之 113.2% 及 278.3%。Monascin 及 ankaflavin 可有效抑制 3T3-L1 前脂肪細胞之分化與增生，展現了改善體脂肪之功效 (Jou et al., 2010)。

2. 降血脂之功效

將紅麴山藥 (低劑量：96 mg/kg、中劑量：192 mg/kg 與高劑量：480 mg/kg)、monascin (5.30 mg/kg BW) 與 ankaflavin (0.77 mg/kg BW) 之純物質，分別餵食高血脂之倉鼠 8 週，探討 monascin 與 ankaflavin 是否具有降血脂之功能。實驗結果顯示，monascin 與 ankaflavin 可顯著降低血清中之膽固醇、三酸甘油酯、低密度脂蛋白膽固醇以及動脈脂質斑塊之效果。Monascin 與 ankaflavin 可分別降低血清總膽固醇達 29.9% 及 28.6%，三酸甘油酯則為 63.4% 及 58.2%，且降低低密度脂蛋白膽固醇達 33.9% 及 42.3%，動脈脂質斑塊則可分別下降 40.1% 及 59.6%。除此之外，monacolin K 會降低血清中高密度脂蛋白膽固醇 (high-density lipoprotein cholesterol, HDL-C) 含量，而 monascin 與 ankaflavin 卻可分別使 HDL-C 之含量上升 14.1% 及 17.3%，展現良好之調節血脂能力 (Lee et al., 2010)。

3. 抗動脈粥狀硬化功效 (anti-atherosclerosis)

Monacolin K 長期以來被認為是紅麴降血脂之主要有效成分，monacolin K 亦是一種著名之降血脂藥物 (lovastatin)，但卻有造成肌肉病變 (myopathy) 之可能不良副作用。因先前研究得知 monascin 與 ankaflavin 具有降血脂功能，進一步探討給予倉鼠同劑量 (0.624 mg/kg BW) 之 monascin、ankaflavin 與 monacolin K 並餵食高膽固醇飲食 6 週，對降血脂及抗動脈粥狀硬化之影響。實驗結果顯示，monascin、ankaflavin 與 monacolin K 可分別顯著降低血清及心臟主動脈脂質斑塊中之總膽固醇 24.6%、33.5% 及 39.3% 、降低三酸甘油酯 24.7%、32.4% 及 47.4% 及降低低密度脂蛋白 29.1%、44.1% 及 37.8%。相較於 monacolin K，ankaflavin 對於預防脂肪肝及心臟主動脈脂質堆積具有更顯著

doi:10.6342/NTU201700799

之影響。此外，monascin 與 ankaflavin 可分別提升高密度脂蛋白濃度達 32.3% 及 10.1%。在副作用之探討中，monacolin K 會提升與橫紋肌溶解症發展高度相關的肌酸磷酸激酶 (creatinine phosphokinase, CPK) 之活性，而 monascin 和 ankaflavin 並不會引起 CPK 之提升 (Lee et al., 2013)。

4. 改善糖尿病

Hsu 等人 (2013) 使用甲基乙二醛 (methylglyoxal, MG) 誘導 Wistar 大鼠糖尿病，發現 monascin 可增強 nuclear factor erythroid 2 related factor 2 (Nrf-2) 所調控的抗發炎與抗氧化之能力進而預防由發炎反應所引起的第二型糖尿病。此外，monascin 可做為過氧化物酶體增殖物活化受體 (peroxisome proliferator-activated receptor gamma, PPARγ) 之促效劑 (agonist)，藉由與 PPARγ 受器結合，穩定結構並且抑制其磷酸化，進一步抑制 p-JNK 蛋白質活性，同時活化 AKT/PI3K 路徑，提升胰島素敏感性，而具有改善糖尿病病癥之潛力 (Hsu et al., 2013a)。

Ankaflavin 藉由影響 Nrf-2 而調節肝臟中 glyoxalase 及 HO-1，進而降低由甲基乙二醛所誘導糖尿病之大鼠血清、肝臟和胰臟中的進階醣化終產物 (advanced glycation end-products, AGEs)。此外 ankaflavin 可活化 PPARγ 提升大鼠的胰島素敏感性，故 ankaflavin 具有進一步為開發為減緩糖尿病之機能性食品潛力 (Lee et al., 2012)。

五、山藥之功效研究

(一) 山藥簡介、成分與營養價值

山藥 (*Dioscorea* spp.) 為薯蕷科 (Dioscoreaceae) 薯蕷屬 (*Dioscorea*) 多年生蔓性根莖類植物，英文名為 Yam，又名山藷、長薯、田薯、淮山等 (黃及蔡，1995)。山藥在我國被栽培與利用之歷史極早，如山海經、本草衍義、圖經本草、新修本草、本草綱目及齊民要術等本草古籍均有記載，最早本草藥典神農本草中還將山藥列為上品藥材，不只可供食用還可用於藥用或保健用，具有高產能及富含營養之特色。山藥利用部位為其塊莖，有滋養強壯、促進消化、止瀉、增強免疫力等功效。薯蕷屬植物除了是糧食中獲取澱粉及蛋白質之重要來源外，在傳統

doi:10.6342/NTU201700799

醫藥方面，則為相當重視之藥用植物之一 (劉等，1995；劉等，1999；劉等，2000a)。

研究學者 (李等，1979；劉等，1995) 指出目前台灣山藥之種植栽培面積約為 500 公頃，最常見的品種多以大薯 (田薯) (*D. alata*)、長薯 (家山藥) (*D. batatas*)、山薯 (日本山藥) (*D. japonica* Thunb. var. *pseudojaponica* (Hay.) Hamam.)、紫田薯 (條薯) (*D. alata* L. var. *purpurea* (Roxb.) M. Pouch.)、恆春薯蕷 (戟葉田薯) (*D. doryophora* Hance) 為主。

山藥分佈於台灣的種原約有 14 種及 5 變種 (劉等，1995)，較常見之品種多源自下列六大類:(1) 大薯又稱田薯 (*D. alata*)，為所有山藥中分佈最廣者，於 1991 年命名推廣之本省第一個山藥品種，山藥臺農一號即屬 *D. alata* 之優良品種；(2) 長薯又稱家山藥 (*D. batatas*)，原產大陸，較為耐寒，可供藥用，主要分佈於台灣北部；(3) 山薯又稱日本山藥 (*D. japonica*)，原產日本，現栽培於大陸、日本及東南亞一帶；(4) 條薯又稱紫田薯 [(*D. alata*L. var. *purpurea* (Roxb.) M. Pouch.)]，現栽培於臺灣中部；(5) 恆春山藥又稱戟葉田薯 (*D. doryophora* Hance)，原產於臺灣恆春半島及屏東一帶；(6) 基隆山藥之學名為 *D. japonica* Thunb. var. *pseudojaponica* (Hay.) Yamamoto，通常簡稱為 *D. pseudojaponica*，為臺灣北部原產之特有品種 (劉等，1995；劉等，2000c)。

山藥塊莖一般成份隨品種不同及種植地區不同而有所差異，一般水分含量在 65-77%，澱粉約佔乾重 75-84%，粗蛋白在 6-8% 之間，粗纖維為 1.2-1.8%，粗灰份在 2.8-3.8% 之間 (Wanasundera and Ravindran, 1994)。

山藥除了可做為糧食中澱粉與蛋白質之重要供應源外，尚含有維生素 A、C、E、B_1、B_2 及磷 (P)、鈣 (Ca)、鉀 (K)、鎂 (Mg) 等礦物質和微量元素鐵 (Fe)、鋅 (Zn)、鍶 (Sr)，此外還含有膽鹼 (choline)、醣蛋白 (高黏性複合多醣，glycoproteins)、黏液質 (mucin)、澱粉酵素 (diastase)、多酚氧化酵素 (polyphenol oxidase)、薯蕷皂苷配基 (diosgenin)、多種必需胺基酸及抗氧化酵素等成分 (劉等，1995；王等，1999；潘，2000；Wanasundera and Ravindran, 1994)。

山藥塊莖特具黏質多醣類 (viscous polysaccharides)，此成分主要含碳水化合物 (carbohydrates)、甘露糖 (mannose)、阿拉伯膠糖 (arabinose)、葡萄糖 (glucose)、半乳糖 (galactose) 及少量之木糖 (xylose) 與鼠李糖 (rhamnose)，以及蛋白質等，

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而黏質多醣類含量之多寡，以及其內含物分子間相互作用之強弱，均會影響到塊莖肉質黏度大小 (林等，1998；劉等，2000b)。

(二) 山藥生理機能

特殊的山藥品種如 *D. colettii* 等含有薯蕷皂苷配基 (diosgenin)，是一種固醇類皂素配質 (steroidal sapogenins)，可做為合成固醇類荷爾蒙 (steroidal hormones)、醫療用類固醇 (medicinal steroids) 及生產口服避孕藥、腎上腺皮質賀爾蒙等多種藥物的原料 (劉等，1994；劉等，1995)。劉等 (2000a) 曾述及山藥在歷代本草中都視為補虛佳品，神農本草經等本草典籍謂其性平、涼潤、味甘而無毒，能健脾胃、補肺腎，主治久痢、消渴、虛勞、去痰等功能，民間多用為滋養強壯藥。近年來，國內外也有醫藥臨床實驗證實，山藥確有增進食慾、改善人體消化功能及增強體質等多種功能，其中 diosgenin 等成分具有增強免疫功能、抗關節炎、抗腫瘤之作用，可促進干擾素之誘生與 T 淋巴球細胞數之增生 (劉等，2000a)。

六、酒精性肝損傷

(一) 酒精性肝損傷的臨床病徵

過去的研究顯示重度酒精使用患者 (平均攝入量，約 100 克/天)，其酒精性肝損傷的臨床綜合徵狀為黃疸和肝衰竭 (Naveau *et al.*, 1997)。雖然酒精性肝損傷中女性較男性有更高的風險，但實際上男性飲酒過量的情況更為嚴重，因此患有酒精性肝病者男性多於女性，同時研究顯示飲用酒的種類並不會影響酒精性肝損傷的風險。酒精性肝損傷主要症狀是黃疸的產生，其他常見症狀包括發燒、腹水、肝臟腫大病變和肌肉的流失，而重度的酒精性肝損傷患者可能產生腦病變。實驗室研究顯示，酒精性肝損傷的患者其血清中 AST 是正常值兩倍以上，而血清中 ALT 含量較低。AST 與 ALT 的比例通常大於 2，酒精會導致肝臟中 ALT 活性降低 (Sidhu *et al.*, 2017)，同時造成肝臟的 pyridoxal 5'-phosphate 下降並升高肝臟細胞中 mitochondrial aspartate (Matloff *et al.*,1980)。同時酒精導致周邊血液白血球、嗜中性白血球、總血清膽紅素、血清肌酐和凝血時間升高。這些數值的升高與肝腎疾病具相關性 (Mutimer *et al.*, 1993)。酒精性肝損傷患者肝臟切片顯示，酒精會造成肝臟細胞損傷並腫脹，此種肝臟細胞稱為 Mallory bodies，其周

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邊聚集嗜中性白血球 (MacSween and Burt, 1986)。這些病徵在酒精性肝損傷中常見，其肝臟細胞腫大累積脂肪並造成脂肪肝疾病，並且造成肝臟星狀細胞的活化產生肝臟纖維化，提高肝硬化及肝癌的發生率。非酒精性脂肪性肝損傷 (nonalcoholic steatohepatitis)，與肥胖和胰島素阻抗相關的疾病，此疾病許多組織學結果與酒精性肝損傷相似，包括肝臟細胞腫大、脂肪肝、mallory bodies、發炎反應、纖維性膠原蛋白累積、纖維化及肝硬化。但是酒精性肝損傷其嚴重性更大於非酒精性脂肪性肝損傷。

(二) 酒精性肝損傷的診斷

臨床診斷中，酒精性肝損傷患者其血清中 AST 含量升高 (但小於 300 IU/mL)，與 ALT 的比例大於 2，總血清膽紅素含量超過 5 毫克/毫公升 （86 μmol/L），凝血時間 INR (international normalized ratio) 升高並且造成嗜中性白血球增多 (Sidhu *et al*., 2017)。造成肝臟損傷的途徑包括非酒精性脂肪性肝損傷、急性或慢性的病毒性肝損傷、藥物導致的肝損傷、自體免疫性肝疾病和肝癌相關等等疾病 (Lucey *et al*., 2008)。因此酒精性肝損傷的鑑別診斷，可由肝臟組織切片檢查結果可以證實病徵，且可幫助排除其他原因的肝臟疾病，經頸靜脈檢查可降低出血的風險，但肝臟組織切片檢查不是必要性診斷。患者診斷亦應利用血液、尿液和腹水培養、或是胸部 X 光攝影來排除細菌感染的可能性，如肺炎、自發性細菌腹膜炎和尿道感染。肝臟超音波的檢查有助於辨別肝膿腫、肝癌和膽道阻塞。肝臟超音波也可與腹水培養結合，並且配合都普勒血流檢查，因為肝動脈升高的收縮速度或直徑的增加都有助於確認及診斷 (Han *et al*., 2002)。

(三) 酒精性肝損傷的相關分子機制

酒精在肝臟細胞中通過代謝氧化成乙醛，隨後從乙醛氧化至乙酸酯。酒精在肝臟中的氧化代謝，主要是利用 NADH 的形式。NADH 及 NAD^+ 其氧化還原電位反應在肝臟中進行，此反應會抑制脂肪酸氧化、三羧酸循環 (tricarboxylic acid cycle) 並促進脂質合成 (You and Crabb, 2004b)。此外，酒精抑制 peroxisome-proliferator–activated receptor α (PPAR-α)、AMP kinase 並刺激轉錄因子 sterol regulatory element-binding protein 1 (SREBP-1) 促進脂質生合成 (Ji *et al*., 2006)。綜合以上機制，導致肝臟內脂肪的累積。經由了解酒精性肝損傷的發病機制，以

doi:10.6342/NTU201700799

及利用酒精誘導肝損傷的動物模式，有助於開發新的治療方法。許多研究使用管餵大鼠或小鼠酒精和脂肪 (Tsukamoto *et al.*, 1984)，使其導致肝臟輕度酒精性肝損傷的病變，但此模式誘導肝臟纖維化的情況較不容易產生 (Uesugi *et al.*, 2001)。內毒素脂多醣 (lipopolysaccharide, LPS) 為革蘭氏陰性菌外壁成分，是酒精性肝臟發炎的關鍵因子。長期吸收酒精會導致腸道滲透性上升，造成內毒素由腸腔進入肝門靜脈血液，而造成持續性的發炎反應 (Wiest and Garcia-Tsao, 2005)。在酒精性肝損傷的動物模式中，利用抗生素清潔腸道菌群或是乳酸菌重新定殖於腸道，都可能減緩腸道內增加的內毒素，並且可以減緩酒精性肝損傷 (Uesugi *et al.*, 2001)。同樣，在人類酒精性肝損傷患者中，酒精的吸收也會造成腸道滲透性升高，而使內毒素增加並產生發炎反應 (Urbaschek *et al.*, 2001)。在酒精性肝損傷患者體內，其腸道內毒素進入肝門靜脈血液時，與 LPS-binding protein 結合造成發炎反應。LPS-LPS-binding protein 複合物與肝臟中 Kupffer cells 細胞膜上的 CD14 受體結合。Kupffer cells 為肝臟中的免疫細胞，對於酒精的發炎反應至關重要。酒精性肝損傷的模式中 Kupffer cells 經由內毒素造成活化需要三種細胞蛋白質的協助：CD14 (單核球分化抗原)、toll-like receptor 4 (TLR4) 及 MD2 蛋白可協助 TLR4 與 LPS-LPS-binding protein 結合 (Akira *et al.*, 2001)。TLR4 分子機制傳導的下游途徑包括活化轉錄因子 early growth response 1 (EGR1)、NF-κb 和 TLR4 配體 (toll-interleukin-1-receptor domain-containing adapter-inducing interferon-beta, TRIF) (Zhao *et al.*, 2008)。酒精性肝損傷小鼠模式中，EGR1 經由 LPS 刺激活化後可促進 TNF-α 細胞激素產生，若將其阻斷可防止酒精誘導的肝損傷。在酒精性肝損傷的患者中，其氧化壓力會因酒精的攝取而升高，動物模式中短期或長期攝取酒精的大鼠和小鼠，會產生大量的自由基造成 Kupffer cells 及肝星狀細胞的活化 (Bailey and Cunningham, 1998)。於肝臟細胞中，酒精誘導的氧化壓力造成 cytochrome P-450 2E1 活化導致粒線體損傷、誘導內質網細胞凋亡產生並促進脂質合成 (Lu *et al.*, 2008)。活化的 Kupffer cells 產生 TNF-α，為導致酒精性肝損傷的關鍵細胞激素。在酒精性肝損傷的動物模式研究中，若是剔除 TNF receptor 1 （TNF-R1）、使用 TNF-α 抗體或 thalidomide 處理 （其減少 TNF-α 的產生），可以有效的降低肝臟發炎的反應 (Enomoto *et al.*, 2002)。酒精的攝取導致粒線體 cytochrome c 及 Fas ligand 蛋白的產生，導致 caspase-3 活化造成肝臟細胞凋亡。此外，TNF-α 和 Fas 蛋白的協同作用促使細胞凋亡，

doi:10.6342/NTU201700799

使得肝臟細胞損傷更嚴重，同時導致肝臟中自然殺手 Tcell 活化引發免疫反應，過度的免疫發炎反應造成肝臟星狀細胞的活化，造成肝臟纖維化病變 (Minagawa *et al*., 2004)。

(四) 酒精性肝損傷的治療方法

1. 禁止酒精攝取

禁止酒精攝取為防止酒精造成的肝臟發炎主要方法。可利用 naltrexone 藥物減少患者對酒精的攝取，naltrexone 其主要藥理學作用為阻斷 opioid receptor，naltrexone 也會改變下視丘、腦垂體及腎上腺軸抑制對於乙醇的消耗。Naltrexone 的通常劑量為 50 mg/day (Jonas *et al*., 2014)。經由分析酒精依賴的臨床試驗發現 naltrexone 組別與安慰劑組別相比可有效減少酒精消耗。Acamprosate 這種新藥的結構與抑制性神經物質傳遞的 γ-氨基丁酸 (γ-aminobutyric acid, GABA) 具有相似性，臨床顯示可減少對酒精的渴望及酒精的戒斷症狀 (Mason, 2015)。而 baclofen (γ-GABA-β receptor agonist) 藥物的使用，在飲酒過量的患者中，具有更高的安全性，且可以長期的減低對酒精攝取慾望，並且改善肝臟功能減緩酒精性的損傷 (Frazier *et al*., 2011)。

2. 類固醇

類固醇 (corticosteroid, CS) 為最廣泛的治療藥物，其主要功能為抑制發炎反應，其抑制轉錄因子如 activator protein 1 （AP-1） 和 NF-κB，並且達到減少血清中 interleukin-8 及 TNF-α 等促發炎細胞激素，減低酒精誘導的發炎反應 (Barnes and Karin, 1997)。其標準療程為口服 prednisolone 藥物每天 40 mg 或是每天靜脈注射 32 mg 的 methylprednisolone 藥物為期四周 (Sidhu *et al*., 2017)。但使用類固醇 (prednisolone) 治療酒精性肝損傷一直有爭議，因為治療效果並不是相當有效 (Lucey *et al*., 2009)。

3. 抗 TNF-α 治療

目前有兩種抗 TNF-α 藥劑的酒精性肝損傷之療法：infliximab 及 etanercept。而 etanercept 可增加酒精性肝損傷患者的存活率，安慰劑與對照組試驗研究顯示存活率相差 6 個月 (Boetticher *et al*., 2008)。

4. 抗氧化劑治療

N-Acetylcysteine (NAC):在最近的隨機臨床試驗中對 174 例酒精性肝損傷的

doi:10.6342/NTU201700799

患者進行分析，使用 NAC 與 CS 併用組別其效果與單獨 CS 相比，可改善患者一個月的存活時間，並且改善傷口化膿的病症 (Nguyen-khac *et al*., 2011)。研究也指出 metadoxine 與 glucocorticoid 共用可改善酒精性肝損傷患者的生存率和發展減少腦病變和肝腎病癥 (Higuera-de la Tijera *et al*., 2014)。

5. 營養的補充

酒精性肝損傷患者都有顯著蛋白質與熱量的營養不良，以及許多維生素和微量礦物質的缺乏，並且營養不良的程度與死亡風險密切相關。建議可口服營養素並且達到最低熱量標準為每天 21.5 kcal / kg (Sidhu *et al*., 2017)。於臨床實驗中，若是可以改善營養狀況，其治療效果與使用類固醇一樣有效 (Cabre *et al*., 2000)。

七、肝臟纖維化

肝纖維化 (hepatic fibrosis) 形成原因主要為慢性的肝臟損傷如：病毒引起的肝損傷 (hepatitis B and C)、酒精濫用、藥物、代謝疾病與自體免疫疾病等 (Friedman, 2000)。纖維化在肝臟中是一個修補的機制，當肝臟受到傷害時，會有一些訊號啟動組織中的修補細胞大量分泌細胞外間質及膠原蛋白 (collagen) 等，來填補這些受傷的部位 (Olaso and Friedman, 1998)。肝纖維化是一種可逆的現象，在還沒有結節的產生，若能移除這些傷害的物質，肝臟本身能將纖維化的情形修復，如果傷害因素無法排除則肝纖維化無法停止，同時纖維性的胞外間質 (extracellular matrix, ECM) 沉積，產生過多的纖維化妨礙肝細胞 (hepatocyte) 正常的運作，無法停止的纖維化也造成結節的堆積，最後導致肝臟硬化的現象，一旦進入了肝硬化時期，則為一個不可逆的現象，進而有機會轉變成肝癌 (Shimizu, 2000)。

(一) 肝星狀細胞

肝臟內的細胞分為實質細胞 (parenchymal cells) 及非實質細胞 (non-parenchymal cells)。肝臟實質細胞主要為肝細胞，而肝星狀細胞約佔肝臟中細胞總數的 15% (Friedman, 2000)。肝星狀細胞 (hepatic stellate cell, HSC) 又稱為 ito cell、lipocyte、perisinusoidal cell 或 fat-storing cell (Iredale *et al*., 1998) 等。星狀細胞在正常肝臟中所扮演的重要功能，包括有：(1) retinoids 的代謝，肝星狀細胞貯存脂溶性維生素 A (retinoids) (Senoo et al. 1998)；(2) 細胞激素 (cytokine) 的

doi:10.6342/NTU201700799

分泌，例如：表皮生長因子 (epidermal growth factor)、肝細胞生長因子 (hepatocytes growth factor) 等，這些因子可以協助維持肝細胞的質與量；(3) 調控血流，由於星狀細胞所在的位置接近神經，故星狀細胞可能受到神經的刺激而收縮 (Friedman, 1996)。

(二) 活化之肝星狀細胞

肝星狀細胞位於血管內皮細胞層 (endothelial cell layer) 和肝實質細胞層 (hepatocyte layer) 之間的迪氏腔 (Disse˙s space)，主要的功能有儲存脂肪、脂溶性維生素 A (retinoids) 及分泌細胞外間質和膠原蛋白。星狀細胞原本是處於未活化的狀態 (quiescent state)，而當肝臟受到外在壓力 (如：氧化作用、自由基攻擊、酗酒、病毒感染-B、C 型肝炎、藥物中毒…等) 的迫害時，會刺激肝星狀細胞的活化而大量增殖 (proliferation)，除了在細胞外型上有著明顯的收縮 (Li *et al.*, 2008)，而在受到外在壓力或毒物對肝臟造成的傷害，也造成肝臟中微環境的改變，如：肝細胞之像手指狀微絨毛的消失 (loss of hepatocyte microvilli)，相連之孔狀內皮細胞 (fenestrated endothelial cells) 之間的孔隙消失 (Friedman, 2000)，庫氏細胞 (kupffer cell) 與內皮細胞活化並釋放細胞激素，肝星狀細胞因此而被活化，並流失大量維生素 A 成為活化態的肝星狀細胞 (activated hepatic stellate cells)。肝星狀細胞的活化可分為兩階段，包括：初始期 (initiation) 與永存期 (perpetuation) (Friedman, 2000)。在起始期的肝星狀細胞，主要為開始反應出對於細胞激素或其他刺激物造成的基因表現或特徵的改變；永存期則是持續表現活化才產生之特徵。在正常肝臟中，肝星狀細胞所產生的細胞外間質為不具纖維狀的膠原蛋白，如：第 IV、VI 型膠原蛋白 (type IV, VI collegan)。然而，被活化的肝星狀細胞，則會產生纖維狀的膠原蛋白，如：第 I、III 型膠原蛋白 (type I, III collegan)，而這些第 I、III 型膠原蛋白是造成肝纖維化的主要成分之一 (Friedman, 1993)。

1. 初始期 (Initiation)

初期星狀細胞受到周圍細胞的旁分泌 (paracrine) 影響，如 kupffer cell、淋巴球、白血球細胞、肝細胞和網狀內皮細胞等多種細胞產生的激素交互作用促使星狀細胞活化造成肝纖維化，而 transforming growth factor-β1 (TGF-β)、tumor necrosis factor (TNF)、matrix metalloproteinase (MMP-9) 以及 interleukin (IL-2、

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4、5、6)、interferon-γ (IFN-γ) (Shi *et al.*, 1997) 等細胞激素會造成星狀細胞活化並大量的增生、表現 α-SMA、釋放 retinoids 並生成累積纖維性膠原蛋白 (collagen) type-1、3 (Li *et al.*, 2008)。另一方面當肝細胞受到損傷時促使活性氧化物產生，使肝星狀細胞活化後，其 cytochrome P450 2E1 (CYP450 2E1) 會過量表現 (Nieto *et al.*, 1999)，而產生更多活性氧化物來刺激第 I 型膠原蛋白基因表現及減弱抗氧化劑的作用，即為自體分泌的運作。因此，若有抗氧化劑的存在，便能阻斷星狀細胞的活化過程。在初期的肝損傷時，網狀內皮細胞 (sinusoidal endothelial cells) 產生的纖維結合蛋白 (fibronectin) 會活化肝星狀細胞，血小板也會生成 platelet-derived growth factor (PDGF)、epidermal growth factor (EGF) 而刺激肝星狀細胞活化 (Friedman, 1999)。星狀細胞也具有 toll-like receptor-4 (TLR-4) 為先天性免疫反應的受器，因此若有配體 (ligand) 接上受器如 lipopolysaccharide (LPS) 則會使 nuclear factor κB (NFκB) 轉錄因子活化，使星狀細胞活化 (Schnabl *et al.*, 2008)。

2. 永存期 (perpetuation)

持續性的旁分泌刺激作用 使得初始期的活化肝星狀細胞進入永存期，此時期自體分泌 (autocrine) 產生的細胞激素對於肝星狀細胞活化相當重要，如：TGF-β1、PDGF、fibroblast growth factor (FGF) 和產生 monocyte chemotactic protein-1 (MCP-1) 及 cytokine-induced neutrophil chemoattractant/IL-8 等細胞激素導致更嚴重的發炎反應 (Marra *et al.*, 1998)。在此時期細胞間質發生成份改變，約有比平常時期高六倍量的纖維性的細胞間質大量產生堆積，如：collagens (I, III, 及 IV)、fibronectin、undulin、elastin、laminin、hyaluronan 及 proteoglycans 等 (Schuppan *et al.*, 1990)，這些纖維性的細胞間質也同時更加強了肝星狀細胞的活化。肝星狀細胞的活化屬於一種修復傷害的機制，當傷害停止肝臟會產生訊號停止肝星狀細胞的活化，如：hepatocyte growth factor (HGF)，而星狀細胞本身也會產生 IL-10 來抑制發炎反應 (Bataller and Brenner, 2005)。因此若是持續性的外來傷害，則會導致肝星狀細胞的調控作用失衡，促使肝纖維化越來越嚴重，而有機會轉變為不可逆的肝硬化、肝癌。

doi:10.6342/NTU201700799

第貳章 研究動機與目的

根據世界衛生組織 (World Health Organization, WHO) 報告指出，酒精的濫用及損害是全球死亡及疾病的主要因子。全球死亡人數中，因酒精濫用造成的傷亡約占 4%，遠超過愛滋病、結核病或是暴力行為所造成的死亡人口，而酒精濫用更會導致 200 種以上的疾病發生。長期酒精攝取造成的損傷，為非常複雜的生化及病理學上的傷害，在酒精肝損傷的發病機制中，長期的氧化壓力和發炎反應的作用扮演重要的角色 (Vidali *et al*., 2008)，其致病機制包括 Kupffer 細胞的活化，產生促發炎細胞激素、趨化因子及自由基，並且與酒精代謝產物、肝臟細胞，細胞激素和免疫系統相互反應 (Arteel, 2003)。而其中受影響最深的器官就是肝臟，因其負責主要的酒精代謝過程，長期攝取酒精所造成的肝臟損傷為脂肪肝、酒精性肝炎、肝硬化等，若不即時治療更會引起肝癌 (Williams, 2006)。脂肪肝主要是因為肝臟中發生脂肪堆積的現象，持續數天的酒精傷害就會使肝臟的氧化壓力增加 (Nordmann *et al*., 1992)，肝臟氧化壓力升高會導致脂肪變性，造成三酸甘油酯微粒累積於肝臟細胞中，並且會造成肝臟細胞死亡而產生酒精性肝炎，引起肝臟廣泛發炎的現象，同時刺激肝臟星狀細胞由休止狀態轉為活化態，分泌並堆積大量細胞外間質，導致死亡的肝臟細胞形成結疤組織，若發炎反應持續使得結疤組織繼續形成，則約有 40% 導致不可修復的肝硬化疾病產生 (Lucey *et al*., 2009)，因此，抑制星狀細胞的活化是治療肝纖維化及避免肝硬化的關鍵步驟。雖然肝硬化疾病只發生在一部分重度飲酒者群體中，但肝硬化的風險與飲用量成比例地增加。每天飲用多於 30 g 酒精就會增加 1% 肝硬化的風險，然而每天飲用超過 120 g 酒精則會達到 5.7% 疾病發生風險 (Bellentani *et al*., 1997)。由於有高達 40% 的患者有嚴重酒精性肝損傷，在臨床綜合徵發病後 6 個月內死亡，另外，肝臟功能發生異常會間接導致其他併發症，如：腎功能的衰退、心血管疾病以及中樞神經系統異常等，這些併發症將會提高病人因長期攝取酒精之死亡率，因此適當診斷和保健治療至關重要 (Lucey *et al*., 2009)。

本研究室先前研究結果顯示，紅麴發酵產物具有多種保健功效，例如調節血脂、預防阿茲海默症、不易形成體脂肪、護肝、調節血糖、抗疲勞及調節血壓等 (Lee *et al*., 2007a; Wu *et al*., 2009; Jou *et al*., 2010; Shi and Pan, 2010)。同時，*M. purpureus* NTU 568 發酵的紅麴米及紅麴山藥經由實驗證實不具有毒性 (Hsu et

doi:10.6342/NTU201700799

al. 2014)，並且具有多種功能性代謝產物 GABA、DMA、及 MK 等 (Lee *et al*., 2010; Chuang *et al*., 2011; Ho *et al*., 2011)

本研究以 *M. purpureus* NTU 568 發酵的紅麴米及紅麴山藥粉末進行實驗，利用米與山藥為主要基質進行發酵。發酵後經乾燥磨粉，動物實驗模式利用液體酒精飼料 (Lieber-DeCarli liquid diet) 模式誘導小鼠形成酒精性肝損傷，並確認其肝臟保護之功效，此方法之優點包括：動物容易取得、流質飲食容易製備、實驗期短，僅需 5 週即可誘發小鼠形成慢性酒精性脂肪肝，而且動物能夠自由進食，符合一般生理狀況。然而，此動物模式之缺點有二：(1) 所誘導的小鼠酒精性脂肪肝與人類酒精性脂肪肝比較之下，小鼠的肝臟脂肪堆積屬於小油滴型，而人類則是屬於大油滴的堆積方式。(2) 無法誘發小鼠發生肝纖維化之發生，因此，無法使用此動物模式進行肝纖維化以及肝硬化之相關研究。因此我們利用體外細胞模式 (HSC-T6 cell line) 進行肝臟纖維化的分析。同時，因發酵基質不同，造成發酵產物成分比例不同，進行紅麴米及紅麴山藥的功效評估比較，並且純化發酵代謝產物中保護肝臟細胞之生物活性物質 (AK 及 MS)，此兩種物質具有抗發炎及抗氧化的功能 (Shi *et al.*, 2012)，經過純化後利用人類肝臟細胞株（HepG2 cell line) 進行酒精誘導肝臟損傷細胞模式。同時進一步研究 AK 及 MS 對肝臟纖維化的保護功能，利用細胞實驗進行分子機制探討 (圖 2-1)，期望開發保健功效及具有實證依據的保健食品。

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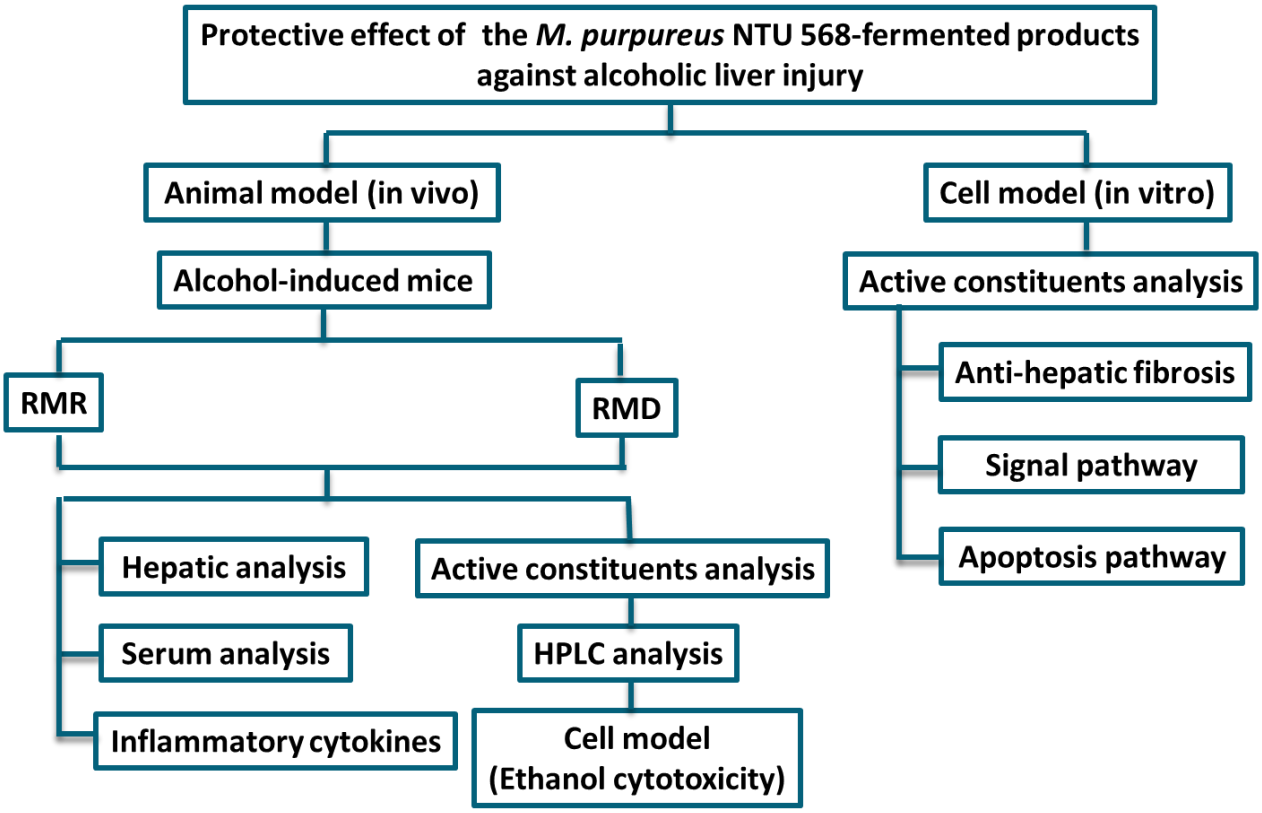

圖 2-1、本論文之研究大綱。

Figure 2-1. The framework of this study.

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第參章 材料與方法

一、儀器及藥品

(一) 藥品

1. 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP)、5,5-dithiobis[2-nitrobenzoic acid] (DTNB)、(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)、Bay117082 (inhibitor of NF-κB)、Bovine serum albumin (BSA)、deoxyribonuclease 1 (DNase 1)、dimethyl sulfoxide (DMSO)、ethylenediaminetetraacetic acid (EDTA)、fetal bovine serum (FBS)、glutathione (GSH)、glutathione reductase (GRd)、glutathione disulfide (GSSG)、LY2940022 (inhibitor of phosphoinositide 3-kinase; inhibitor of PI3K)、$MgCl_2 \cdot 6H_2O$、NaN_3、nicotinamide adenine dinucleotide phosphate (NADPH)、propidium iodide (PI)、sodium chloride (NaCl)、sodium hydroxide (NaOH)、sodium dihydrogen phosphate (NaH_2PO_4)、potassium dihydrogen phosphate (KH_2PO_4)、sodium dodecyl sulfate (SDS)、trypsin、SB203580 (inhibitor of p38 MAPK)、Tween 20 及 Triton-X 100 購自 Sigma 公司 (Sigma Co., St Louis, MO, USA)
2. Anti-p53 antibody、anti-21 antibody、anti-cyclin D1 antibody、anti-extracellular signal-regulated kinases (ERK) antibody、anti-c-Jun amino-terminal kinases (JNK) antibody、anti-p-JNK antibody、anti-NF-κB antibody 及 anti-NF-κB inhibitor of kappa B (IκB) antibody 購自 Abcam Ltd. (Cambridge, UK)
3. Anti-p-ERK antibody、anti-Akt antibody、anti-p-Akt antibody、anti-p38 antibody、anti-p-p38 antibody、anti-Bax antibody 及 anti-Bcl-2 antibody 購自 Cell Signaling Technology, Inc. 公司 (Beverly, MA, USA)
4. Anti-β-actin antibodies 購自 Proteintech Group, Inc. 公司 (Chicago, IL, USA)
5. Aspartate aminotransferase (AST) 及 alanine aminotransferase (ALT) assay kits 購自 BioQuant 公司 (San Diego, CA, USA)
6. F12-K medium 及 Dulbecco's Modified Eagle Medium (DMEM) 培養基購自 HyClone Laboratories 公司 (Logan, UT, USA)
7. FFA assay、alcohol dehydrogenase (ADH) activity assay kits 及 caspase 3 activity assay kit 購自 BioVision 公司 (Mountain View, CA, USA)

doi:10.6342/NTU201700799

8. HepG2 細胞株係購自食品工業發展研究所生物資源保存及研究中心 (Hsinchu, Taiwan)
9. HSC-T6 細胞株係由財團法人生物技術開發中心 (Development Center for Biotechnology, DCB) 提供 (Xizhi, Taiwan)
10. Leptin 及 adiponectin assay kit 購自 AssayPro Inc. 公司 (Brooklyn, NY, USA)
11. L-glutamine、antibiotics (penicillin/streptomycin) 及 sodium pyruvate 購自 Gibco 公司 (Grand Island, NY, USA)
12. Lieber–DeCarli liquid diet 購自 Dyets 公司 (Bethlehem, PA, USA)
13. Interleukin (IL)-1β、IL-6、tumor necrosis factor α (TNF-α) immunoassay kit 及 Annexin-V 購自 eBioscience 公司 (San Diego, CA, USA)
14. Nuclear extraction kit、Peroxisome proliferator-activated receptor-γ (PPAR-γ) transcription factor assay kit、SREBP-1 transcription factor assay kit 及 Superoxide dismutase (SOD) assay kits 購自 Cayman 公司 (Ann Arbor, MI, USA)
15. Protein assay kit 購自 Bio-Rad 公司 (Bio-Rad Laboratories, Inc., Hercules, CA, USA)
16. Total cholesterol (TC) assay kit 購自 Randox 公司 (Crumlin, Co. Antrim, U.K.)
17. Triglycerides (TG) assay kit 購自 Fortress 公司 (Fortress Diagnostics Limited, Antrim, U.K.)
18. Transforming growth factor β1 (TGF-β1) immunoassay kit 購自 R&D Systems 公司 (Minneapolis, MN, USA)

(二) 儀器

1. 加熱板 (Major science Co., Saratoga, CA, USA)
2. 離心機 (Himac CR-21, Hitachi Co., Tokyo, Japan)
3. 水浴恆溫槽 (Model DB, Deng Yng Co., Taipei, Taiwan)
4. 冷凍乾燥機 (Bench Top3R, Virtis Co., Gardiner, NY, USA)
5. 減壓濃縮機 (Rikakikai Co., Tokyo, Japan)
6. 分光光度計 (Model U-2001, Hitachi Co., Tokyo, Japan)
7. 無菌操作台 (Kansin Co., Taipei, Taiwan)
8. 細胞培養箱 (Model RCO 3000 TABB, Revco Techologie, Ayheville, NC, USA)

doi:10.6342/NTU201700799

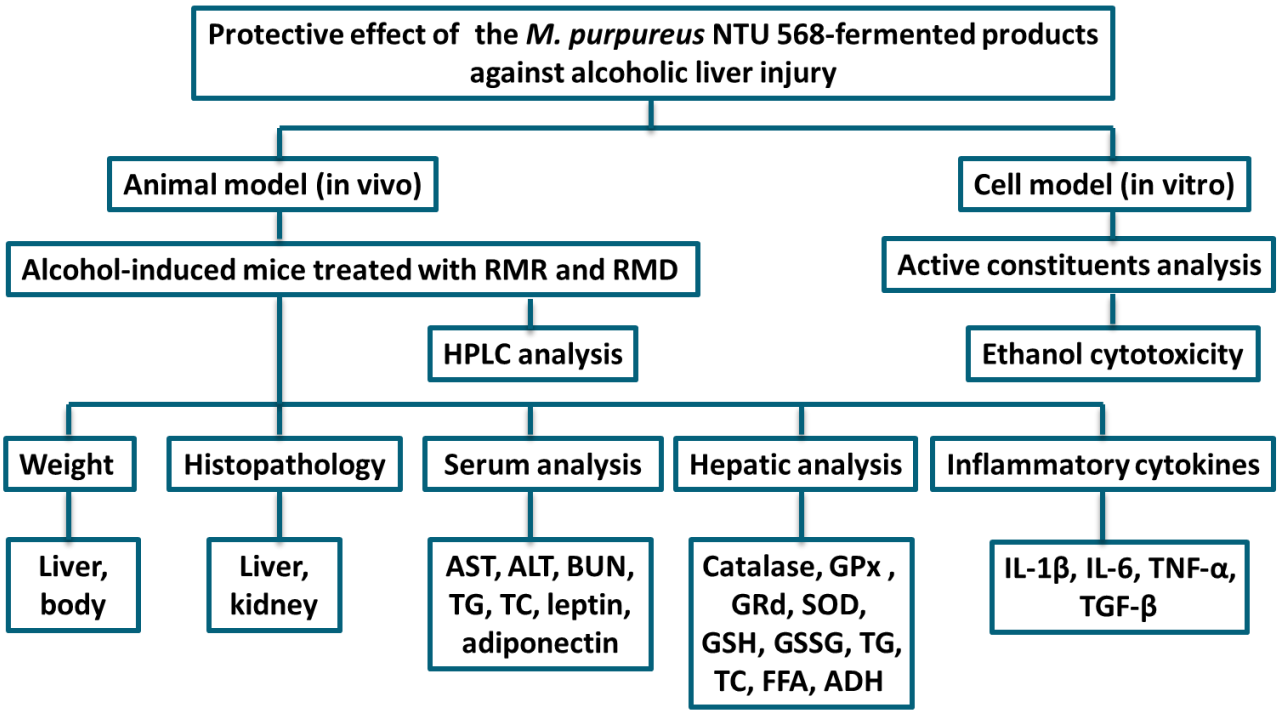
9. 純水製造機 (Milli-Q, Millipore Co., Molsheim, France)
10. 微量分注器 (Rainin Co., Bedford, MA, USA)
11. 高壓蒸氣滅菌釜 (Model SS-320, Tomy Co., Tokyo, Japan)
12. 酵素免疫分析儀 (Thermo Labsystem, Beverly, MA, USA)
13. 倒立式位像差顯微鏡 (ECLIPSE TS 100, Nikon Co., Tokyo, Japan)
14. 冷光螢光影像分析系統 (UVP Co., Upland, CA, USA)
15. 蛋白質電泳槽 (AE-6450, ATTO, Tokyo, Japan)
16. pH meter (Model 6071, Jenco Co., San Diego, CA, USA)
17. 高效液相層析儀 (Model L-6200, Hitachi Co., Tokyo, Japan)
18. HPLC 積分儀 (Model D-2500, Hitachi Co., Tokyo, Japan)
19. HPLC UV/VIS 偵測器 (Model L-4200, Hitachi Co., Tokyo, Japan)
20. HPLC 螢光偵測器 (FL-1, Rainin Co., Wobum, MA, USA)
21. 流式細胞分析儀 (BD FACSCanto II, BD Co., Heidelberg, Germany)

doi:10.6342/NTU201700799

二、實驗方法

(一) 紅麴發酵產物改善 Lieber-DeCarli liquid diet 誘導酒精性肝損傷小鼠模式

本試驗利用兩種基質 (米及山藥) 發酵的紅麴代謝產物，改善 Lieber-DeCarli liquid diet 誘導酒精性肝損傷之保健功效評估，利用廣泛運用的紅麴米發酵代謝產物以及含較多黃色素代謝產物的紅麴山藥進行相關試驗，並且利用分離的黃色素進行細胞實驗確認其功效，實驗架構如下：



1. 紅麴米及紅麴山藥培養及製備 (Su *et al*., 2003)

將市售在來白米 250 g 或市售山藥片 (*Dioscorea batatas* Dence) 250 g，以水洗滌至不呈混濁，浸水 8 小時。用棉布濾水，置於麴盤上，於高壓殺菌釜內蒸煮 15 分鐘，取出冷卻後灑水攪拌均勻再蒸煮 15 分鐘，取出冷卻至 40°C，完成蒸煮之程序。所使用之菌株 *M. purpureus* NTU 568 菌株，為由紅麴分離之 *M. purpureus* HM105 進行變異所得菌株 (Lee *et al*., 2006b)，將紅麴種菌液 (5%)，接種入蒸過之米或山藥中，並充分攪拌均勻後，開始培養於木製之麴盤 (30 × 20 × 5 cm)。期間經翻拌、頭水、次水、完水及後熟等步驟完成培養 (圖 2-2)，培養條件為 30°C，8 天。之後將培養所得之紅麴米或山藥進行各項分析。

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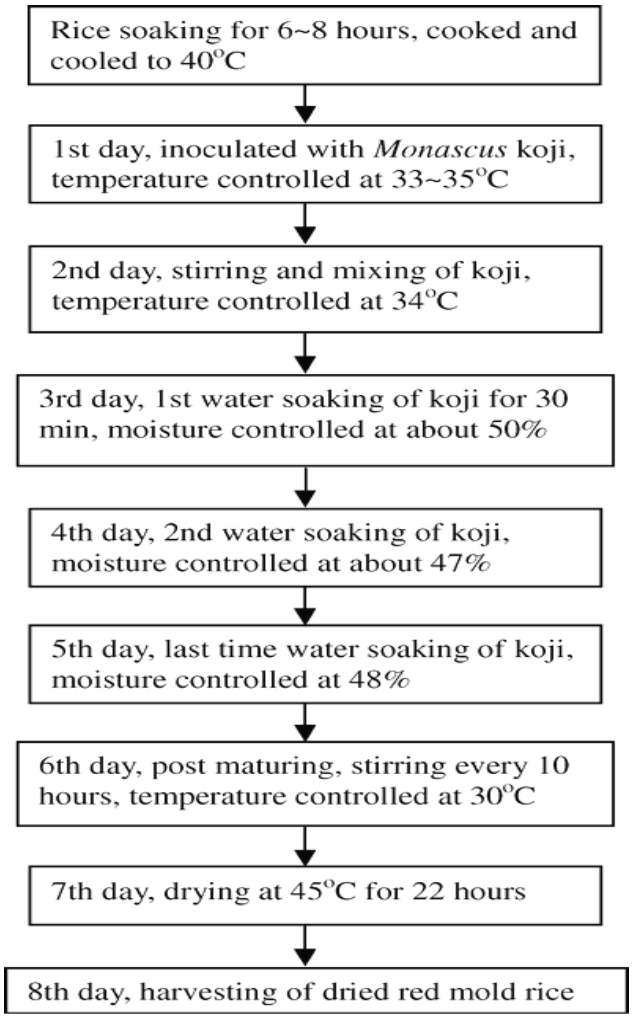

圖 2-2 紅麴米傳統製造流程 (Chiu *et al*., 2006)。

Fig. 2-2 Red mold rice production procedure by traditional process.

doi:10.6342/NTU201700799

2. 紅麴產物之次級代謝物分析

2-1 紅麴中 citrinin 濃度的分析方法 (Blanc *et al*., 1995)

稱取 1 g 紅麴發酵產物粉末，加入 10 mL 的 95% ethanol，60 °C 水浴中加熱 30 分鐘進行萃取，萃取後於室溫靜置取上清液，以 0.45 μm 濾膜過濾，以備 HPLC 分析，計算方式為將 5 個相同理論濃度之樣品以高效液相層析分析所測得之積分面積代入 citrinin 之濃度對面積標準曲線，可求得該樣品實際濃度。

HPLC 分析條件：

Column：Beckman Ultrasphere ODS (150 × 4.6 mm)，guard column 為 Beckman Ultrasphere ODS (45 × 4.6 mm)

Mobil phase：water：acetonitrile：trifluoroacetate (450：550：0.5)

Flow rate：1.0 mL/min

Detector : fluorescence detector，excitation λ_{max} = 330 nm，emission λ_{max} = 500 nm，retention time: 30 min。

2-2 紅麴中 MS 及 AK 濃度的分析方法 (Yu *et al*., 2006)

稱取 1 g 紅麴發酵產物粉末，加入 10 mL 的 95% ethanol，60 °C 水浴中加熱 30 分鐘進行萃取，萃取後於室溫靜置取上清液，以 0.45 μm 濾膜過濾，以備 HPLC 分析，計算方式為將 5 個相同理論濃度之樣品以高效液相層析分析所測得之積分面積代入 monascin 或 ankaflavin 之濃度對面積標準曲線，可求得該樣品實際濃度。

HPLC 分析條件：

Column: HPLC PU2089 (Jasco Co., Tokyo, Japan). C18 column, 25 cm × 4.6 mm i.d., 5 μm (Bellefonte, PA)

Mobil phase：water：acetonitrile：trifluoroacetate (450：550：0.5)

Flow rate: 1.0 mL/min

Detector: UV2075 plus detectors (Jasco Co.) 偵測波長為 238 nm

doi:10.6342/NTU201700799

2-3 色素分析 (Lin and Iizuka, 1982)

色素濃度分析採用分光光度計分析法，於 400 nm、470 nm 或 500 nm 波長下測其吸光值，做為黃色、橘色與紅色色素之指標。取 1 g 紅麴發酵產物粉末，加入 10 mL 的 95% ethanol，37°C 水浴中加熱 90 分鐘進行萃取，萃取後於室溫靜置取上清液，以 0.45 μm 濾膜過濾，經適當稀釋後測定之。色素濃度以每公克粉末可測得的吸收光密度 (A_{abs}/g rice) 表示之。

3. 動物實驗

實驗動物為 8 週齡之 C57BL/6J 雄性小鼠，購自樂斯科生物科技股份有限公司將實驗老鼠隨機分組，64 隻小鼠分別隨機分成 8 組，每組 8 隻。採取自由進食方式，隨時保持液體飼料 (Lieber-DeCarli diet) 充足，每週記錄攝食量及體重。飼養時控制相對溼度 60%，溫度 25±2°C，光照時間為 8:00-20:00 之 12 小時光照循環，實驗為期 35 天，樣品餵食係採用管餵法供給固定量的樣品給小鼠。實驗動物之照護遵循中華民國行政院農業委員會於 1988 年 11 月 4 日發布，且於 2016 年 5 月 18 日修訂之動物保護法 (華總一義字第 10500042801 號令)。本實驗飼料分為兩種：佔總熱量 36% 之酒精流質飲食以及正常流質飲食。為了使兩種流質飲食達到等熱量，正常流質飲食以麥芽糖糊精 (Maltodextrin) 取代酒精所佔的熱量部分 (Lieber and DeCarli, 1989)。實驗飼料組成如表下 (Dyets, Bethlehem, PA, USA) :

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Liquid diet data

	Control Liquid Diet	Ethanol Liquid Diet
Ingredient	g/Liter of Diet	g/Liter of Diet
	#710027	#710260
Casein(100 Mesh)	41.4	41.4
L-Cystine	0.5	0.5
DL-Methionine	0.3	0.3
Corn Oil	8.5	8.5
Olive Oil	28.4	28.4
Safflower Oil	2.7	2.7
Maltose Dextrin	115.2	25.6
Cellulose	10	10
Salt Mix #210011	8.75	8.75
Vitamin Mix #310011	2.5	2.5
Choline Bitartrate	0.53	0.53
Xanthan Gum	3	3
	Lieber and DeCarli Alcoholism-Clinical and Experimental Research 6, 523-531(1982)	
	Note: This diet contains 1.0 Kcal/ml. of which 35% are fat derived, 47% are derived from carbohydrate and 18% are derived from protein.	Note: This diet contains 1.0 Kcal/ml. of which 35% are fat derived, 11% are derived from carbohydrate, 18% are derived from protein, and 36% are derived from ethanol.
	DIRECTIONS: Quantity sufficient to one liter with water 221.78 grams of Diet #710027 and mix for thirty seconds in a blender.	DIRECTIONS: To 132.18 grams of Diet #710260 add: (1) Ethanol (67.3 ml of 95% Ethanol) and (2) Quantity Sufficient to one liter with cold water and mix for thirty seconds in a blender.

3-1 分組及實驗方法

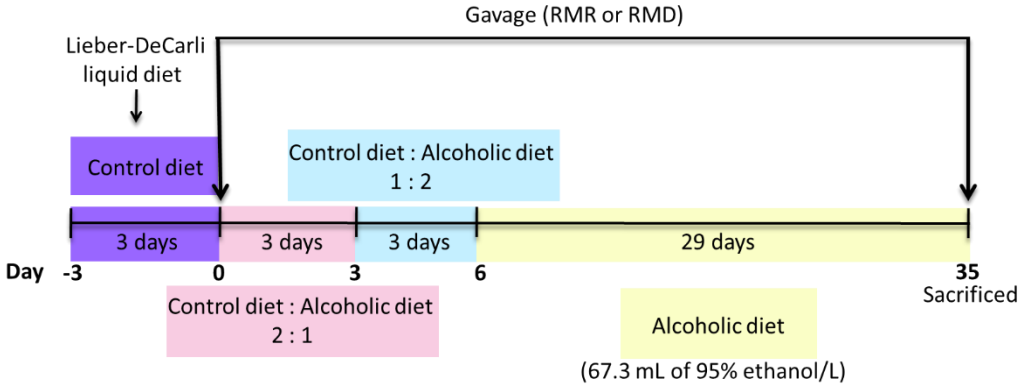
本實驗以長期 (14 天以上) 口服方式投予受試物；受試物劑量除控制組 (control group) 及酒精性肝損傷組 (alcoholic liver disease, ALD) 外， 以成人 (身高 170 公分及體重 65 公斤) 每日建議攝取量 1 到 2 g (Heber *et al*. 1999; Wang *et al*. 2004) 平均值 1.5 g 紅麴米或紅麴山藥為一倍劑量，利用 Food and Drug Administrtion (FDA) 所提供之體表面積換算公式 (Boyd, 1935)，其換算係數 (human equivalent dose, HED) 為：HED = animal dose in mg/kg x (animal weight in kg/human weight in kg)$^{0.33}$，得出係數為 12.3，口服餵食劑量公式為：小鼠每公斤體重之攝取劑量 ＝ 人體建議攝取量 ÷ 體重 (65 kg) × 12.3，計算出餵食劑量。並區分為一倍劑量 (307.5 mg/kg)、二倍劑量 (615.0 mg/kg) 及五倍劑量組 (1537.5 mg/kg)。本實驗以紅麴米或紅麴山藥管餵樣品組老鼠，管餵劑量總體積為 200 μL。

3-2 酒精性肝損傷小鼠模式建立 (Lieber and DeCarli, 1989)

本誘導架構根據衛生福利部制定之健康食品護肝功能評估方法，利用液態飼料 Lieber-DeCarli diet 誘導 C57BL/6J 雄性小鼠酒精性肝損傷，其誘導流程如下：利用 control liquid diet 預養全部組別小鼠 3 天使其習慣液態飼料，三天後為實

doi:10.6342/NTU201700799

驗開始第 0 天，樣品組及酒精性肝損傷組別飼料之 1/3 置換為 alcoholic liquid diet 並同時開始管餵樣品，3 天後飼料之 2/3 置換為 alcoholic liquid diet，餵養 3 天後，樣品組及酒精性肝損傷組別全部開始使用 alcoholic liquid diet (最終濃度每升液態飼料含有 95 % 酒精 67.3 mL) 進行誘導，總實驗天數為 35 天，誘導架構如下：



4. 組織病理切片及判讀

實驗結束後將老鼠犧牲，稱量體重後採血並取其臟器，以 3.7% 中性福馬林固定，以 Hematoxylin-Eosine (H&E) 染色法進行染色，之後委由國立中興大學獸醫病理生物學研究所廖俊旺教授協助病理組織切片分析與判讀。

5. 安全性指標之評估

小鼠犧牲後取血清進行腎功能分析，利用自動血清生化儀 (Beckman-700, Fullerton, CA) 測試血清中的尿素氮 (blood urea nitrogen, BUN) 以評估其腎功能。

6. 肝臟損傷指標之評估

依照衛生福利部公布之護肝保健功效評估方法，小鼠犧牲後取血清進行肝功能分析，利用自動血清生化儀 (Beckman-700, Fullerton, CA) 測試血清中的天門冬胺酸轉胺酶 (aspartate aminotransferase, AST)、丙胺酸轉胺酶 (alanine aminotransferase, ALT) 以評估其肝功能。

doi:10.6342/NTU201700799

7. 肝臟均質液製備

取 5 g 肝臟組織以 150 mM Tris-EDTA (pH 7.4) 緩衝溶液以組織均質機磨碎，製成 10 倍稀釋之均質液。將均質液離心 (12000 × g, 4°C, 10 min)，取上清液儲存於 -80°C 備用，以進行各項試驗測定。

8. 麩胱甘肽過氧化酶 (glutathione peroxidase, GPx) 活性分析

依 Lawrence 與 Burk 之方法進行 (Lawrence and Burk, 1976)。取 5 μL 肝臟均質液及 95 μL 20 mM 磷酸鉀緩衝液 (pH 7.0)，並加入 0.8 mL 100 mM 磷酸鉀緩衝液 (pH 7.0) 之反應混合液 (含 1 mM EDTA、1 mM NaN_3、0.2 mM NADPH、1 U/mL GSH Rd 及 1 mM GSH)，室溫下靜置 5 分鐘，再加入 0.1 mL 2.5 mM H_2O_2，以分光光度計於 340 nm 下測定吸光值變化，計算 NADPH 減少之速率，計算麩胱甘肽過氧化酶的活性，另以去離子水 5 μL 當作空白組。結果以比活性 (specific activity) 表示法 (nmole NADPH/min/mg protein) 表示之。

9. 麩胱甘肽還原酶 (glutathione reductase, GRd) 活性分析

依 Bellomo 等人之方法進行 (Bellomo *et al.*, 1987)。取 10 μL 肝臟均質液及 90 μL 20 mM phosphate buffer (pH 7.0) 加入 0.9 mL 100 mM phosphate buffer (pH 7.0) 之反應混合液 (含 1.1 mM $MgCl_2 \cdot 6H_2O$、5.0 mM GSSG 及 0.1 mM NADPH)，再以分光光度計在 340 nm 下測定五分鐘內吸光值之變化，計算 NADPH 減少的速率，另以 10 μL 之 PBS 作為空白組。結果以比活性 (specific activity) 表示法 (nmole NADPH/min/mg protein) 表示之。

10. 觸酶 (catalase, CAT) 測定

本試驗依 Aebi 之方法進行 (Aebi, 1984)。取 50 μL 肝臟均質液加入 950 μL 0.02 M H_2O_2，混勻，以分光光度計於 240 nm 下測量吸光值之變化，每隔 15 秒測量一次，共測 3 分鐘。結果以 nmole H_2O_2/min/mg protein 表示之。

11. 還原型麩胱甘肽 (glutathione, GSH) 含量測定

本試驗依 Hu 之方法進行 (Hu, 1994)。肝組織中的還原 GSH 濃度，以 GSH（0-100 μM） 為標準品。取肝臟均質液或標準品 (10 μL) 與 95 μL 試劑 (2

doi:10.6342/NTU201700799

U/mL GRd、200 μM NADPH 及 2 mM EDTA 混合於 50 mM 磷酸鹽緩衝液，pH 7.2)，加入 100 mL 的試劑 (10 mM 5,5-dithiobis[2-nitrobenzoic acid] DTNB 混合於 50 mM 磷酸鹽緩衝液，pH 7.2)。反應混合物在室溫下，每一分鐘測定一次共反應五分鐘，以分光光度計於 405 nm 測其吸光值，換算肝臟均質液中 GSH 之含量。

12. 麩胱甘肽雙硫氧化物 (glutathione disulfide, GSSG) 含量測定

本試驗依 Tietze 之方法進行 (Tietze, 1969)。肝組織中的 GSSG 的濃度，以 GSSG（0-100 μM）作為標準品。取肝臟均質液或標準品 (70 μL) 與 4 μL M2VP 混合。反應混合物在室溫下靜置 1 小時。將反應混合物 (10 μL) 與 95 μL 的試劑 （2 U/mL GRd，200 μM NADPH，和 2 mM EDTA，混合於 50 mM 磷酸鹽緩衝液，pH 7.2） 加入 100 μL 試劑（10 mM DTNB 混合於 50 mM 磷酸鹽緩衝液，pH 7.2)。反應混合物在室溫下，每一分鐘測定一次共反應五分鐘，以分光光度計於 405 nm 測其吸光值，換算肝臟均質液中 GSSG 之含量。

13. GSH/GSSG 比例計算 (Owen and Butterfield, 2010)

GSH/GSSG = (total GSH−2 x GSSG)/GSSG.

14. 超氧歧化酵素 (superoxide dismutase, SOD) 活性測定

使用市售生化試劑分析肝臟組織萃取液中的 SOD 活性。其操作原理為基質試劑 (mixed substrate) 內含 xanthine，操作時加入 xanthine oxidase，使之迅速產生超氧根離子 (O^{2-})，而超氧根離子遇到基質試劑所含的 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) 即作用產生一紅色的 formazan dye。SOD 能與 INT 競爭，將超氧根離子作用生成氧，抑制紅色形成。分析時以分光光度計測量紅色染劑濃度，代表 SOD 抑制超氧根產生的程度，間接推估 SOD 活性。於 cuvette 加入 850 μL mixed substrate (內含 0.05 mmol/L xanthine 與 0.025 mmol/L INT)、25 μL 的標準品或肝臟組織萃取液，最後再加入 125 μL 的 xanthine oxidase (80 U/L)，於加入後第 30 sec 及第 210 sec 分別記錄波長 505 nm 之吸光值，依以下公式計算抑制率 (%)，再由標準樣品測出檢量線來推算 SOD 之活性。所得活性再以組織萃取液之總蛋白質量為基準計算每 mg 的

doi:10.6342/NTU201700799

蛋白質量所含 SOD 活性。

計算公式：

Inhibition % = 100 − (ΔAsample/min × 100)/(ΔAS1/min)

ΔAsample/min：樣品每 min 吸光值之改變

ΔAS1/min：phosphate buffer 每 min 吸光值之改變

SOD 之活性 (U/mL) 為抑制百分比之反對數值 (against Log10) 乘以稀釋倍數

15. 酒精去氫酶 (alcohol dehydrogenase, ADH) 活性測定

酒精去氫酶存在於許多生物體中，利用 NAD^+ 轉化為 NADH 而促進醇轉換為醛或酮。使用市售生化試劑 (BioVision, Mountain View, CA, USA) 分析肝臟組織萃取液中的 ADH 活性，BioVision 的酒精去氫酶測定套組利用異丙醇作為基質，導致試劑顯色來測定 ADH 的活性，偵測極限為 0.01 mU 的樣品，操作方法如套組說明書所示，敘述如下:於 96 孔盤中加入 20 μL 肝臟均質液及 100 μL reaction mix buffer 混勻，反應混合物在 37°C 下，每五分鐘測定一次共反應 30 分鐘，以分光光度計於 450 nm 測其吸光值，利用標準曲線換算肝臟均質液中 ADH 之含量。

16. 血清脂質含量測定

小鼠犧牲後取其血清，使用市售生化試劑總膽固醇分析套組 (CH 200, Randox, Crumlin, Co. Antrim, U.K.) 和三酸甘油酯分析套組 (BXC0272C, Fortress Diagnostics, Antrim, U.K.)。操作方法如套組說明書所示，敘述如下:將 500 μL 混合試劑加入 24 孔盤中，加入 5 μL 血清或標準品至樣品孔中。使用 5 μL 的去離子水作為空白樣品。於 37°C 反應 30 分鐘後測量吸光值，利用標準曲線換算含量，分析血液中的總膽固醇和三酸甘油酯量。

17. 肝臟脂質含量測定

將肝組織 (0.5 g) 在 10 mL 冰冷的 Folch 溶液中研磨 (氯仿/甲醇，2:1，v/v)，並在室溫下靜置 30 分鐘。抽除水層並將有機層蒸發乾燥。將乾燥的脂質層用等體積的 DMSO 溶解，然後用分析套組測定總膽固醇 (CH 200, Randox, Crumlin,

doi:10.6342/NTU201700799

Co. Antrim, U.K.)、三酸甘油酯 (BXC0272C, Fortress Diagnostics, Antrim, U.K.) 及游離脂肪酸 (BioVision, Mountain View, CA, USA) 含量。總膽固醇及三酸甘油酯測定，將 500 μL 混合試劑加入 24 孔盤中，加入 5 μL 樣品或是標準品到樣品孔中。使用 5 μL 的去離子水作為空白樣品。於 37ºC 反應 30 分鐘後測量吸光值，利用標準曲線換算含量，分析肝臟中的總膽固醇和三酸甘油酯含量。游離脂肪酸含量測定，將 48 μL 混合試劑加入 96 孔盤中，加入 2 μL 樣品或標準品到樣品孔中。使用 2 μL 的去離子水作為空白樣品。於 37ºC 反應後測量吸光值，利用標準曲線換算肝臟中的游離脂肪酸含量

18. 肝臟發炎因子含量測定

肝臟中 TNF-α、IL-1β、IL-6 (eBioscience, San Diego, CA, USA) 和 TGF-β1 (R&D Systems, Minneapolis, MN, USA) 含量，利用市售 ELISA 分析套件測定，將分析方法敘述如下:將 capture antibody 分別 coating 於 96 盤中，4ºC 放置隔夜。將 assay buffer 於室溫下 blocking 至少一小時，再於各孔洞中加入樣品或標準品，並加入 detection antibody，室溫下反應一小時，再加入 avidin-HRP 反應後加入基質 TMB solution 顯色，利用吸光值計算其含量。

19. 血清中瘦體素及脂聯素 (leptin, adiponectin) 含量測定

血清中 leptin 和 adiponectin 含量，利用市售 ELISA 分析套件 (AssayPro, Brooklyn, NY, USA) 測定含量，操作方法如套組說明書所示，即於每孔加入 50 μL 標準品或樣品，反應 2 小時，加入 50 μL biotinylated antibody，反應 2 小時，加入 50 μL SP conjugate，反應 30 分鐘。，加入 50 μL 的 chromogen substrate，反應 20 分鐘。每孔加入 50 μL stop solution，利用 450 nm 吸光值換算含量。

20. 紅麴發酵產物之分離純化

本研究所使用之紅麴菌株為本研究室分離之突變株 *M. purpureus* NTU 568，並以在來米作為發酵基質。將所得之紅麴米先以甲醇進行萃取，萃取物利用矽膠（Silica gel；Kiesilgel 60，70-230 mesh 及 230-400 mesh）材質做為固定相，再配合製備 HPLC （高壓液相層析儀） 等儀器來進行分離、純化，可分離出 MS

doi:10.6342/NTU201700799

及 AK 等活性物質 (Hsu *et al.*, 2010)，以 DMSO 回溶 MS 及 AK 進行細胞實驗，且最終 DMSO 濃度低於 0.1%。

21. MTT 細胞存活率檢測

將 HepG2 細胞培養於含有 10% FBS 的 DMED 培養基中。將細胞以 2×10^5 cell/well 種於 24 孔培養盤中，並在 1 mL 生長培養基中生長 24 小時。HepG2 細胞利用含有乙醇 (100、200、300 和 500 mM) 之培養基培養 24 小時，或含有乙醇 (300 mM) 及 AK 或 MS (5、10 和 20 μM) 之培養基培養細胞 24 小時。隨後洗滌並測試其細胞存活率。MTT 為一種 tetrazolium salt，可被活細胞吸收並被粒線體中的 succinate tetrazolium reductase 還原成藍色的 formazan，常用來檢測細胞的增殖。經過處理的 HepG2 細胞去除培養基後，加入新鮮配製的 MTT 溶液，最終濃度為 0.5 mg/mL，於 37°C 避光培養 3-4 小時，將多餘的 MTT 溶液取出，加入 DMSO 回溶沉澱結晶 formazan，以酵素免疫分析儀檢測波長 570 nm 之吸光值並換算細胞在各樣品中之存活率。細胞存活率計算方式如下：細胞存活率 (% of control) = ($A_{570\ nm}$ (加藥組)/$A_{570\ nm}$ (未加藥組))×100% (Mosmann, 1983)。

22. 蛋白質定量

利用 Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) 進行蛋白質濃度之測定。將 5 μL 細胞萃取液與 25 μL reagent A 及 200 μL reagent B 混合均勻後，置於室溫下反應 10 分鐘。利用分光光度計，於波長 750 nm 下，測定溶液之吸光值。以 BSA 作為標準品製作蛋白質濃度標準曲線。將樣品之吸光值對照標準曲線，即可換算蛋白質濃度。

23. 細胞三酸甘油酯含量測定

將 HepG2 細胞培養於含有 10% FBS 的 DMED 培養基中。將細胞以 1×10^6 cell/dish 種於 6 公分細胞培養盤中，並在 6 mL 生長培養基中生長 24 小時。HepG2 細胞利用含有乙醇 (100、200 和 300 mM) 之培養基培養 24 小時，或含有乙醇 (300 mM) 及 AK 或 MS (5 和 10 μM) 之培養基培養細胞 24 小時。取出細胞，以 PBS 清洗細胞並收集至微量離心管中，加入 50 μL lysis buffer 置

doi:10.6342/NTU201700799

於液態氮中 3 分鐘，再置於 25°C 水浴中超音波震盪 5 分鐘，重複此步驟三次，再以 12,000 × g 離心 10 分鐘，取上清液進行蛋白質定量並測定三酸甘油酯含量，使用市售生化試劑三酸甘油酯分析套組 (BXC0272C, Fortress Diagnostics, Antrim, U.K.)。操作方法如套組說明書所示，敘述如下：將 500 μL 混合試劑加入 24 孔盤中，加入 5 μL 細胞蛋白或標準品至樣品孔中。使用 5 μL 的去離子水作為空白樣品。於 37°C 反應後測量吸光值，利用標準品換算含量，分析細胞中三酸甘油酯含量。

24. 細胞核蛋白萃取

將 HepG2 細胞培養於 10% FBS 的 DMED 培養基中。將細胞以 2×10^6 cell/dish 種於 10 公分細胞培養盤中，並在 10 mL 生長培養基中生長 24 小時。HepG2 細胞利用含有乙醇 (300 mM) 之培養基培養，或含有乙醇 (300 mM) 及 AK 或 MS (5 μM 和 10 μM) 之培養基培養細胞 24 小時。核蛋白萃取利用市售 nuclear extraction kit 萃取套件 (Cayman, Ann Arbor, MI, USA)，操作方法如套組說明書所示，用細胞刮勺收集 2×10^6 細胞於預冷的 15 mL 離心管中以 PBS 洗滌兩次，離心 (300 × g/5 分鐘/4°C) 後去除上清液隨後加入 1 mL 冰的 PBS/phosphatase inhibitor solution，離心 (300 ×g/5 分鐘/4°C) 後去除上清液，加入 100 μL complete hypotonic buffer，溫和的 pipetting 後移至預冷的 1.5 mL 微量離心管中，將細胞於冰上靜置 15 分鐘。加入 20 μL 10% NP-40 assay reagent，溫和地 pipetting，用桌上型離心機離心 (30 秒/4°C) 後將上清液 (cytosolic fraction) 移至新的 1.5 mL 微量離心管中存放於 -80°C，將沈澱物懸浮於 20 μL 冰的 complete nuclear extraction buffer 中，劇烈 vortex 15 秒後在於冰上搖晃 15 分鐘，重複兩次。離心 (14,000 × g/10 分鐘/4°C) 後將上清液 (nuclear fraction) 移至新的 1.5 mL 微量離心管中存放於 -80°C。

25. 細胞核內 PPAR-γ 含量測定

將 HepG2 細胞培養於含有 10% FBS 的 DMED 培養基中。將細胞以 2×10^6 cell/dish 種於 10 公分細胞培養盤中，並在 10 mL 生長培養基中生長 24 小時。HepG2 細胞利用含有乙醇 (300 mM) 之培養基培養，或含有乙醇 (300 mM) 及 AK 或 MS (5 μM 和 10 μM) 之培養基培養細胞 24 小時。萃取核蛋白後進

doi:10.6342/NTU201700799

行蛋白質定量並利用市售 PPAR-γ transcription factor assay kit 套件 (Cayman, Ann Arbor, MI, USA)，操作方法如套組說明書所示，即於每孔加入 CTFB 溶液 90 μL 及 10 μL 標準品或樣品，於 4ºC 下反應過夜，每孔加入 200 μL 的 wash buffer 清洗五次，每孔加入 100 μL 的 PPAR-γ primary antibody 室溫下反應一小時，每孔加入 200 μL 的 wash buffer 清洗五次，每孔加入 100 μL 的 goat anti-rabbit HRP antibody 室溫下反應一小時，每孔加入 200 μL 的 wash buffer 清洗五次，每孔加入 100 μL 的 transcription factor developing solution 混合均勻後室溫下反應 15 到 45 分鐘 (避光)，測量 655 nm 數值為 0.4 至 0.5 時 (或 450 nm 數值為 1)，每孔加入 100 μL 的 stop solution，利用 ELISA reader 以 450 nm 吸光值測定細胞核內 PPAR-γ 含量，計算方式如下：PPAR-γ 含量 (% of control)＝($A_{450\ nm}$ (加藥組)/$A_{450\ nm}$ (未加藥組))×100%。

26. 細胞核內 SREPB-1 含量測定

將 HepG2 細胞培養於含有 10% FBS 的 DMED 培養基中。將細胞以 2×10^6 cell/dish 種於 10 公分細胞培養盤中，並在 10 mL 生長培養基中生長 24 小時。HepG2 細胞利用含有乙醇 (300 mM) 之培養基培養，或含有乙醇 (300 mM) 及 AK 或 MS (5 μM 和 10 μM) 之培養基培養細胞 24 小時。萃取核蛋白後進行蛋白質定量並利用市售 SREBP-1 transcription factor assay kit 套件 (Cayman)，操作方法如套組說明書所示，即於每孔加入 CTFB 溶液 90 μL 及 10 μL 標準品或樣品，於 4ºC 下反應過夜，每孔加入 200 μL 的 wash buffer 清洗五次，每孔加入 100 μL 的 SREBP-1 primary antibody 室溫下反應一小時，每孔加入 200 μL 的 wash buffer 清洗五次，每孔加入 100 μL 的 goat anti-rabbit HRP antibody 室溫下反應一小時，每孔加入 200 μL 的 wash buffer 清洗五次，每孔加入 100 μL 的 transcription factor developing solution 混合均勻後室溫下反應 15 到 45 分鐘 (避光)，測量 655 nm 數值為 0.4-0.5 時 (或 450 nm 數值為 1)，每孔加入 100 μL 的 stop solution，利用 ELISA reader 以 450 nm 吸光值測定細胞核內 SREBP-1 含量，計算方式如下：SREBP-1 含量 (% of control)＝($A_{450\ nm}$ (加藥組)/$A_{450\ nm}$ (未加藥組))×100%。

27. 生物統計分析方法

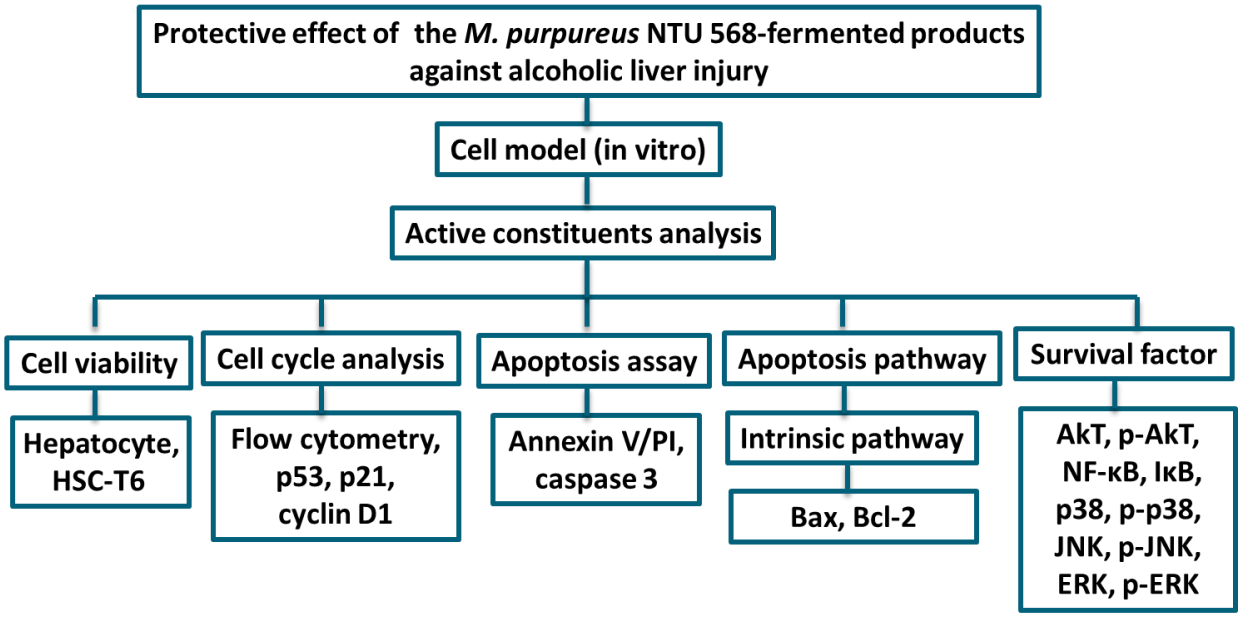
doi:10.6342/NTU201700799

所有試驗皆進行三重複。以 SPSS 系統之雙因子變方分析 (two-way ANOVA) 進行統計處理，$P < 0.05$ 表示具有顯著性差異。

doi:10.6342/NTU201700799

(二) 紅麴發酵產物改善肝臟纖維化之分子路徑探討

本試驗目的為利用紅麴發酵代謝產物，評估減緩肝臟纖維化的功效，並且確認可誘導 HSC-T6 細胞株產生細胞凋亡，同時瞭解其分子路徑，以紅麴發酵代謝產物 AK 及 MS 進行相關試驗，實驗架構如下：



1. HSC-T6 肝臟星狀細胞培養

HSC-T6 細胞為活化態的大鼠肝臟星狀細胞株 (rat hepatic stellate cell)，其細胞特性與正常大鼠活化的肝臟星狀細胞相似，經常用作肝纖維化的體外試驗 (Vogel *et al.*, 2000)。以 DMEM 培養基含 10% 熱去補體胎牛血清，培養於 37°C、5% CO_2 之培養箱中，每 2 至 3 天更換培養基。HSC-T6 為活化之肝臟星狀細胞，經由細胞激素刺激會產生纖維性膠原蛋白造成肝臟纖維化，同時也會分泌 TNF-α、platelet-derived growth factor (PDGF) 及 TGF-β 等細胞激素，造成更多星狀細胞活化並導致持續性肝臟發炎反應。

2. 肝臟細胞的萃取分離

購自樂斯科生物科技股份有限公司 450-550 g 的雄性 Sprague-Dawley (SD) 大鼠，並在室溫 (25°C) 和 12 小時光/暗循環，可自由飲用標準顆粒飲食和水。實驗操作之前將所有解剖器具先用 70% 酒精消毒、UV 燈照光隔夜至第二天方可使用。引流管、穿刺針需經酒精消毒、無菌蒸餾水徹底洗淨後才能使用。大鼠

doi:10.6342/NTU201700799

利用乙醚麻醉後仰臥固定置於乾淨工作台，迅速開刀呈 U 字型剖胸，並於肝門靜脈處插入並留置穿刺針，再以細線結紮住包含穿刺針的肝門靜脈，確認灌注的溶液不會從穿刺的洞口流出。再於上腔靜脈埋沒引流管固定後引流。從肝門靜脈處注入前灌溶液 (10 mL/min)，邊灌注邊按摩肝臟，讓前灌溶液充分流入肝臟每一個角落，洗去血水，並由上腔靜脈流出，直至整個肝臟呈現淡褐色。接著注入膠原蛋白酶溶液，分段注入約 15-20 分鐘後，肝臟呈現解離狀。剪碎肝臟並過濾離心去除組織，培養在 37°C、5% CO_2 環境下，在含有 10% 熱去補體的 FBS、0.15% 胰島素、100 mg/L 鏈黴素和 70 mg/L 青黴素的 DMEM 中培養細胞 (LaBrecque and Howard, 1976)。

3. 紅麴發酵產物對肝臟初代細胞及肝臟星狀細胞 HSC-T6 之毒性分析

將 5×10^4 cell/well 肝臟初代細胞及肝臟星狀細胞種於 24 孔盤中，經 24 小時穩定後，加入不同濃度之紅麴發酵代謝產物樣品 (AK 及 MS)，以 MTT 法測試細胞存活率，以得知紅麴代謝產物對於肝臟初代細胞及肝臟星狀細胞之毒性。

4. 細胞週期檢測

將 HSC-T6 細胞種於 6 cm 盤中 (1 mL, 5×10^5 cells/mL)，經 24 小時穩定後，利用含有 0.5% 胎牛血清的 DMEM 培養基培養 24 小時，使其細胞週期於 G1 期，再回復含有 10% 胎牛血清的 DMEM 培養基並加入不同濃度之 AK 及 MS 處理 0、6、12 及 24 小時後，取出細胞，利用 PBS 沖洗細胞，以 250 × g、4°C 離心 10 分鐘，沉澱加入 1 mL 冰凍的 75% 乙醇固定細胞，小心混合均勻後，置於 4°C 隔夜，以 250 × g、4°C 離心 10 分鐘後，利用 PBS 清洗，加入 1 mL PI DNA 染色劑 (PBS 中含有 50 μg/mL PI、50 μg/mL RNase A、0.1 mM EDTA disodium 及 0.1% Triton X-100)，放在冰上待測，以流式細胞儀進行分析 (Islam *et al.*, 2008)。

5. Annexin V 分析

將 HSC-T6 細胞種於 6 cm 盤中 (1 mL, 5×10^5 cells/mL)，經 24 小時穩定後，加入不同濃度之 AK 及 MS 處理 24 小時後，取出細胞，利用 PBS 沖洗

doi:10.6342/NTU201700799

細胞，以 250 × g、4°C 離心 10 分鐘，利用 Annexin V-FITC 染色劑以流式細胞儀進行分析 (Vermes *et al.*, 1995)。

6. Caspase-3 活性分析

將 HSC-T6 細胞種於 6 cm 盤中 (1 mL, 5×10^5 cells/mL)，經 24 小時穩定後，加入 30 μM 之 AK 及 MS 處理 24 小時後，以 caspase-3 assay kit (Biovision Co., Mountain view, CA, USA) 進行活性測定，其測試方法依說明書操作，將細胞收集並打破細胞，加入可被 caspase-3 辨識及剪切之特殊蛋白序列受質，利用吸光值換算 caspase-3 之活性。

7. 西方墨點法 (Western blot assay) (Renart *et al.*, 1979)

將 HSC-T6 細胞種於 6 cm 盤中 (1 mL, 5×10^5 cells/mL)，經 24 小時穩定後，加入不同濃度之 AK 及 MS 處理 24 小時後，取出細胞，以 PBS 清洗細胞，加入 200 μL extraction buffer，放置於 -20°C 過夜，以 10,000 g 離心 10 分鐘，取上清液進行蛋白質定量並進行蛋白質電泳、轉漬 (immunoblotting)。轉漬完成後，將轉漬膜浸於含 5% 脫脂奶粉之 PBS 中，於室溫震盪 2 小時，加入一次抗體溶液置於 4°C 靜置隔夜，以 PBS 清洗三次後再加入二次抗體，置於室溫下震盪 1 小時，清洗三次，以 chemiluminescence detection reagents (Sigma Chemical Co.) 呈色照相。訊號強度以 Image J 軟體進行分析比對。

8. 生物統計分析方法

所有試驗皆進行三重複。以 SPSS 系統之雙因子變方分析 (two-way ANOVA) 進行統計處理，$P < 0.05$ 表示具有顯著性差異。

doi:10.6342/NTU201700799

第肆章 結果與討論

一、紅麴發酵產物改善 Lieber-DeCarli liquid diet 誘導酒精性肝損傷小鼠模式

(一) 紅麴米及紅麴山藥對於體重及肝臟的改善功能

如表 4-1 所示，酒精性肝損傷、紅麴米樣品組及控制組小鼠於 0、14、28 和 35 天監測體重，控制組的小鼠體重隨著天數增加而上升，但酒精性肝損傷小鼠組於第 28 天體重上升開始明顯減緩，並與控制組有顯著差異，餵食不同劑量紅麴米組體重沒有顯著提升的功效，但是紅麴山藥樣品組顯著提升體重的功效(表 4-2)，並於第 28 天開始與酒精性肝損傷組有顯著差異。體重減輕為長期飲酒所產生的現象之一，由於長期酒精攝取會阻礙腸道對於維生素及礦物質的吸收，同時細胞受到長期酒精及其代謝物乙醛的干擾，使得蛋白質合成下降，造成營養不良、體重下降的情況，由以上結果得知，紅麴山藥對於體重的回復有顯著的功效，且山藥與米的熱量相當 (衛生福利部食品藥物管理署)，因此推論紅麴山藥可能具有改善營養吸收的功效。比較肝臟及體重的比例可得知，酒精性肝損傷組的肝臟重量/體重比例較控制組高，而紅麴米及紅麴山藥五倍劑量組，則有恢復正常肝重量/體重的功效（圖 4-1A 及 4-2A）。長期攝取酒精會造成脂肪肝而增加肝臟重量，而紅麴米及紅麴山藥可以減緩脂肪肝的產生，並且使得肝臟內三酸甘油酯含量下降，使得肝臟重量/體重比例下降。然而各組腎臟重量之間的比較，酒精性肝損傷組、控制組和樣品組都沒有顯著差異（圖 4-1B 及 4-2B）。

(二) 紅麴米及紅麴山藥對於肝臟及腎臟組織影響

利用肝臟切片及組織染色，控制組為正常肝臟細胞結構並有良好的細胞質、細胞核和核仁（圖 4-3A），而酒精性肝損傷組可觀察到肝臟細胞產生脂肪變性並且呈現氣泡狀 Mallory bodies（圖 4-3B）。餵食紅麴米組，肝臟的型態明顯的回復正常型態，於兩倍劑量樣品組中脂肪變性的程度下降（圖 4-3D），餵食五倍劑量其肝臟沒有明顯脂肪變性及氣泡狀細胞產生（圖 4-3E）。餵食紅麴山藥後，可發現一倍劑量樣品組脂肪變性的程度明顯下降（圖 4-4C），兩倍及五倍紅麴山藥組別則完全未發現脂肪變性氣泡（圖 4-4D 及 4-4E）。酒精性肝損傷患者肝臟切片顯示，酒精會造成肝臟細胞累積脂肪造成損傷死亡，此種腫大的肝臟細胞稱為

doi:10.6342/NTU201700799

表 4-1 餵食紅麴米對於酒精性肝損傷之體重改善情形

Table 4-1 Effect of RMR on mouse body weight after various weeks of alcohol diet feeding.

Groups	Body weight (g)			
	Day 0	Day 14	Day 28	Day 35
Control	20.0 ±1.7	21.9 ±1.7	24.5 ± 1.7	26.1 ±1.3
ALD	20.0 ±1.3	20.9 ±0.5	22.5 ± 0.9 *****	22.7 ±0.5 *****
1x RMR	20.0 ±1.1	21.5 ±0.6	22.8 ± 1.4	23.3 ±1.1
2x RMR	20.0 ±0.8	21.1 ±0.9	23.3 ± 1.5	23.8 ±1.1
5x RMR	20.0 ±0.7	21.6 ±0.5	23.0 ± 1.1	23.3 ±1.5

Data presented as mean ± SD (n = 8). *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with ALD group. Control, normal control group; ALD, alcohol-induced disease group; RMR, red mold rice treatment group. The alcohol-induced mice were orally treated with RMR at 307.5 mg/kg (1-fold), 615 mg/kg (2-fold), and 1537.5 mg/kg (5-fold).

doi:10.6342/NTU201700799

表 4-2 餵食紅麴山藥對於酒精性肝損傷之體重改善情形

Table 4-2 Effect of RMD on mouse body weight after various weeks of alcohol diet feeding.

Groups	Body weight (g)			
	Day 0	Day 14	Day 28	Day 35
Control	23.0 ±0.7	24.5 ±2.2	26.9 ± 1.2	28.0 ± 1.5
ALD	23.4 ±1.9	24.0 ±0.5	25.2 ± 1.3*	25.9 ± 1.3*
1x RMD	23.8 ±1.9	24.4 ±1.0	27.0 ± 1.4#	27.5 ± 1.7#
2x RMD	23.6 ±1.7	25.3 ±1.5	27.1 ± 1.3#	28.4 ± 1.7#
5x RMD	23.7 ±1.7	24.3 ±1.5	27.3 ± 0.6#	27.8 ± 1.5#

Data presented as mean ± SD (n = 8). *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with ALD group. Control, normal control group; ALD, alcohol-induced disease group; RMD, red mold dioscorea treatment group. The alcohol-induced mice were orally treated with RMD at 307.5 mg/kg (1-fold), 615 mg/kg (2-fold), and 1537.5 mg/kg (5-fold).

doi:10.6342/NTU201700799

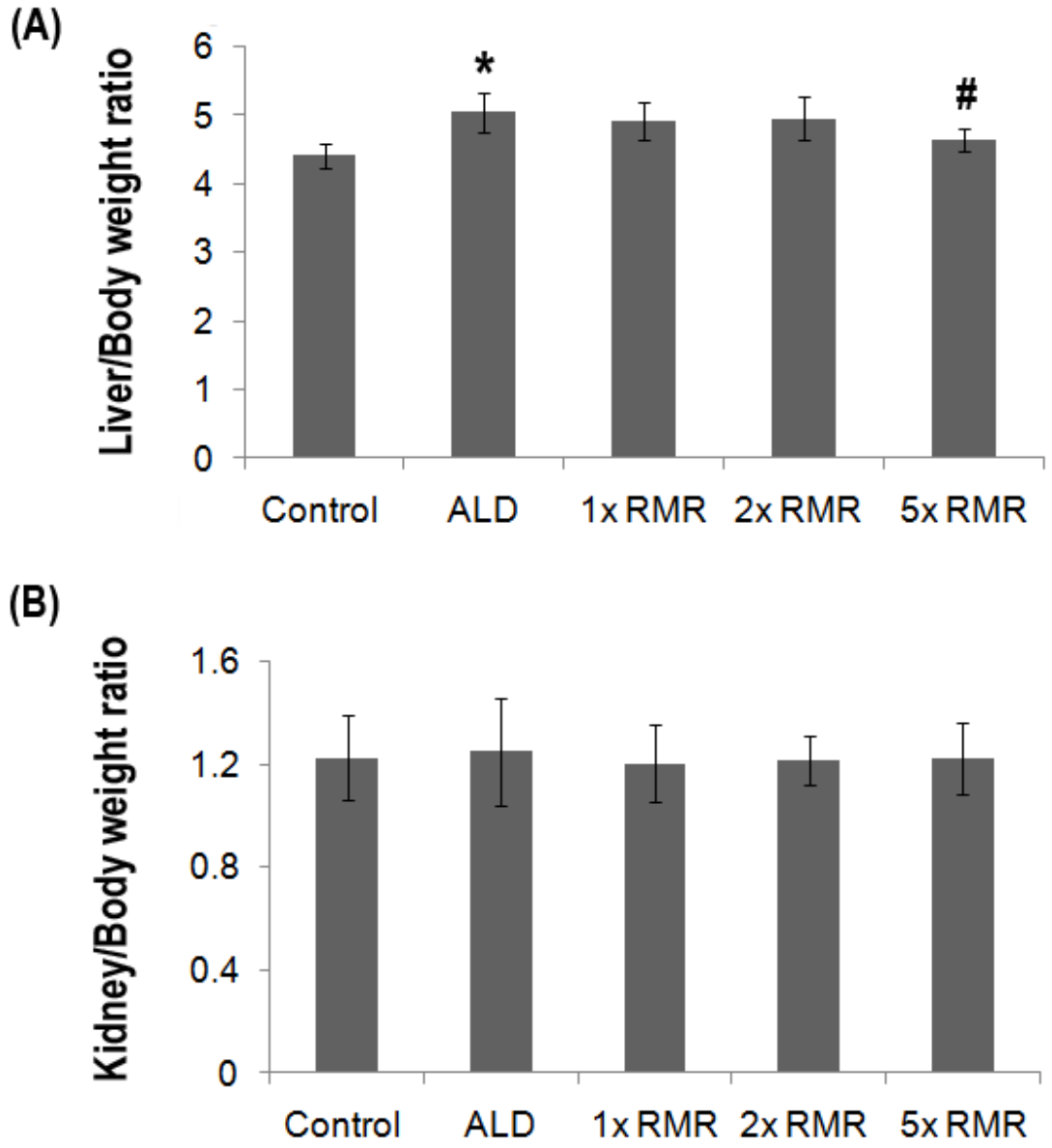


圖 4-1 紅麴米對於肝臟及腎臟之影響 (A) 肝臟及體重之比例 (B) 腎臟及體重之比例。

Figure 4-1 Effects of RMR on liver and kidney: (A) liver and body weight ratio; (B) kidney and body weight ratio. Results are expressed as the mean ± SD (n = 8). *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with ALD group. ALD, alcoholic liver disease; RMR, red mold rice treatment.

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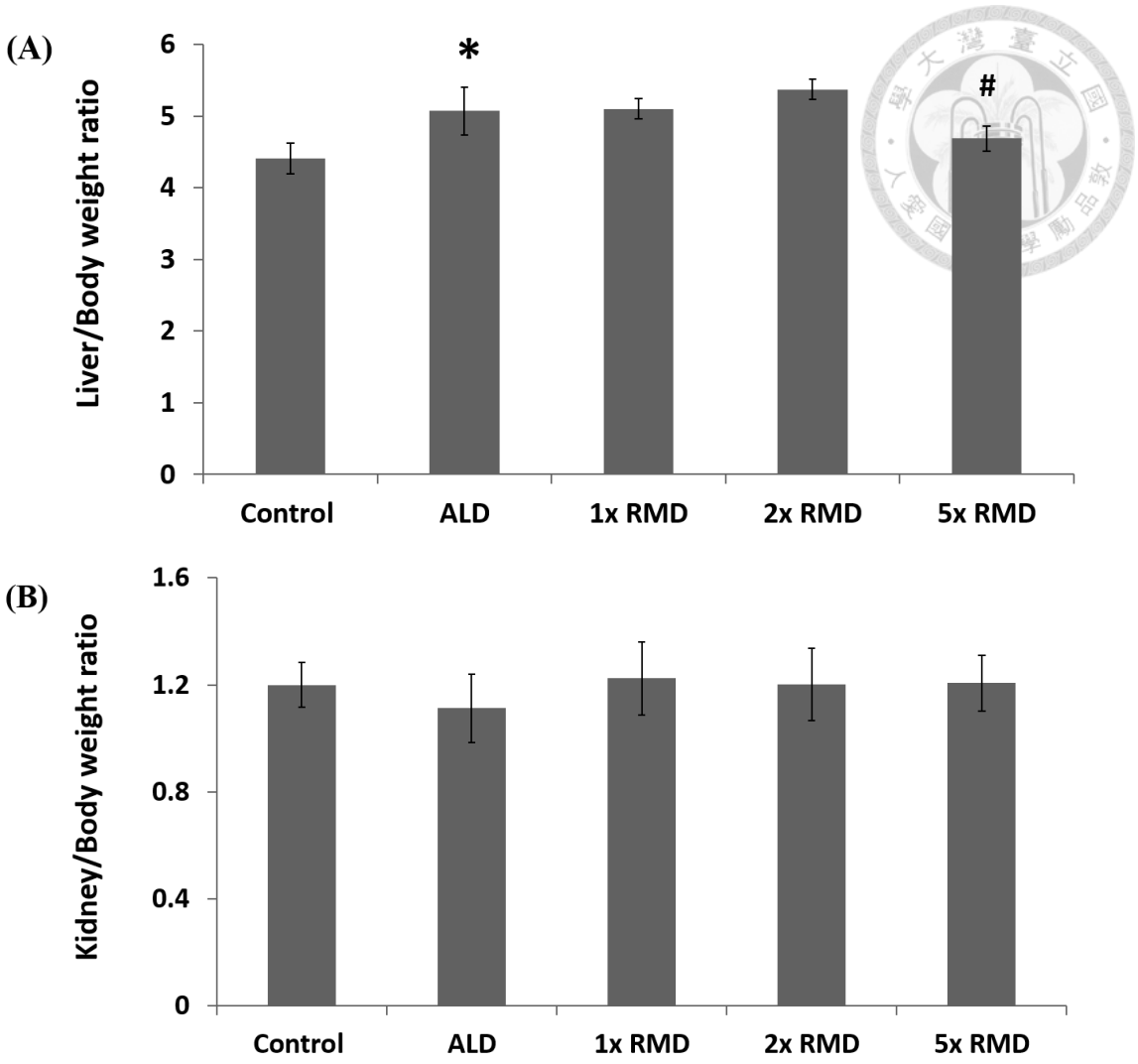

圖 4-2 紅麴山藥對於肝臟及腎臟之影響 (A) 肝臟及體重之比例 (B) 腎臟及體重之比例。

Figure 4-2 Effects of RMD on liver and kidney: (A) liver and body weight ratio; (B) kidney and body weight ratio. Data presented as mean ± SD (n = 8). *, $P < 0.05$ compared with normal control group; #, $P < 0.05$ compared with ALD group. Control, normal control group; ALD, alcohol-induced disease group; RMD, red mold dioscorea treatment group.

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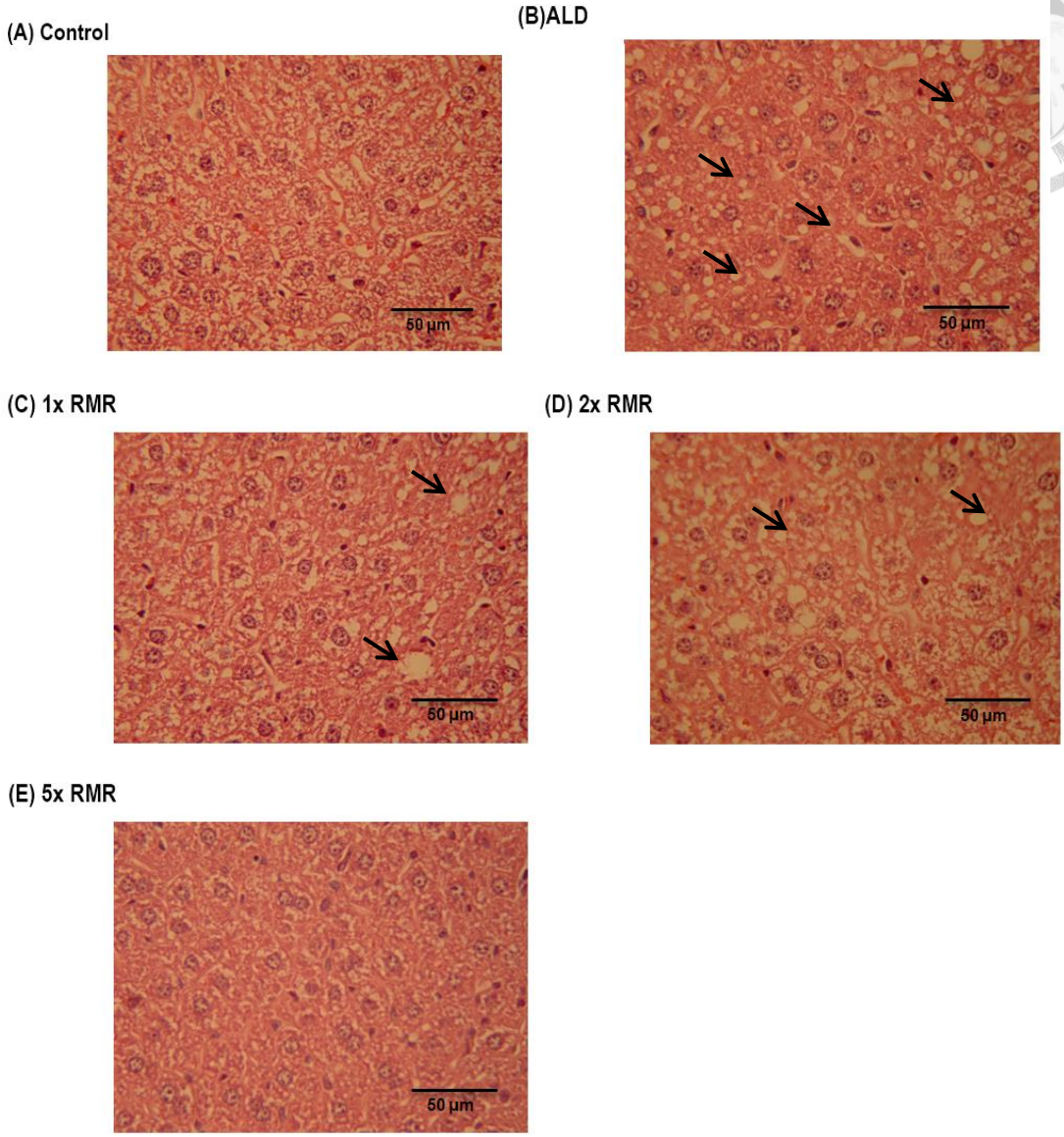

圖 4-3 紅麴米對於酒精性肝損傷組織之影響 (A) 控制組肝臟切片 (B) 酒精性肝損傷組肝臟切片 (C) 一倍紅麴米劑量組 (D) 兩倍紅麴米劑量組 (E) 五倍紅麴米劑量組。→標示為肝臟脂肪變性之氣泡狀組織。

Figure 4-3 Effects of RMR on liver histopathology of ALD mice using H&E staining: liver tissue of (A) control mice; (B) ALD group; (C) 1 × RMR group; (D) 2 × RMR group; (E) 5 × RMR group (scale bar = 50 μm).

doi:10.6342/NTU201700799

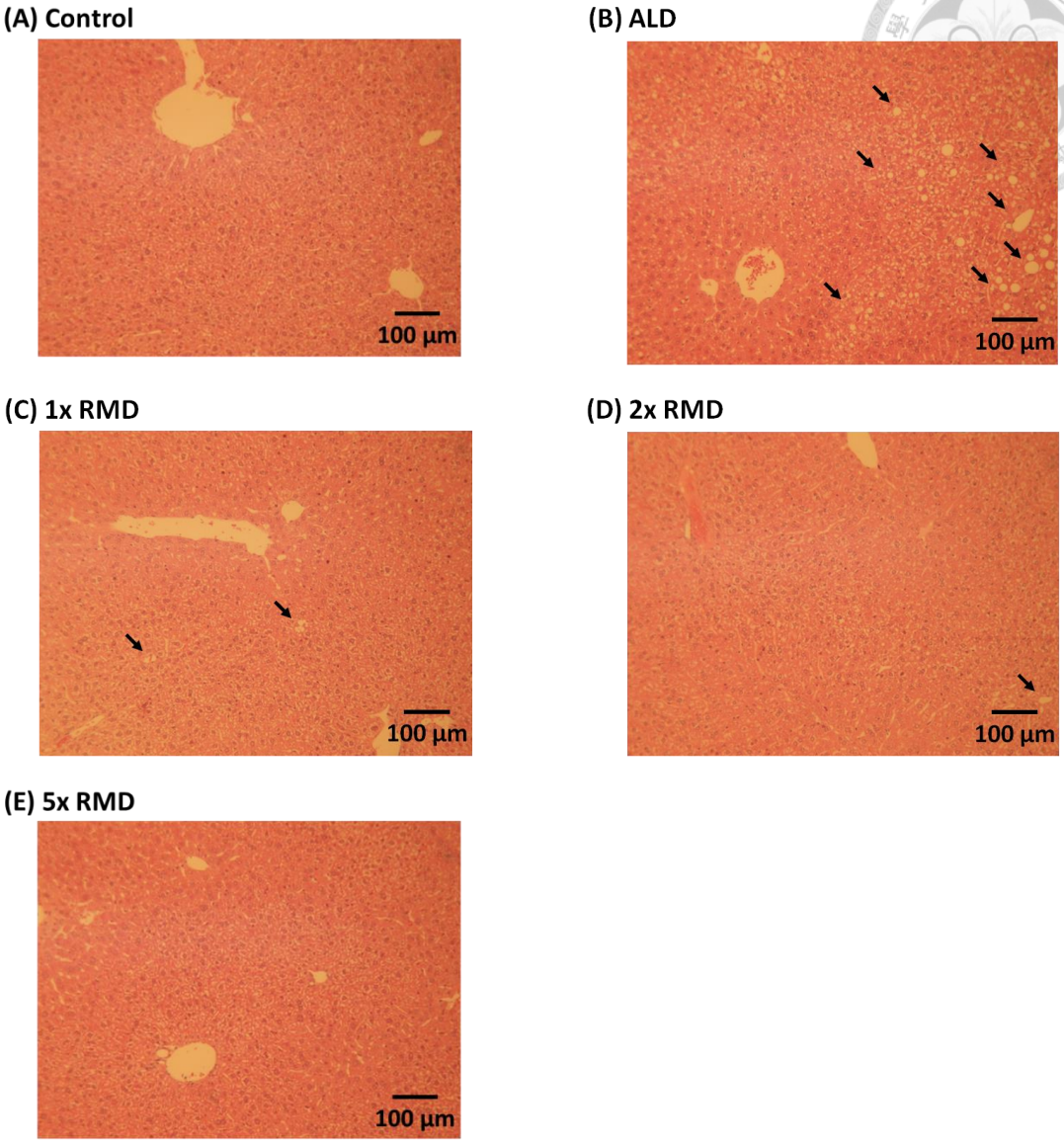

圖 4-4 紅麴山藥對於酒精性肝損傷組織之影響 (A) 控制組肝臟切片 (B) 酒精性肝損傷組肝臟切片 (C) 一倍紅麴山藥劑量組 (D) 兩倍紅麴山藥劑量組 (E) 五倍紅麴山藥劑量組。→標示為肝臟脂肪變性之氣泡狀組織。

Figure 4-4 Effects of RMD on liver histopathology of ALD mice using H&E staining: (A) normal control group; (B) ALD group; (C) 1× RMD group; (D) 2× RMD group; and (E) 5× RMD group. → Ultrastructural analysis depicting microvesicular and macrovesicular steatosis. (scale bar: 100 μm)

doi:10.6342/NTU201700799

Mallory bodies，由於細胞死亡造成發炎反應，其周邊聚集嗜中性白血球 (MacSween and Burt, 1986)。這些病徵在酒精性肝損傷中常見，其肝臟細胞腫大累積脂肪進而造成脂肪肝疾病，並且造成肝臟星狀細胞的活化產生肝臟纖維化，提高肝硬化及肝癌的發生率。由以上結果得知，紅麴米及紅麴山藥可以改善酒精性肝損傷及保護肝臟細胞之功效，且紅麴山藥於一倍劑量就有改善之效果，顯示紅麴山藥代謝物中之有效成分可能較紅麴米多。利用腎臟組織切片染色，觀察紅麴發酵物是否對於腎臟具有傷害，可發現酒精性肝損傷並不會造成腎臟破壞，而紅麴米及紅麴山藥也不具有腎臟毒性（圖 4-5 及 4-6）。

(三) 紅麴米及紅麴山藥改善酒精性肝損傷之功效

天門冬胺酸轉胺酶與丙胺酸轉胺酶為肝臟細胞中常見的酵素，在正常肝臟細胞中 AST 及 ALT 為胞內酵素，因此在血液中活性很低，當肝臟細胞受損則會釋放到血液中，使得血液中 AST 及 ALT 活性增高，所以可以藉由 AST 及 ALT 作為診斷肝臟健康狀態的指標。先前研究顯示，酒精性肝損傷的患者由於肝臟細胞死亡，因此其血清中 AST 可達到正常值兩倍以上，而血清中 ALT 活性較低或不受影響，且 AST 與 ALT 的比例通常大於 2，但酒精會導致肝臟中 ALT 活性降低 (Sidhu *et al*., 2017)。日常飲酒導致慢性肝損傷，可利用血清中肝臟酵素活性及組織病理學變化了解損傷情況。表 4-3 與 4-4 顯示紅麴米及紅麴山藥對酒精性肝損傷小鼠的保護作用，測量血清中 ALT 和 AST 的活性和 AST/ALT 比值可得知酒精性肝臟的損傷的情況。控制組血清中 ALT 和 AST 活性分別為 55.6 ± 4.9 及 81.3 ± 11.0 U/L。餵食酒精性飼料 5 週後，酒精性肝損傷組造成血清中 AST 的活性和 AST/ALT 比例上升，與控制組相比較 ALT 活性沒有顯著上升的情況，也符合酒精性肝損傷的病理性質。餵食兩倍及五倍劑量的紅麴米組，其血清中 AST 活性顯著下降 (104.0 ± 11.3 降至 75.1 ± 9.4 U / L)，而五倍劑量紅麴米組也可以降低 AST/ALT 比值 (AST/ ALT 比率從 1.9 ± 0.3 降至 1.5 ± 0.2 U/L)。然而在紅麴山藥的動物實驗中，也可發現其控制組，血清中 ALT 和 AST 活性分別為 56.6 ± 5.5 及 82.8 ± 13.2 U/L。酒精性肝損傷組血清中 AST 的活性和 AST/ALT 比例顯著上升，餵食紅麴山藥後，一倍、兩倍及五倍劑量組均有降低血清中 AST 活性的功效，而兩倍及五倍劑量也可以顯著降低 AST/ALT 比率。由以上實驗結果顯示，紅麴山藥一倍劑量即可顯著降低血清 AST 活性 ，

doi:10.6342/NTU201700799

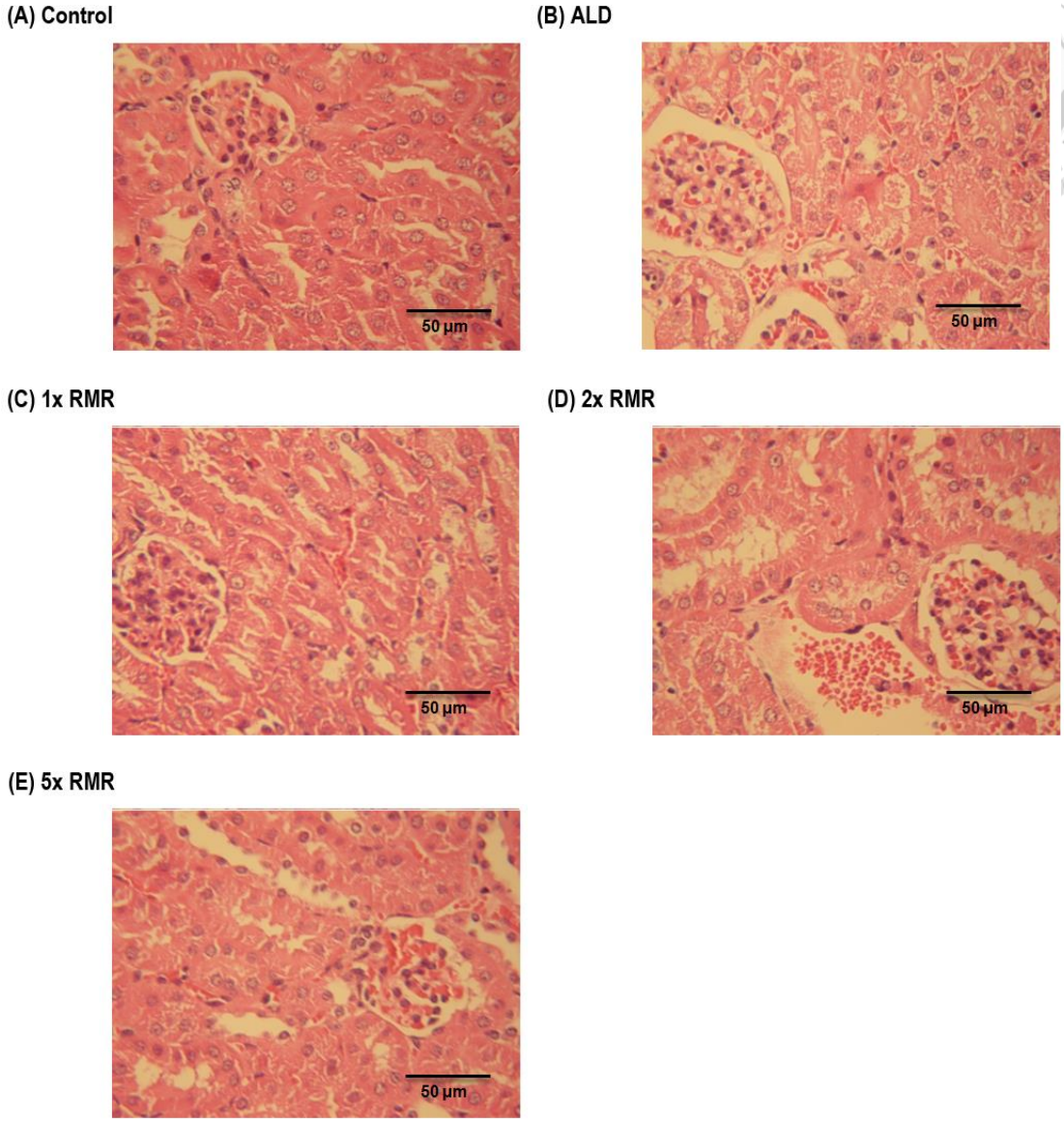

圖 4-5 紅麴米對於酒精性肝損傷之腎臟組織影響 (A) 控制組腎臟切片 (B) 酒精性肝損傷組腎臟切片 (C) 一倍紅麴米劑量組 (D) 兩倍紅麴米劑量組 (E) 五倍紅麴米劑量組。

Figure 4-5. Effects of RMR on kidney histopathology of ALD mice using H&E staining: kidney tissue of (A) control mice; (B) ALD group; (C) 1 × RMR group; (D) 2 × RMR group; (E) 5 × RMR group (scale bar = 50 μm).

doi:10.6342/NTU201700799

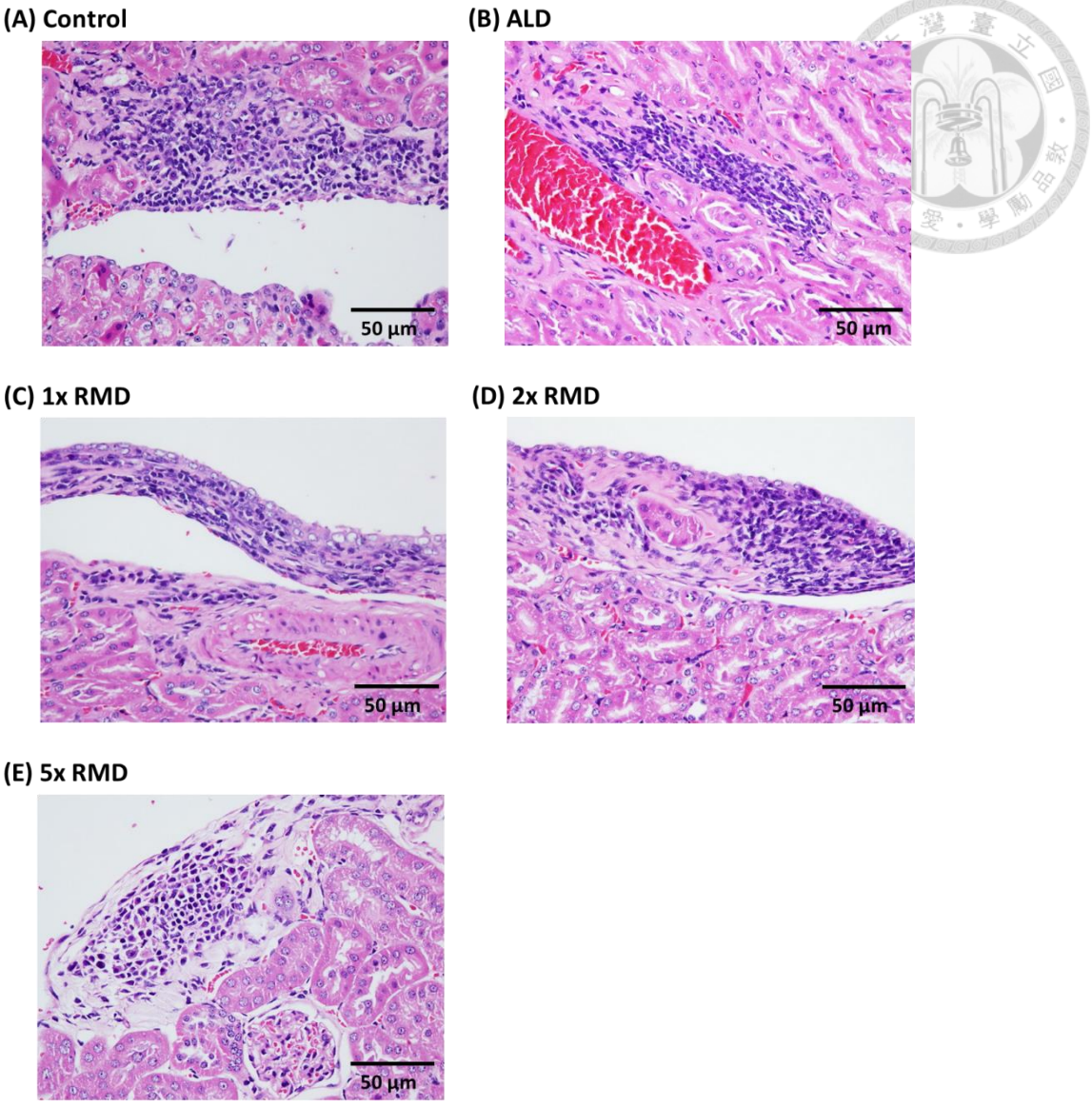

圖 4-6 紅麴山藥對於酒精性肝損傷之腎臟組織影響 (A) 控制組腎臟切片 (B) 酒精性肝損傷組腎臟切片 (C) 一倍紅麴山藥劑量組 (D) 兩倍紅麴山藥劑量組 (E) 五倍紅麴山藥劑量組。

Figure 4-6 Effects of RMD on kidney histopathology of ALD mice using H&E staining: kidney tissue of (A) control mice; (B) ALD group; (C) 1 × RMD group; (D) 2 × RMD group; (E) 5 × RMD group (scale bar = 50 μm).

doi:10.6342/NTU201700799

表 4-3 紅麴米對於酒精性肝損傷之小鼠改善肝臟功能評估

Table 4-3 Effect of RMR on serum enzymes and BUN in alcohol-induced liver damage in mouse

Groups	AST	ALT	AST/ALT	BUN
	U/L	U/L	ratio	mg/dL
Control	81.3 ± 11.0	55.6 ± 4.9	1.5 ± 0.2	20.4 ± 2.6
ALD	104.0 ± 11.3 *	57.8 ± 8.6	1.9 ± 0.3 *	20.5 ± 4.4
1xRMR	96.7 ± 12.2	53.8 ± 4.6	2.0 ± 0.3	21.2 ± 3.1
2xRMR	82.7 ± 10.3 #	52.2 ± 3.5	1.7 ± 0.2	23.8 ± 3.9
5xRMR	75.1 ± 9.4 #	51.4 ± 1.7	1.5 ± 0.2 #	19.5 ± 3.7

Results are expressed as the mean ± SD (n = 8). *, $P < 0.05$ with control group; #, $p < 0.05$ compared with ALD group. Control, normal control group; ALD, alcohol-induced disease group; RMR, red mold rice treatment; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

doi:10.6342/NTU201700799

表 4-4 紅麴山藥對於酒精性肝損傷之小鼠改善肝臟功能評估

Table 4-4 Effect of RMD on serum enzymes and BUN in alcohol-induced liver damage in mouse

Groups	AST	ALT	AST/ALT	BUN
	U/L	U/L	ratio	mg/dL
Control	82.8 ± 13.2	56.6 ± 5.5	1.5 ± 0.2	19.8 ± 2.7
ALD	107.4 ± 9.6*	58.1 ± 9.7	1.9 ± 0.3*	20.1 ± 4.1
1xRMD	88.4 ± 13.1#	52.6 ± 2.6	1.7 ± 0.3	18.5 ± 2.7
2xRMD	78.8 ± 7.0#	49.7 ± 3.2	1.6 ± 0.1#	19.2 ± 2.6
5xRMD	79.1 ± 8.5#	48.9 ± 2.7#	1.6 ± 0.1#	16.6 ± 2.7

Data presented as mean ± SD (n = 8). *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with ALD group. Control, normal control group; ALD, alcohol-induced disease group; RMD, red mold dioscorea treatment; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

doi:10.6342/NTU201700799

而兩倍劑量即可顯著降低 AST/ALT 比率，由此可知紅麴山藥效果較紅麴米為佳，具有保護肝臟細胞避免損傷的功效。檢測組織病理切片及血清測定顯示均無傷害腎臟的情況產生，因此顯示紅麴米及紅麴山藥對於酒精性肝損傷，具有肝臟保護功能且具有高安全性。

(四) 紅麴米及紅麴山藥增強肝臟中抗氧化酵素的能力

肝臟是代謝藥物及酒精的主要器官，肝臟中 CAT、SOD、GPx 和 GRd 在維持細胞中氧化還原平衡具有關鍵作用，並且酒精毒性與這些重要的抗氧化酶具有相關性 (Deleve, 1994)，這些抗氧化成分在肝臟中的活性可做為肝臟受損程度的評估。此外酒精代謝過程中會使得氧化壓力上升，在此狀態下 GSH 被轉化為 GSSG，導致 GSH 含量下降，造成脂質過氧化及肝臟細胞死亡，因此酒精的毒性與 GSH 的不足具有直接相關性 (Skibola and Smith, 2000; Galati *et al*., 2002)，此外先前的研究中指出，肝臟中若有 20% 或更多的 GSH 消耗則會導致組織損傷和發炎反應 (Yao *et al*., 2007)。酒精代謝除了造成氧化壓力上升外，同時造成肝臟內 CAT、SOD、GRd、GPx 和 GSH 的活性下降造成肝臟細胞受損，因此表 4-5 顯示酒精性肝損傷小鼠肝臟抗氧化酵素活性顯著比控制組低，餵食紅麴米及紅麴山藥後，其肝臟內抗氧化酵素 CAT、SOD、GRd 和 GPx 的活性都有顯著提升的效果，藉此可提升肝臟細胞抗氧化能力，避免細胞因氧化壓力而受損死亡。在五倍紅麴米劑量餵食組中，有顯著提升酒精性肝損傷小鼠肝臟 GSH/GSSG 比例的效果，但一倍及兩倍劑量則沒有顯著提升的效果，然而紅麴山藥餵食組，一倍、兩倍及五倍劑量都有顯著提升肝臟內 GSH/GSSG 比例的效果 (表 4-6)。由以上結果得知，一倍劑量紅麴山藥餵食組即可顯著提升肝臟 GSH/GSSG 比例，而紅麴米則需要五倍劑量才可達到顯著改善效果。ADH 為肝臟中代謝酒精的主要酵素 (Lieber, 2004)，酒精性肝損傷會降低此酵素在肝臟中活性使得酒精代謝異常 (Bhopale *et al*., 2006)，若提高此酵素活性並且配合 GSH 協助氧化還原反應進行，則使酒精代謝過程順暢 (Wu and Cederbaum, 2009)，因此測量紅麴山藥組肝臟中 ADH 的活性，發現本研究中紅麴山藥五倍劑量組也具有提升 ADH 活性的功效 (表 4-6)。由以上結果得知紅麴米及紅麴山藥都可提升肝臟內抗氧化酵素活性，而紅麴山藥在一倍劑量就可提升 GSH/GSSG 比例

doi:10.6342/NTU201700799

表 4-5 紅麴米對於酒精性肝損傷之小鼠提升肝臟抗氧化酵素功能

Table 4-5 Effect of red mold rice on antioxidase and GSH activities of liver in control and experimental animals

Groups	CAT	GPx	GRd	SOD	GSH/GSSG	ADH
	nmol H_2O_2/min/ mg protein	nmol NADPH/min/mg protein		U/mg protein	ratio	U/mg protein
Control	317.0 ± 90.8	19.2 ± 6.1	17.1 ± 6.2	23.0 ± 3.3	33.7 ± 6.5	141.5 ± 38.6
ALD	97.0 ± 35.7 *	13.3 ± 3.4 *	11.8 ± 3.2 *	19.6 ± 2.2 *	13.7 ± 8.6 *	87.5 ± 57.3*
1× RMR	207.4 ± 68.0 #	16.9 ± 0.7 #	14.1 ± 3.8 #	23.5 ± 4.1 #	19.3 ± 15.7	82.2 ± 52.5
2× RMR	218.9 ± 83.8 #	18.2 ± 1.8 #	15.3 ± 3.7 #	24.4 ± 7.2 #	22.3 ± 8.2	116.2 ± 49.6
5× RMR	251.5 ± 59.3 #	18.0 ± 4.2 #	15.4 ± 4.7 #	24.2 ± 12.8 #	24.7 ± 8.7 #	144.1 ± 41.5#

Data presented as mean ± SD (n = 8). *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with ALD group.

CAT, catalase; GPx, glutathione peroxidase; GRd, glutathione reductase; SOD, superoxide dismutase; GSH, glutathione; GSSG, glutathione disulfide; Control, normal control group; RMD, red mold rice treatment group; ALD, alcohol-induced disease group.

doi:10.6342/NTU201700799

表 4-6 紅麴山藥對於酒精性肝損傷之小鼠提升肝臟抗氧化酵素及酒精代謝酵素功能

Table 4-6 Effect of red mold dioscorea on antioxidase, GSH, and alcohol metabolism enzyme activities in the livers of control and alcohol-induced liver injury mouse models

Groups	CAT	GPx	GRd	SOD	GSH/GSSG	ADH
	nmol H_2O_2/min/ mg protein	nmol NADPH/min/mg protein		U/mg protein	ratio	U/mg protein
Control	297.2 ± 47.4	18.0 ± 1.7	16.8 ± 3.8	24.0 ± 3.5	35.8 ± 7.8	141.5 ± 38.6
ALD	73.0 ± 17.9*	13.0 ± 2.0*	12.0 ± 3.0*	17.5 ± 1.4*	10.7 ± 5.4*	87.5 ± 57.3*
1× RMD	164.2 ± 65.5#	19.0 ± 4.9#	14.6 ± 3.9#	24.7 ± 1.7#	21.3 ± 8.6#	95.3 ± 26.1
2× RMD	217.7 ± 108.6#	20.3 ± 4.0#	14.5 ± 3.7#	24.9 ± 4.6#	20.7 ± 11.2#	100.0 ± 29.1
5× RMD	240.0 ± 62.8#	19.0 ± 3.5#	14.3 ± 3.0#	24.2 ± 4.0#	24.7 ± 2.8#	138.0 ± 19.3#

Data presented as mean ± SD (n = 8). *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with ALD group.

CAT, catalase; GPx, glutathione peroxidase; GRd, glutathione reductase; SOD, superoxide dismutase; GSH, glutathione; GSSG, glutathione disulfide; ADH, alcohol dehydrogenase; Control, normal control group; RMD, red mold dioscorea treatment group; ALD, alcohol-induced disease group.

doi:10.6342/NTU201700799

並且在五倍劑量可提升 ADH 活性，可能具有提升肝臟內酒精的代謝能力並且避免肝臟細胞的死亡。

(五) 紅麴米及紅麴山藥肝臟脂質及血清生化數值評估

早期的慢性酒精性肝損傷及可觀察到脂質累積和脂肪變性，使得酒精代謝紊亂及脂肪激素下降 (You and Crabb, 2004a)，酒精造成肝臟內氧化壓力升高，並且刺激脂質生合成轉錄因子 SREBP-1 活化進入細胞核中進行轉錄，導致 FFA 及 TC 合成酶活化 (Browning and Horton, 2004)，造成脂質在肝臟細胞質中累積，而脂質累積的形式大多為 TG 及 FFA 而 TC 則會累積在粒線體內並且造成粒線體 GSH 活性降低 (Fernandez *et al.*, 2008)，因此，減少或防止肝臟中的脂肪蓄積為預防脂肪肝的主要步驟。酒精性肝損傷經由餵食紅麴米，可以有效降低肝臟 TG 含量，餵食兩倍及五倍劑量紅麴米，其肝臟 TC 顯著降低 (圖 4-7A 和 4-7B)。慢性酒精餵養小鼠血清中 TG 也顯著升高，紅麴米餵食後可以顯著降低血清中 TG 含量 (圖 4-7C)，而酒精性肝損傷不影響血清中 TC 含量，於各組間沒有顯著差異 (圖 4-7D)。圖 4-8 顯示肝臟中 TG、TC 及 FFA 數值，酒精性肝損傷組與控制組相比肝臟中 TG、TC 及 FFA 的含量顯著增加。在所有紅麴山藥樣品組中肝臟 TG 累積均顯著減少，然而，肝臟 TC 含量僅在兩倍及五倍劑量紅麴山藥組中顯著降低。此外，五倍劑量紅麴山藥組亦具有顯著降低肝臟 FFA 的功效。慢性酒精損傷除了造成胰島素拮抗和肝臟脂肪變性，也會導致脂肪組織發生發炎反應 (Sebastian *et al.*, 2011)。因此，酒精性肝損傷的致病機制應包括脂肪組織分泌和脂肪激素 (leptin、TGF-α、IL-6 及 adiponectin) 的分析，而酒精性肝損傷會導致 leptin 的表現量增加，並會增強肝臟發炎反應，同時促使肝臟星狀細胞活化產生 type I 膠原蛋白，增強活化星狀細胞的 TGF-β1 受器敏感度，最終使得肝臟纖維化的反應增強 (Purohit and Brenner, 2006)。Adiponectin 可以調節脂質、葡萄糖代謝以及肝臟細胞脂質的累積，並且可以避免酒精造成的肝臟腫大及脂肪肝的形成，具有保護肝臟的功能 (Bethanis and Theocharis, 2006)，adiponectin 表現量的上升則可以減緩肝臟損傷的程度 (Chuang *et al.*, 2004)。圖 4-9 顯示血清中 TG、TC、leptin 和 adiponectin 數值。各組血清中 TC 含量沒有顯著差異，而酒精性肝損傷組的血清 TG 含量顯著高於正常控制組。所有紅麴山藥樣品組血清 TG 含量顯著減低。

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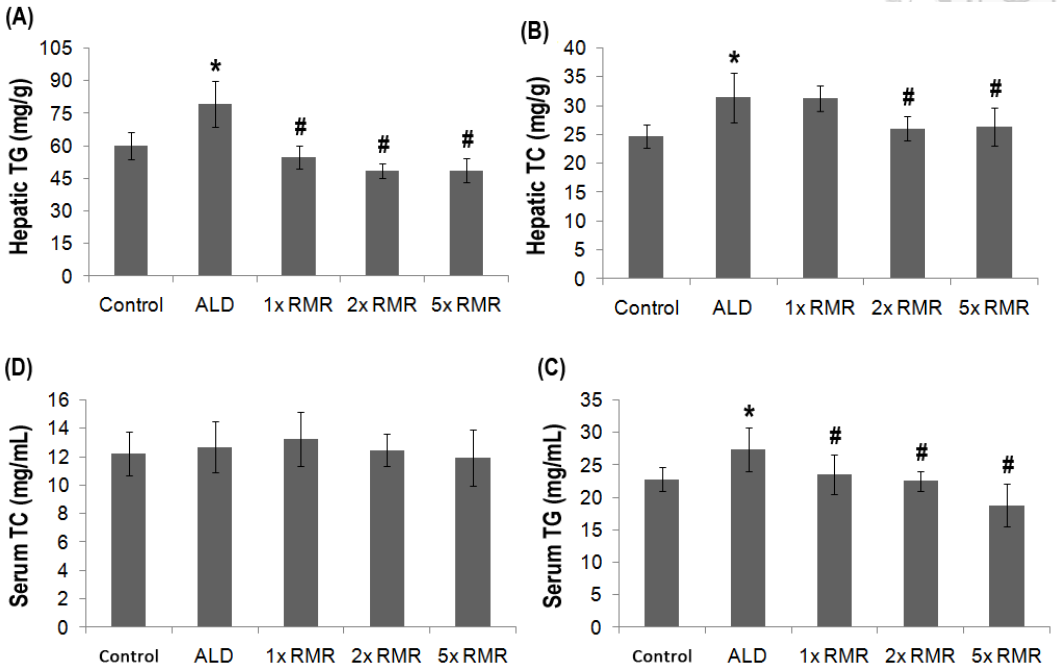

圖 4-7 紅麴米降低酒精性脂肪肝 (A) 肝臟內三酸甘油酯含量 (B) 肝臟內總膽固醇含量 (C) 血清中三酸甘油酯含量 (D) 血清中總膽固醇含量。

Figure 4-7 RMR alleviates alcohol-induced liver fatty level. (A) Hepatic triglyceride content (B) Hepatic total cholesterol content (C) Serum triglyceride content (D) Serum total cholesterol content. Results were expressed as the mean ± SD (n = 8), *P ＜ 0.05 compared with control group; #P ＜ 0.05 compared with ALD group. TG: triglyceride; TC: total cholesterol.

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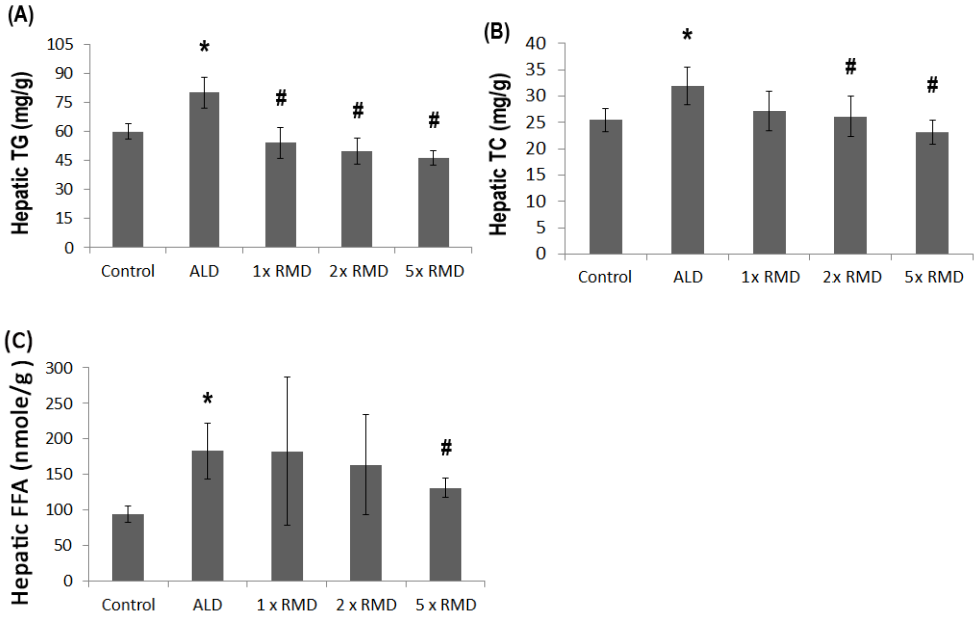

圖 4-8 紅麴山藥改善肝臟脂質累積之功效 (A) 肝臟內三酸甘油酯含量 (B) 肝臟內總膽固醇含量 (C) 肝臟中游離脂肪酸含量。

Figure 4-8 Effect of RMD supplementation on the hepatic TC, TG, and FFA levels in mice with alcoholic liver disease. (A) Hepatic triglyceride content (B) Hepatic total cholesterol content (C) Hepatic FFA. Results were expressed as the mean ± SD (n = 8), *P ＜ 0.05 compared with control group; #P ＜ 0.05 compared with ALD group. TG: triglyceride; TC: total cholesterol; FFA, free fatty acid.

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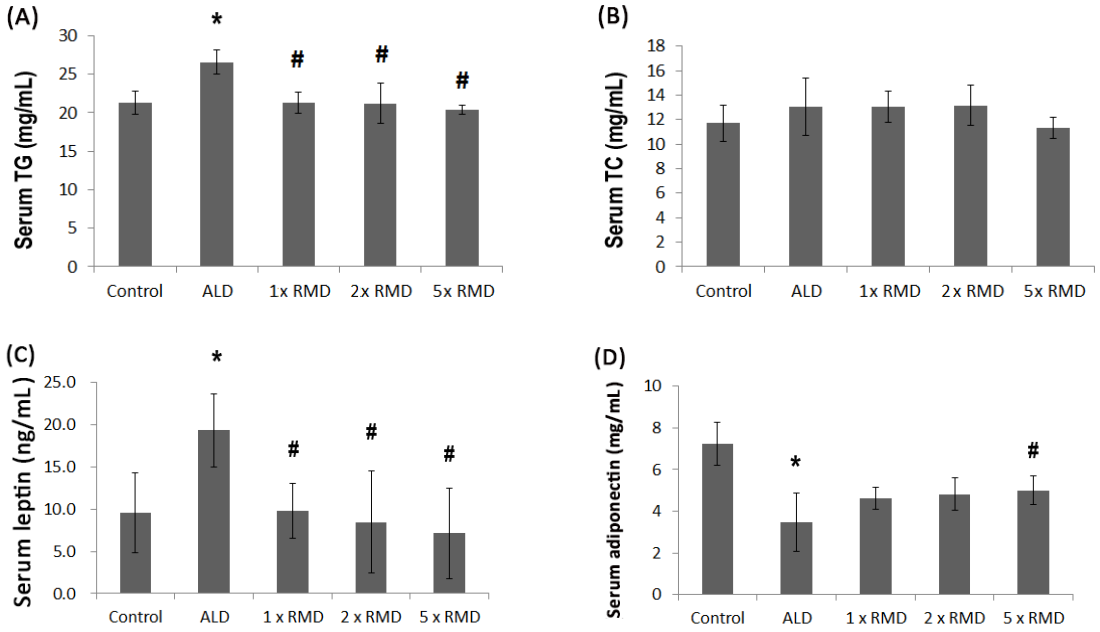

圖 4-9 紅麴山藥對血清脂質累積及瘦體素與脂聯素的影響 (A) 血清中三酸甘油酯含量 (B) 血清中總膽固醇含量 (C) 血清中瘦體素含量 (D) 血清中脂聯素含量。

Figure 4-9 Effect of RMD supplementation on the serum TC, TG, leptin, and adiponectin levels in mice with alcoholic liver disease. (A) Serum triglyceride content (B) Serum total cholesterol content (C) Serum leptin content (D) Serum adiponectin content. Results were expressed as the mean ± SD (n = 8), *P ＜ 0.05 compared with control group; #P ＜ 0.05 compared with ALD group. TG: triglyceride; TC: total cholesterol.

doi:10.6342/NTU201700799

酒精傷害會導致酒精性肝損傷組中血清 leptin 含量增加及 adiponectin 含量降低。所有紅麴山藥樣品組血清 leptin 濃度均降低，而餵食五倍劑量紅麴山藥，則顯著使血清中 adiponectin 濃度增加，顯示餵食紅麴山藥可以改善肝損傷。

(六) 紅麴米及紅麴山藥肝臟細胞激素影響

根據先前的研究，長期吸收酒精會導致腸道滲透性上升，造成內毒素由腸腔進入肝門靜脈血液，而造成持續性的發炎反應 (Urbaschek *et al*., 2001)。肝臟在先天免疫中具有重要作用，為穿過腸屏障的腸道微生物和內毒素提供第一道防線。Kupffer cells 為肝臟內的巨噬細胞可快速清除血液中的細菌。Kupffer cell 的活化釋放細胞激素和趨化因子 （TNF-α 和 IL-1β）以及 ROS 和 NO。在酒精性肝損傷中 TNF-α 和 IL-6 的增加會導致肝硬化發生機率 (Daniluk *et al*., 1996)。此外，活化的肝星狀細胞 (hepatic stellate cell, HSC) 也在肝損傷期間釋放許多細胞激素，其中 TGF-β 被認為是主要造成肝臟纖維化的細胞激素。細胞激素和趨化因子的過度表現，使得白血球聚集造成持續性的發炎反應，而進一步使肝臟纖維化 (Friedman, 2004; Bataller and Brenner, 2005)。因此降低發炎反應對修復肝臟相當重要。在此動物模式中，酒精性肝損傷的小鼠其肝臟內 IL-6、IL-1β、TNF-α 和 TGF-β 含量顯著比控制組高，但紅麴米餵食後可顯著降低肝臟 IL-6、IL-1β 和 TNF-α 含量而兩倍及五倍劑量紅麴米可降低肝臟 TGF-β 含量 (圖 4-10)。各紅麴山藥劑量組均可以有效降低 IL-6、IL-1β、TNF-α 及 TGF-β 含量 (圖 4-11)，由於 TGF-β 可造成肝臟星狀細胞的活化，並造成肝臟纖維化，由以上數據可得知對於 TGF-β 的抑制作用，紅麴山藥有更顯著的效果，總和以上實驗結果，紅麴米及紅麴山藥代謝產物亦具有抑制肝臟星狀細胞的效果。

(七) 利用 HPLC 測定紅麴米及紅麴山藥的二次代謝物含量

未發酵山藥具有許多生理活性，餵食山藥具有改善脂肪代謝 (Chen *et al*., 2003) 及改善肝腎毒性 (Lee *et al*., 2002) 的功效，但其研究中使用的山藥餵食量 (30-90 g/kg) 遠超過本研究使用劑量，或是採用山藥萃取物餵食以達到有效劑量。然而山藥及米經過 *M. purpureus* NTU 568 紅麴菌種發酵後，使用低劑量餵食就

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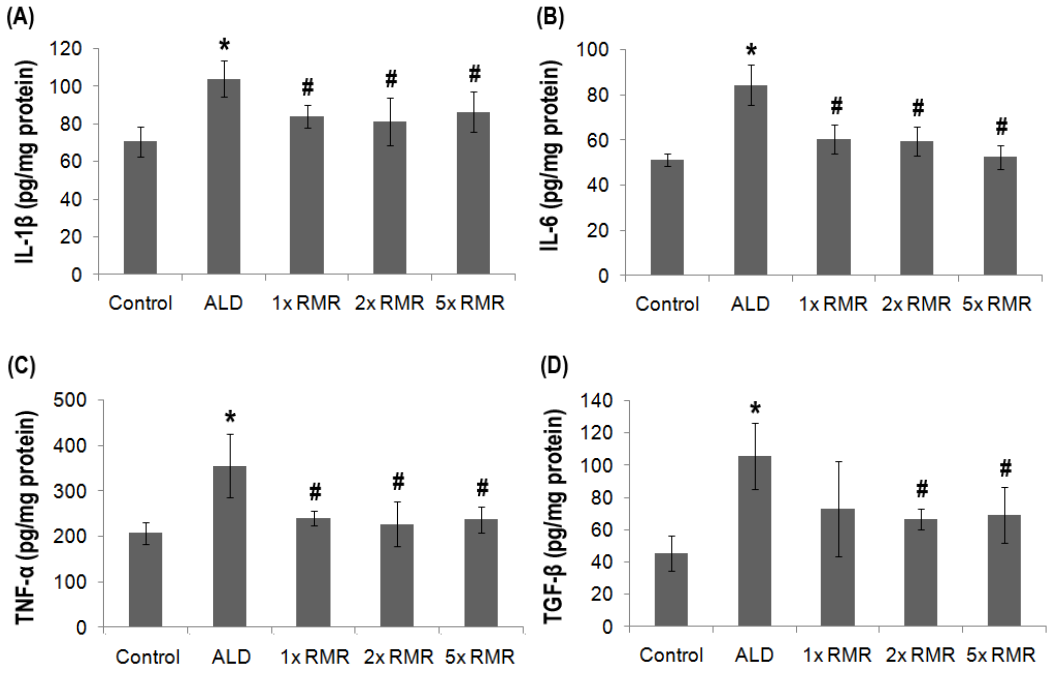

圖 4-10 紅麴米對酒精性肝損傷動物肝臟中細胞激素的影響。

紅麴米可降低細胞激素（A）IL-1β，（B）IL-6，（C）TNF-α 和（D）TGF-β

Figure 4-10 Effects of RMR on inflammatory cytokine expression in the liver from experimental animals. RMR attenuates (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) TGF-β in experimental animals. Data presented as mean ± SD (n = 8). *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with ALD group. Control, normal control group; ALD, alcohol-induced disease group; RMR, red mold rice treatment group; IL: interleukin; TNF: tumor necrosis factor; TGF: transforming growth factor.

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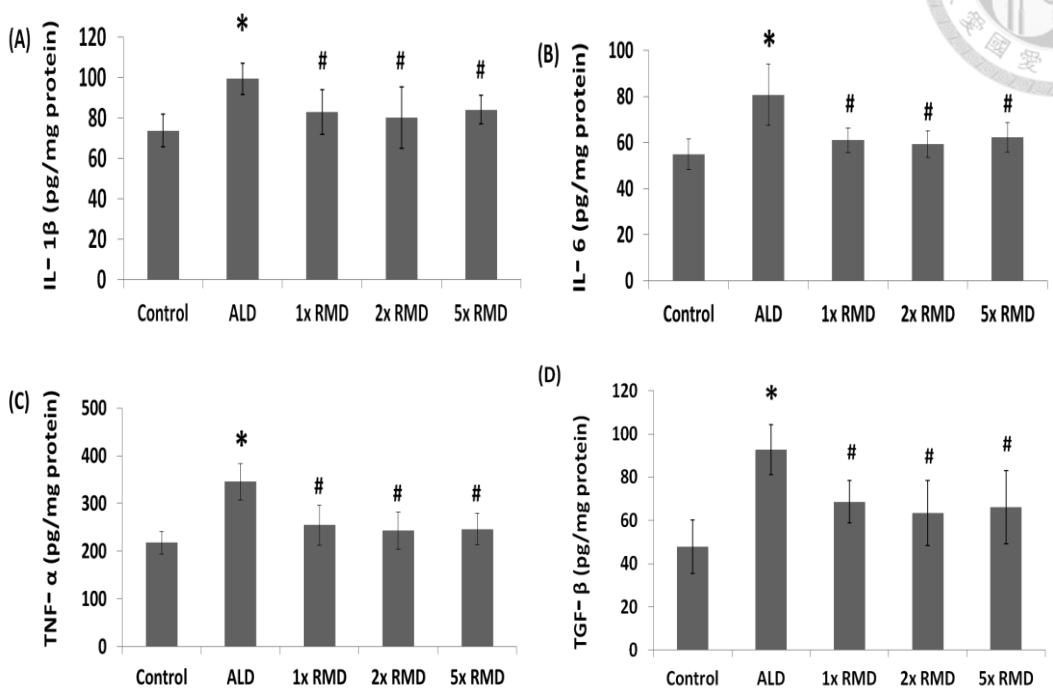

圖 4-11 紅麴山藥對酒精性肝損傷動物肝臟中細胞激素的影響。

紅麴山藥可降低細胞激素（A）IL-1β，（B）IL-6，（C）TNF-α 和（D）TGF-β

Figure 4-11 Effects of RMD on inflammatory cytokine expression in the liver from experimental animals. RMD attenuates (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) TGF-β levels in a chronic alcoholic liver model. Data presented as mean ± SD (n = 8). *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with ALD group. Control, normal control group; ALD, alcohol-induced disease group; RMD, red mold dioscorea treatment group; IL: interleukin; TNF: tumor necrosis factor; TGF: transforming growth factor.

doi:10.6342/NTU201700799

可顯著改善酒精造成的肝臟損傷，因此猜測經過 *M. purpureus* NTU 568 紅麴菌種發酵後，其發酵產物具有改善酒精性肝損傷的功效成分。在先前研究中發現，使用不同基質其最佳條件不同，主要發酵產物含量也不同 (Lee *et al*., 2007b)，本研究使用不同基質但同一條件下進行發酵，發現餵食紅麴山藥組別在體重回復、血清 AST/ALT、GSH/GSSG 比值及細胞激素 TGF-β 的數據都比紅麴米更具有減緩酒精性肝損傷的功效，並利用 HPLC 分析測量兩種基質發酵後其功效成分的差異，可發現紅麴山藥的總黃色素較高 (表 4-7)，其中紅麴山藥 MS 的含量約是紅麴米的 1.66 倍。總和以上實驗結果顯示，紅麴山藥比紅麴米具有更顯著改善酒精性肝損傷的功能，可能因為基質不同，而山藥發酵後可產生大量的黃色素，因此我們分離黃色素 (AK 及 MS) 利用體外細胞實驗證實其功效，並且了解其改善酒精性肝損傷的分子路徑。

(八) 紅麴發酵產物 AK 及 MS 對慢性酒精性肝損傷之影響

酒精使得肝臟細胞膜通透性改變，鈣離子不平衡肝臟細胞的死亡，因此酒精性肝損傷細胞模式，為慢性誘導細胞累積三酸甘油酯並且造成細胞死亡，而非急性快速死亡 (Schanne *et al*., 1981)，因此我們利用 HepG2 細胞在 100-500 mM 的酒精濃度範圍內培養 24 小時後，測定 HepG2 細胞的存活率及三酸甘油酯含量作為實驗劑量篩選 (圖 4-12A-C)。發現在乙醇誘導下於 300 mM 濃度下，其細胞存活率沒有顯著降低但三酸甘油酯含量顯著上升，因此在後續實驗中選擇乙醇濃度 300 mM，模擬慢性酒精性肝損傷細胞模式。由實驗室先前未發表的論文得知 MS 與 AK 之口服生物可利用率分別為 15.67% 與 20.36%，MS 與 AK 皆可分佈至肝臟中。換算發酵產物中含有 MS 與 AK 的純物質含量分別為，一倍、兩倍及五倍劑量的紅麴米中含有 MS 劑量為 8.5 μg、17.0 μg 及 42.6 μg，而紅麴山藥含有 MS 劑量為 14.2 μg、28.4 μg 及 70.9 μg。一倍、兩倍及五倍劑量紅麴米含有 AK 劑量為 13.3 μg、26.6 μg 及 66.6 μg，而紅麴山藥含有 AK 劑量為 13.6 μg、27.1 μg 及 67.8 μg。經由生物可利用率計算 (MS 最小劑量 8.5 μg x 15.67% 至最大劑量 70.9 μg x 15.67%) MS 劑量範圍約為 1.3 μg 到 11.1 μg (3.6 μM 到 31 μM) 而計算 (AK 最小劑量 13.3 μg x 20.36% 至最大劑量 67.8 μg x 20.36%) AK 範圍約為 2.7 μg 到 13.8 μg (7.0 μM 到 35.7 μM)，因此利用此劑量範圍進行細胞存活率實驗。利用含有乙醇濃度 300 mM 的培養基與 MS 或 AK 藥物 (5 μM、10 μM 和 20 μM) 共培養 24 小時後，發現 MS 及 AK 於 5 μM 和 10 μM 濃度下不會對細胞存活率造成顯著抑制 (圖 4-12C)，因此選用 5 μM 和 10 μM 濃度作為後續實驗劑量。

doi:10.6342/NTU201700799

表 4-7、紅麴米及紅麴山藥中二次代謝產物含量

Table 4-7 Production of various secondary metabolites by *Monascus*-fermented red mold rice and red mold dioscorea

	MS (mg/kg)	AK (mg/kg)	Citrinin (mg/kg)	Red pigment (A_{500}/g)	Orange pigment (A_{470}/g)	Yellow pigment (A_{400}/g)
RMR	1385 ± 242	2166 ± 177	ND	168.5 ± 6.6	109.2 ± 14.0	224.1 ± 31.6
RMD	2307 ± 252 *	2205 ± 144	ND	147.7 ± 25.3	156.8 ± 17.2	324.8 ± 57.2

Data presented as mean ± SD (n = 3). ND, non-detected. *, $P < 0.05$ compared with RMR group

MS: monascin; AK: ankaflavin

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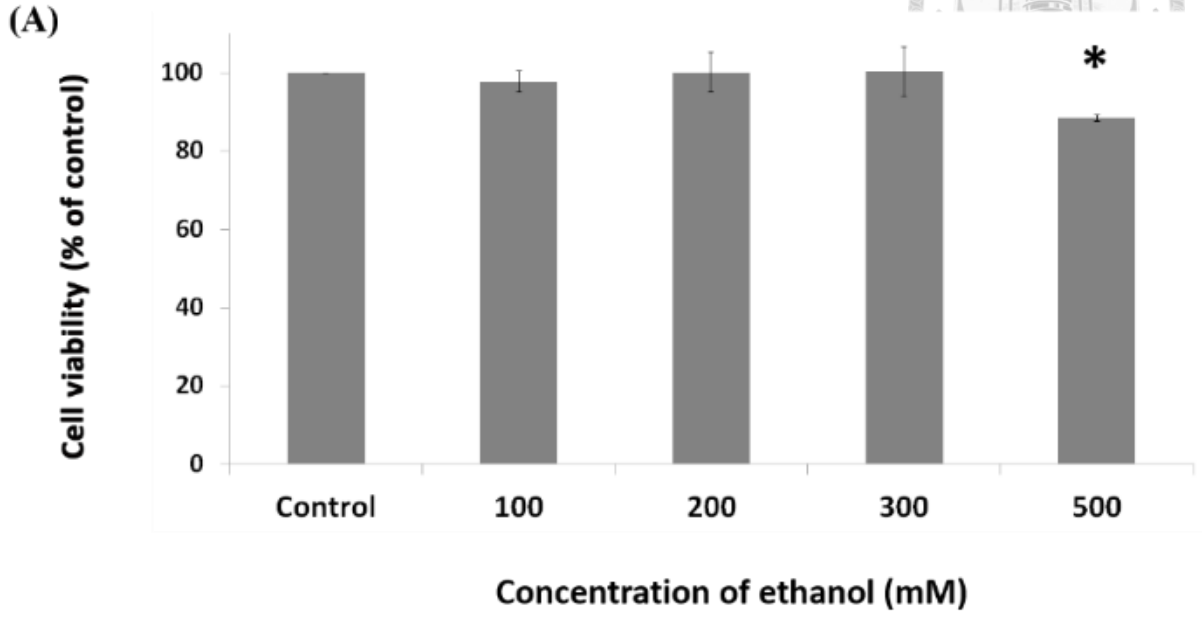

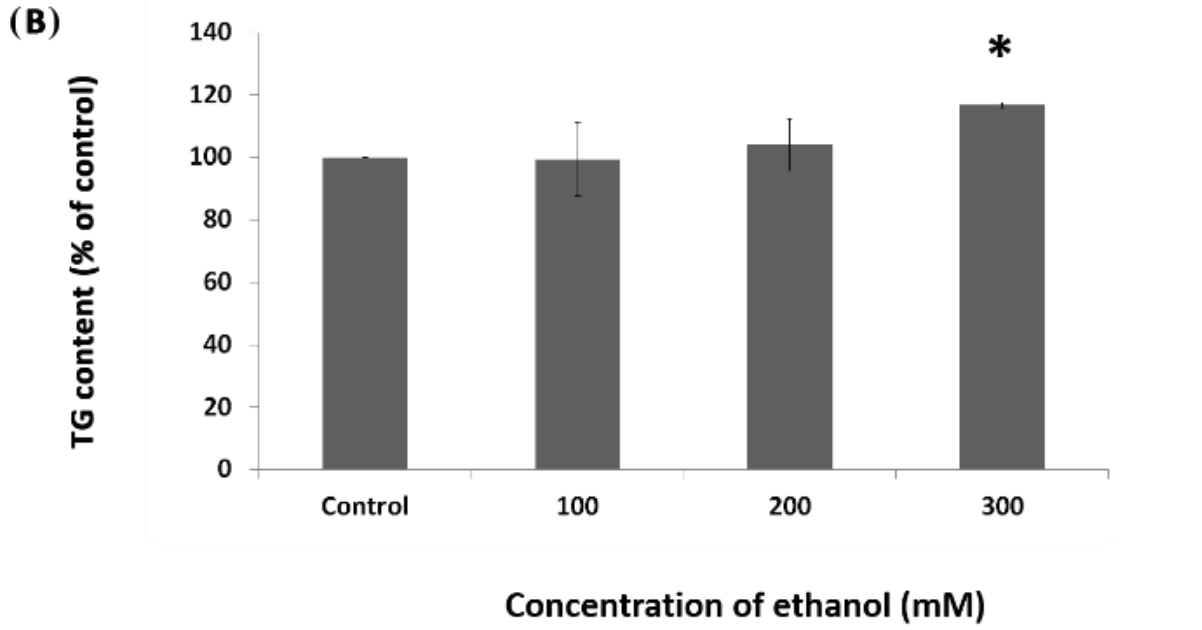

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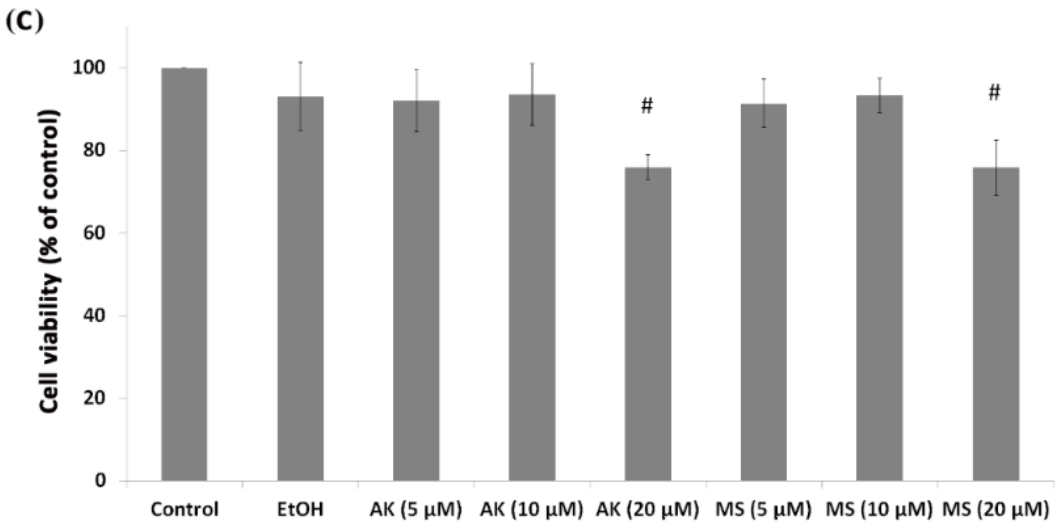

圖 4-12 乙醇及紅麴發酵產物 AK 及 MS 對於肝臟細胞 HepG2 影響 (A) 用不同劑量的乙醇處理 HepG2 細胞，並利用 MTT 測定乙醇毒性 (B) 不同劑量乙醇處理後，HepG2 細胞內三酸甘油酯含量 (C) 乙醇 300 mM 與不同劑量 (5、10 和 20 μM) 的 AK 和 MS 處理 HepG2 細胞培養 24 小時。

Figure 4-12 Effect of ethanol on triglycerol accumulation and cell viability in the absence and presence of AK or MS. (A) HepG2 cells were treated with different doses of ethanol (mM) and the cytotoxicity evaluated by the MTT assay. (B) triglycerol quantification in cells exposed to different doses of ethanol (mM). (C) HepG2 cells were treated with 300 mM ethanol (EtOH) alone or combined with varying doses (5, 10, and 20 μM) of AK or MS for 24 h. Data presented as mean ± SD (n = 3). *, $P < 0.05$ compared to the control; #, $P < 0.05$ compared to the EtOH. MS: monascin; AK: ankaflavin.

doi:10.6342/NTU201700799

(九) 紅麴發酵產物 AK 及 MS 提升轉錄因子 PPAR-γ 並降低 SREBP-1 及三酸甘油酯含量

長期的酒精攝取會造成多種器官的傷害包括酒精性肝損傷、胰臟炎和高血壓，酒精代謝的過程中由酒精去氫酶將乙醇代謝成乙醛並減少 nicotinamide adenine dinucleotide (NADH) 含量，並經由 cytochrome-P450 2E1 協助酒精代謝，造成 ROS 增加及氧化壓力上升，這些因子促使脂質生合成基因啟動，而 PPARs 和 SREBPs 則為脂肪生合成重要的轉錄因子 (Ansari *et al.*, 2016)。PPARs 為細胞核內受器家族，包含有三亞種 PPAR-α、PPAR-γ 及 PPAR-β/δ 與脂肪酸代謝及脂質生合成相關 (Grygiel-Gorniak, 2014)，而 PPAR-γ 可經由基因剪切產生 γ1 及 γ2 兩種異構物 (Vidal-Puig *et al.*, 1997)，除了為脂質生合成重要的轉錄因子，並且對脂肪細胞成熟、脂肪累積及 insulin-sensitive glucose transporter 相當重要 (Fajas *et al.*, 1999)。PPAR-γ 主要高度表現在脂肪細胞、造血細胞及肝臟星狀細胞，而肝臟細胞的表現量較低 (Spiegelman, 1998)，雖然肝臟細胞的表現量不高，但研究指出活化 PPAR-γ 可抑制 NF-κB，減少巨噬細胞及 Kupffer cells 產生 IL-6, TNF-α, IL-1β 及 iNOS，並且抑制發炎反應 (Desreumaux *et al.*, 2001; Ricote *et al.* 1998; Uchimura *et al.*, 2001)。先前研究指出肥胖及營養過剩會導致肝臟中 PPAR-γ 表現增加 (Boelsterli and Bedoucha, 2002)，但長期酒精攝取的大鼠實驗則是導致肝臟中 PPAR-γ 降低 (Wan, 1995)，而動物實驗中 PPAR-γ agonists pioglitazone 則可以抑制肝臟 Kupffer cells 的 NF-κB 活性降低細胞激素的分泌，達到改善酒精性肝損傷及發炎反應的功效 (Enomoto *et al.*, 2003)。本研究室之前發現 MS 為 PPAR-γ activator 並且可抑制肝臟星狀細胞 (Hsu *et al.* 2013b)，因此我們利用 AK 及 MS 與乙醇同時處理 HepG2 cells，發現單獨乙醇 300 mM 處理組會造成細胞核中轉錄因子 PPAR-γ 表現量顯著下降，而 AK 及 MS 處理後則會使得細胞核內 PPAR-γ 表現量上升 (圖 4-13A)，因此推論 AK 及 MS 會促使轉錄因子 PPAR-γ 進入細胞核中，並且保護肝臟細胞避免酒精的傷害。

SREBPs 為脂肪及膽固醇重要的調控轉錄因子 (Shimano, 2009)，此蛋白質家族具有三種異構物 SREBP-1a、SREBP-1c 及 SREBP-2，而 SREBP-1 基因經過選擇性剪切產生 SREBP-1a 及 SREBP-1c 兩種異構物，可經由調控 fatty acid synthase、stearoyl-CoA desaturase 及 ATP citrate lyase 等酵素活性，來調節肝臟的三酸甘油酯合成 (Eberle *et al,*. 2004)，而 SREBP-2 則是利用 low-density lipoprotein receptor、HMG-CoA synthase、HMG-CoA reductase 及 squalene synthase 調節膽固醇的代謝 (Shimano *et al.*, 1997;

doi:10.6342/NTU201700799

Horton *et al.*, 1998; Pai *et al.*, 1998)，研究指出酒精處理大鼠肝臟細胞及 HepG2 cells 後，因代謝酒精過程產生乙醛，刺激 SREBP 活性並且抑制酒精去氫酶的活性造成酒精性肝損(Lluis *et al.*, 2003; You *et al.*, 2002)。在本研究中 300 mM 乙醇處理組使得細胞核內 SREBP-1 含量上升，而 AK 及 MS 處理後則會使得 SREBP-1 含量顯著下降(圖 4-13B)，同時肝臟細胞內三酸甘油酯的含量也顯著的下降，達到抑制肝臟累積 TG 的功效 (圖 4-13C)。由此實驗得知兩種純物質可以提升 PPAR-γ 活性，達到抑制發炎反應及保護肝臟的功能，而 SREBP-1 則被顯著抑制達到脂肪肝的預防，因此，這兩種純物質均具有抵抗乙醇誘導肝臟細胞毒性及 TG 累積的保護效果，同時細胞實驗結果與動物實驗吻合，因此推論 AK 和 MS 為預防酒精性肝損傷的有效活性成分。然而酒精性肝損傷除了造成肝臟細胞死亡外，更進一步的導致肝臟星狀細胞活化，產生纖維化膠原蛋白使得肝臟纖維化，並且增加肝硬化及肝癌的發生機率，肝硬化為不可修復的肝臟損傷，但肝臟纖維化是可逆及可修復的疾病，因此肝臟纖維化時期的減緩及預防格外重要。減緩肝臟纖維化首要抑制肝臟星狀細胞的活化及發炎反應，而轉錄因子 PPAR-γ 對於肝臟星狀細胞相當重要，促進其活性可以導致肝臟星狀細胞的 NF-κB 被抑制，使得肝臟星狀細胞活性下降，降低肝臟纖維化發生機率 (Hazra *et al.*,2004)，先前的研究指出 MS 為 PPAR-γ 的活化劑 (Hsu *et al.* 2013b)，在本研究中發現 AK 及 MS 可以有效的提升 PPAR-γ 的活性，具有減緩降低酒精性肝損傷及脂肪肝的功效。在接下來的實驗，我們將利用有效成分 AK 和 MS 進行肝臟纖維化的細胞實驗，研究分子路徑驗證紅麴代謝物保護肝臟的功效。

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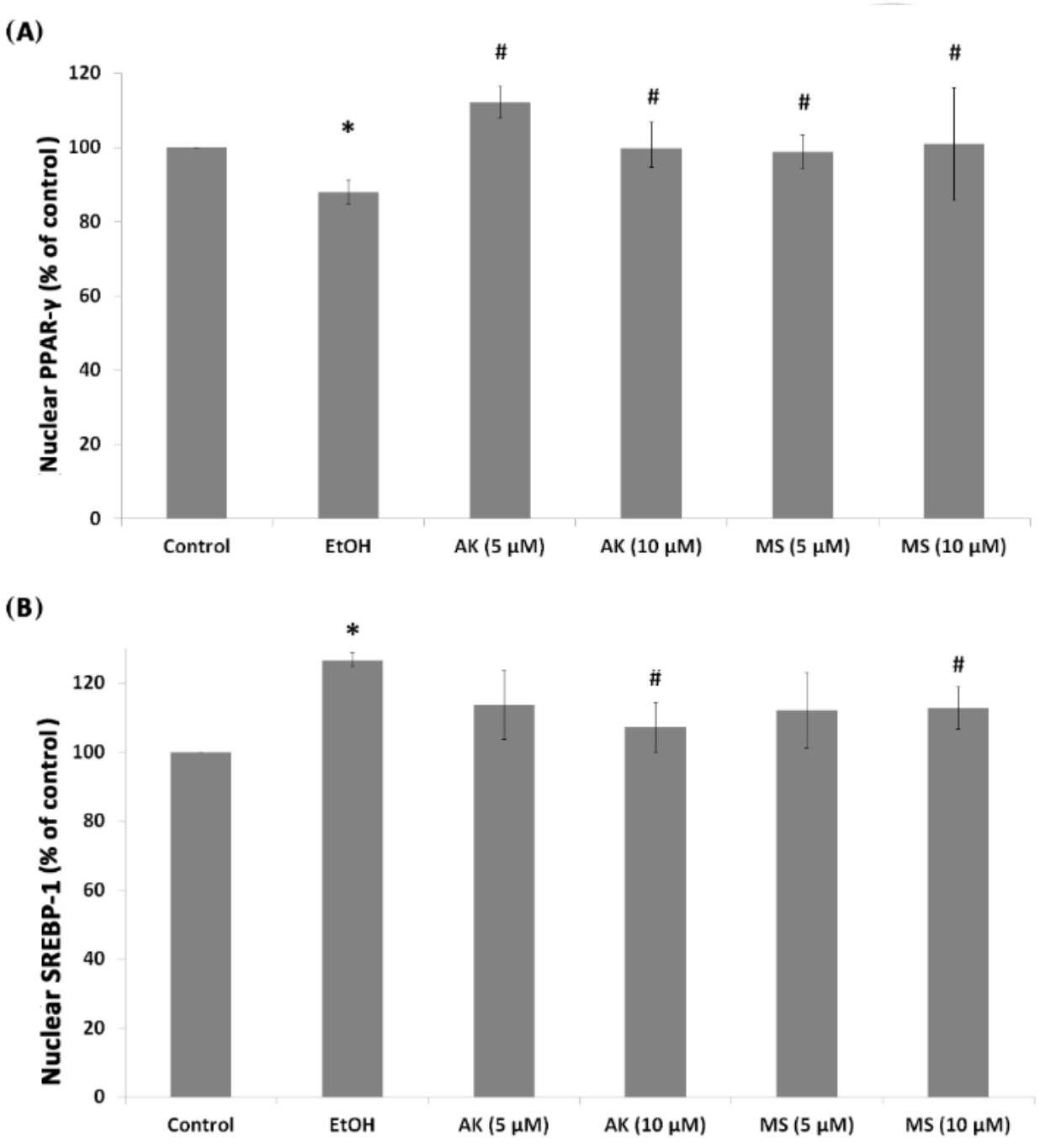

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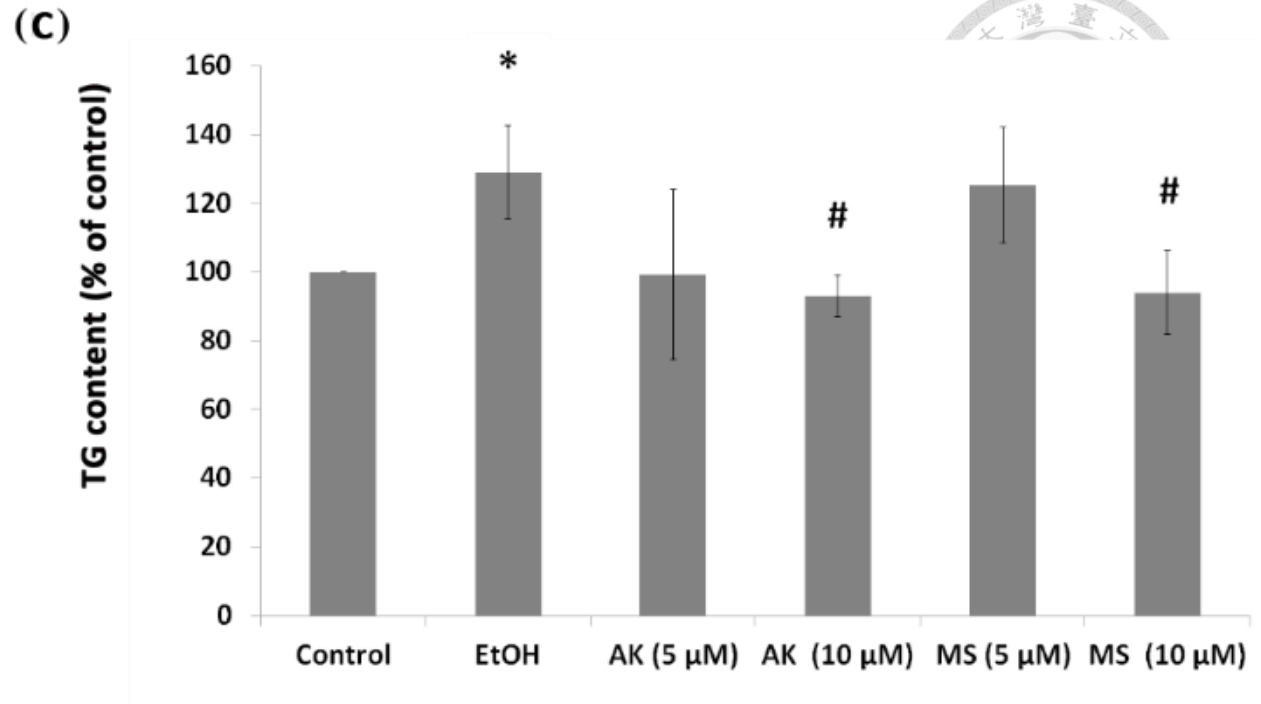

圖 4-13 紅麴發酵產物 AK 及 MS 調控轉錄因子 PPAR-γ、SREBP-1 及三酸甘油酯累積 (A) 乙醇 300 mM 與不同劑量 (5 和 10 μM) 的 AK 或 MS 處理 HepG2 細胞 24 小時後，測量核內轉錄因子 PPAR-γ 及 (B) 核內轉錄因子 SREBP-1 活性 (C) 細胞內三酸甘油酯含量。

Figure 4-13 Effect of ethanol on transcripition factors and triglycerol accumulation in the absence and presence of AK or MS. (A) Nuclear transcripition factor PPAR-γ and (B) SREBP-1 protein expression in HepG2 cells incubated for 24 hr in presence of 300 mM ethanol (EtOH) alone or combined with AK or MS (5 and 10 μM) for 24 h. (C) Triglycerol quantification in cells exposed to 300 mM ethanol (EtOH) alone or combined with AK or MS (5 and 10 μM) for 24 h. Data presented as mean ± SD (n = 3). *, $P < 0.05$ compared to the control; #, $P < 0.05$ compared to the EtOH. MS: monascin; AK: ankaflavin.

doi:10.6342/NTU201700799

二、紅麴發酵產物 AK 及 MS 誘導肝臟星狀細胞凋亡分子路徑探討

(一) AK 和 MS 對於 HSC-T6 細胞及肝臟初代細胞的影響

肝臟星狀細胞的活化及增生是造成肝臟纖維化的主要因素，本研究主要探討 AK 及 MS 抗纖維化功效及抑制肝臟星狀細胞的分子路徑。因此利用 HSC-T6 細胞分別處理 6、15 或 30 μM 的 AK 或 MS 24 小時，可發現 AK 和 MS 在濃度 30 μM 顯著抑制 HSC-T6 細胞增生，並且 MS 抑制效果顯著較 AK 高（圖 4-14A）。此外，我們也實驗 AK 及 MS 對於肝臟細胞是否具有毒性，因此分離肝臟初代細胞處理 AK 和 MS（6、15 和 30 μM 濃度） 24 小時，進行 AK 和 MS 的細胞毒性評估 (圖 4-14B)。實驗證明經由 AK 和 MS 處理 24 小時，不會影響肝臟細胞存活。AK 和 MS 能夠減少活化 HSC 的增生，並且不會造成肝臟細胞的損傷，因此我們使用濃度 15 和 30 μM 進行以下的研究。由以上結果得知，AK 及 MS 可以減低 HSC-T6 的細胞存活率，但不影響正常肝臟細胞的生理功能，因此這兩種純物質具有治療肝臟纖維化的潛力。

(二) AK 和 MS 對於 HSC-T6 細胞週期的影響

細胞週期分為兩大階段:間期 (inter phase) 及細胞分裂期 (mitosis phase, M phase)，其又可分為 G1、S、G2 期，而在細胞週期中具有三個檢查點 (checkpoints) 來決定細胞週期是否進入至下一時期。當 DNA 受損時，細胞週期便無法通過檢查點的檢查而停滯 (cell cycle arrest)，此時細胞內會進行 DNA 的修復動作，一旦修復完成才會進入下一個時期，若無法修補錯誤時，細胞則走向細胞凋亡 (apoptosis)。為了研究 AK 和 MS 抑制活性肝臟星狀細胞的機制，利用含有 0.5% 胎牛血清的培養基培養細胞，將細胞週期統一於 G1 期，經過樣品處理後，以流式細胞儀測定各組細胞週期的影響。細胞與 15 或 30 μM 的 AK 和 MS 共培養 0、6、12 或 24 小時，發現 AK 和 MS 可以將 HSC-T6 細胞停滯於 G1 期，並且具有時間和劑量的相關性 (表 4-8)。用 30 μM 的 AK 和 MS 處理 HSC-T6 細胞 24 小時後，其細胞週期 G1 期的 DNA 含量測定為 76.1% 及 79.9%，而控制組的 G1 期百分比則為 65.9%，顯示 AK 和 MS 可顯著升高細胞 G1 週期的含量，

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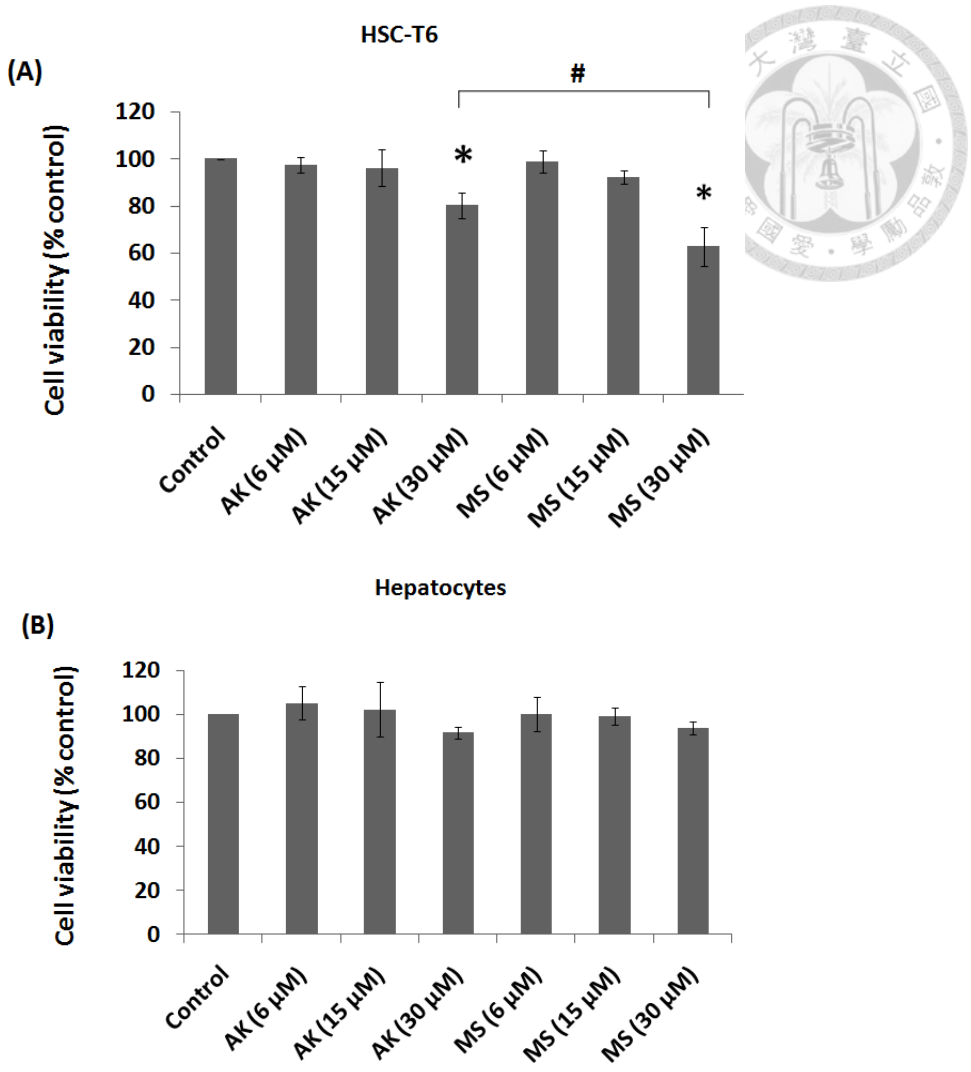

圖 4-14 AK 及 MS 對於肝臟星狀細胞 HSC-T6 與肝臟初代細胞的毒性分析 (A) HSC-T6 (B) 肝臟初代細胞以 AK 及 MS (6、15 或 30 μM) 處理 24 小時的細胞存活率。

Figure 4-14 AK and MS-induced HSC-T6 and rat primary hepatocyte cell death. (A) HSC-T6 and (B) primary hepatocytes were treated with 6, 15, or 30 μM AK or MS for 24 h, and the cell viability was tested using MTT assay. The obtained results are expressed as mean ± standard deviation (SD; n = 3). *, $P < 0.05$, compared with the control group; #, $P < 0.05$, between the two indicated groups. AK, ankaflavin; MS, monascin.

doi:10.6342/NTU201700799

表 4-8 AK 及 MS 誘導 HSC-T6 細胞週期停滯

Table 4-8 AK and MS induce HSC-T6 cell cycle arrest. MS induces G1 phase cell cycle arrest of HSC-T6 cells. Cells were stained with propidium iodide and analyzed using flow cytometry. Results are expressed as mean ± standard deviation (SD; n = 3).

	Control		
Time (h)	G0/G1 (%)	S (%)	G2/M (%)
0	89.6±2.3	6.5±2.0	2.9±1.2
6	83.8±3.2	12.5±3.4	3.7±1.1
12	67.2±3.9	18.6±1.9	14.2±2.7
24	65.9±5.0	20.7±3.7	13.4±3.6

	AK (30 μM)			AK (15 μM)		
Time (h)	G0/G1 (%)	S (%)	G2/M (%)	G0/G1 (%)	S (%)	G2/M (%)
6	83.0±1.6	13.8±1.1	3.2±0.7	82.6±1.2	14.0±2.6	3.3±0.9
12	75.6±5.3	15.0±2.5	9.4±2.5	65.1±4.5	21.5±2.6	13.4±3.0
24	76.1±2.9	15.7±2.5	8.2±0.5	64.8±1.2	22.3±2.8	12.9±4.6

	MS (30 μM)			MS (15 μM)		
Time (h)	G0/G1 (%)	S (%)	G2/M (%)	G0/G1 (%)	S (%)	G2/M (%)
6	83.4±1.2	13.4±0.4	3.0±0.2	82.5±2.7	14.1±1.7	3.4±0.2
12	76.1±3.8	14.6±1.2	9.3±2.9	67.2±3.0	20.2±2.3	13.0±2.1
24	79.9±1.8	15.8±3.1	4.3±0.3	72.3±1.6	16.9±2.2	10.8±1.7

AK: ankaflavin; MS: monascin

doi:10.6342/NTU201700799

而 HSC-T6 細胞在 S 和 G2/M 週期中 DNA 含量百分比也相應減低 (圖 4-15A 和 4-15B)。此外凋亡後的細胞會產生凋亡小體，其 DNA 被切成片段，此時若用 PI 染色細胞核，進行流式細胞儀檢測，觀察其 G0/G1 期前的 sub-G1 期 PI 螢光量會增加，則可得知是否為細胞凋亡。AK 和 MS（30 μM）處理導致 sub-G1 期的細胞數量增高，因此 AK 和 MS 可誘導細胞週期停滯及細胞凋亡現象 (圖 4-15C)。Cyclins 與 CDKs (cyclin-dependent kinases) 為調控細胞週期的主要分子，當 CDKs 與 cyclins 結合形成複合體 (cyclin-CDK complexe) 時，CDKs 具有蛋白質磷酸化激酶的活性，才可磷酸化其下游的分子進行細胞分裂。但當細胞 DNA 受到傷害、缺氧 (hypoxia) 或是致癌因子 (oncogene) 活化的形況下，會促使 p53 大量活化表現。p53 的功能包括有：細胞 DNA 的修復、細胞週期的停止與細胞凋亡 (apoptosis) 等的生理反應。而 p53 主要的下游調控分子是 CDKIs 家族中的 $p21^{CIP1/WAF1}$，p53 的活化會使得 $p21^{CIP1/WAF1}$ 於細胞內的表現量增加，導致細胞週期的停滯 (el-Deiry, 1998)。因此，我們利用西方墨點法分析細胞週期調節蛋白的變化，進一步確認 AK 和 MS 可誘導 HSC-T6 細胞凋亡（圖 4-15D），實驗結果顯示 AK 和 MS 於 15 μM 濃度時，可促使 p53 蛋白質表現量顯著增加，而 30 μM 濃度則進一步誘導 p53 下游蛋白 p21 顯著上升，並抑制細胞分裂重要蛋白質 cyclin D1 的產生，造成細胞週期停滯（圖 4-15E）。

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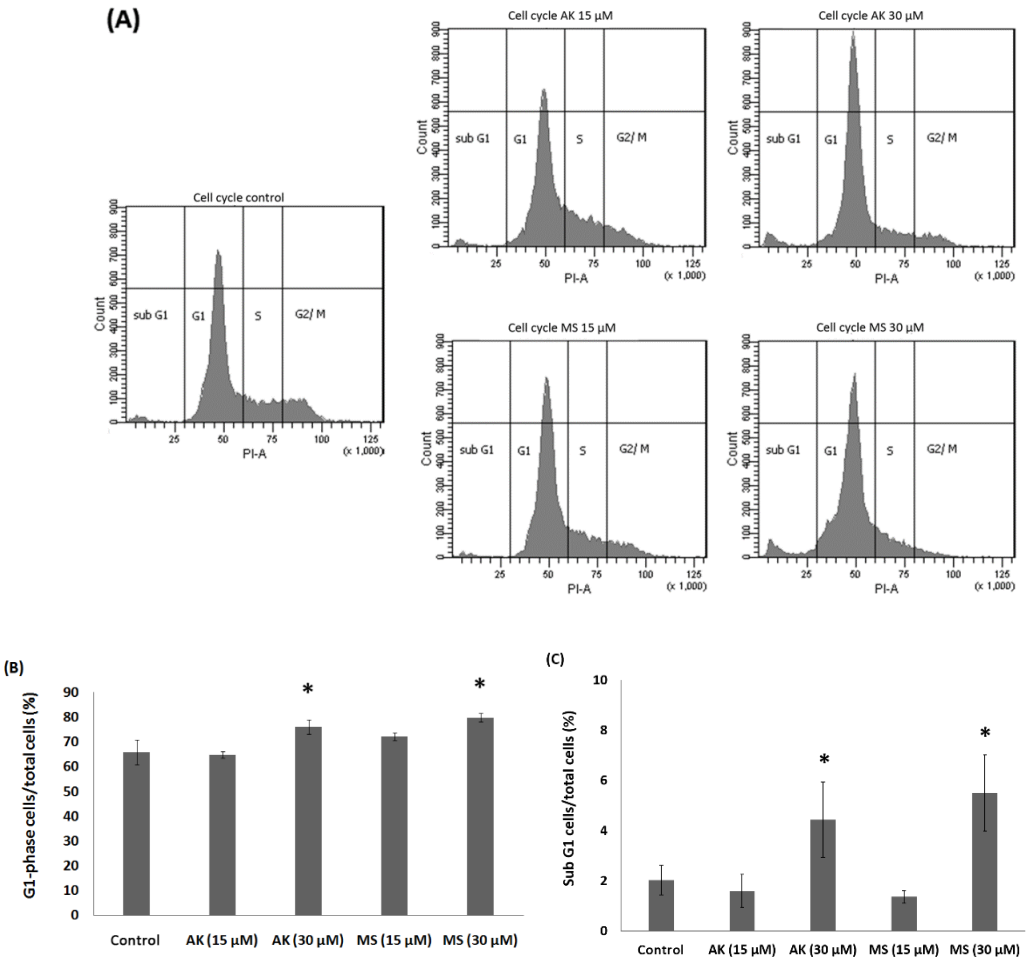

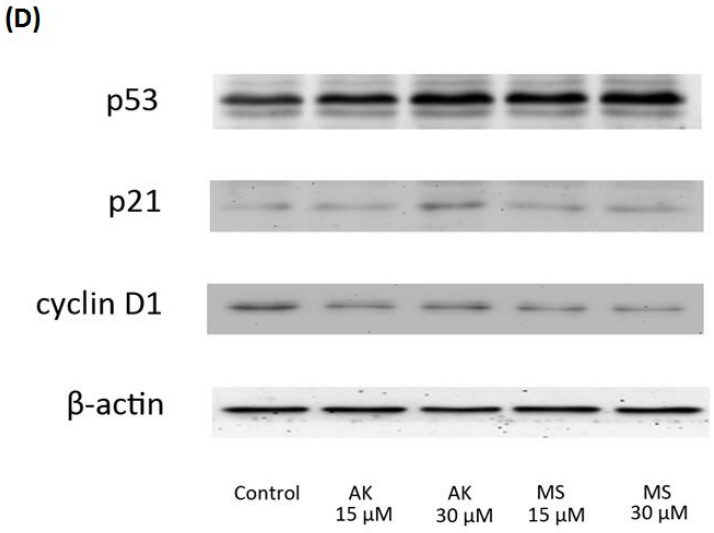

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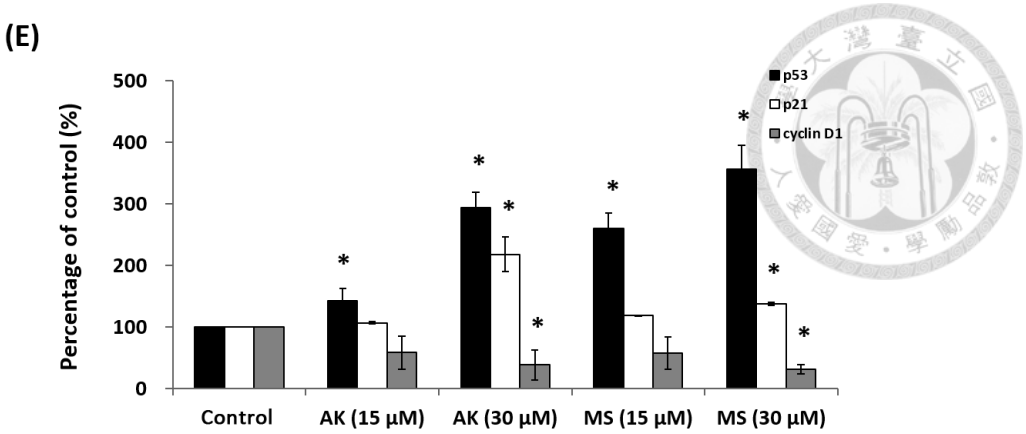


圖 4-15 AK 及 MS 誘導 HSC-T6 細胞週期停滯 (A) HSC-T6 細胞流式細胞儀測定細胞週期 (B) 細胞週期 G1 期百分比 (C) sub-G1 期百分比 (D) 西方墨點法測定 p53、p21 和 cyclin D1 蛋白質含量 (E) 蛋白質含量量化圖。

Figure 4-15 AK and MS induce HSC-T6 cell cycle arrest. (A) HSC-T6 cells, stained with propidium iodide (PI), sorted and analyzed using flow cytometry, following their treatment with AK or MS (15 and 30 μM) for 24 h. (B) The changes in the percentage of cells in G1 phase, following the treatment with AK or MS. (C) Dose-dependent changes in the population of cells in sub-G1 phase. (D) Western blot analysis of p53, p21, and cyclin D1 levels, after the treatments. (E) Densitometric analysis of band intensities. Data are expressed as the fold of control. All obtained results were normalized to β-actin levels. Results are expressed as mean ± standard deviation (SD; n = 3). *, $P < 0.05$, compared with the control. AK, ankaflavin; MS, monascin.

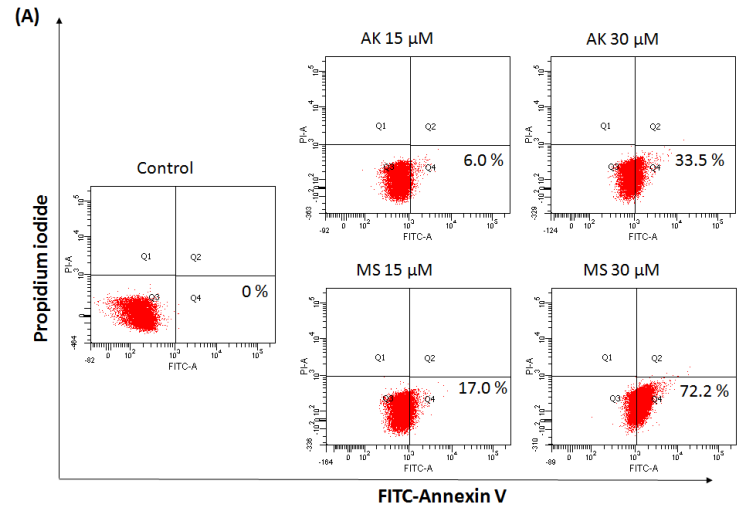
doi:10.6342/NTU201700799

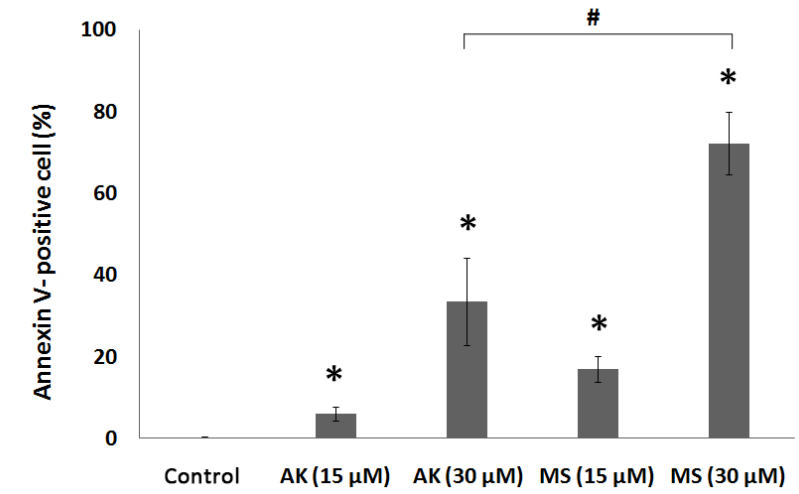
(三) AK 和 MS 誘導 HSC-T6 細胞凋亡

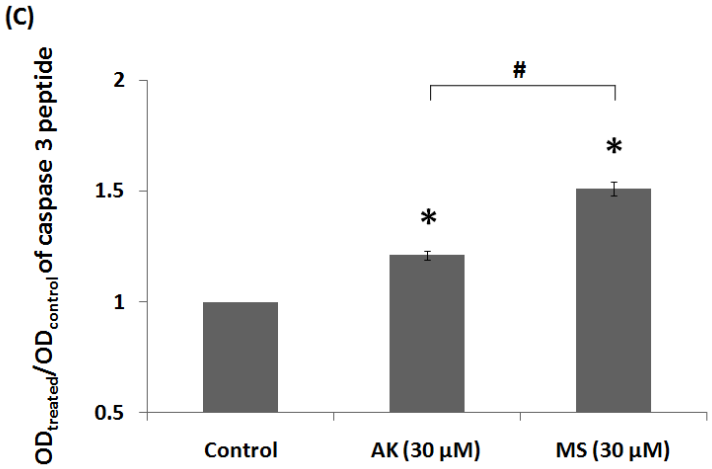
正常細胞中，細胞膜脂質雙層的內膜具有 phosphatidylserine (PS) 成分，在細胞凋亡早期，會造成細胞膜翻轉，使得細胞膜內膜的 PS 由膜內側翻向外側。Annexin V 與 PS 有高度親和力，可與細胞外側暴露的 PS 結合。因此 Annexin V 被作為細胞早期凋亡的檢測方法之一。利用流式細胞儀確認 AK 和 MS 可否誘導細胞凋亡，將 annexin V FITC/PI 染色劑與細胞染色測定，可發現經過 AK 和 MS 處理 24 小時的細胞顯著提升細胞凋亡的數量，並且具有劑量效應（圖 4-16A 及 4-16B）。

當細胞受到外部的訊號或內部的損壞，如：DNA 損傷，而引起 p53 分子大量表現，並經由活化 Bax 來抑制一些 anti-apoptotic family (例如：Bcl-2 與 Bcl-xL) 對粒線體的保護，並使得粒線體的外膜不穩定，造成 cytochrome c 的釋出，與 Apaf-1 及 procaspase-9 形成 apoptosome (Evan and Vousden, 2001)，進而使 caspase-3 活化來裂解細胞中的重要蛋白質，而達到細胞凋亡的目的。利用濃度 30 μM 的 AK 和 MS 進行 24 小時處理後，發現可顯著提升細胞凋亡蛋白 caspase-3 活性 (圖 4-16C)。此外，Bcl-2 蛋白質家族對於 HSCs 存活具有關鍵作用，活化的 HSC 會經由表現 Bcl-2 來抵抗促凋亡因子，這特性在慢性肝纖維化的發展中具有重要作用 (Novo *et al.*, 2006)。因此我們分析了 Bcl-2 和 Bax 蛋白質含量，如圖 4-16D 和 4-16E 所示，Bcl-2 含量經由 AK 和 MS (30 μM) 處理後顯著降低，而 MS (30 μM) 具有顯著提升 Bax 蛋白表現功能。由以上結論，可以確定 AK 和 MS 確實具有誘導活化肝臟星狀細胞凋亡的功能，並且影響多種蛋白質導致細胞凋亡，因此我們進一步利用西方墨點法，研究細胞凋亡的分子路徑。

doi:10.6342/NTU201700799







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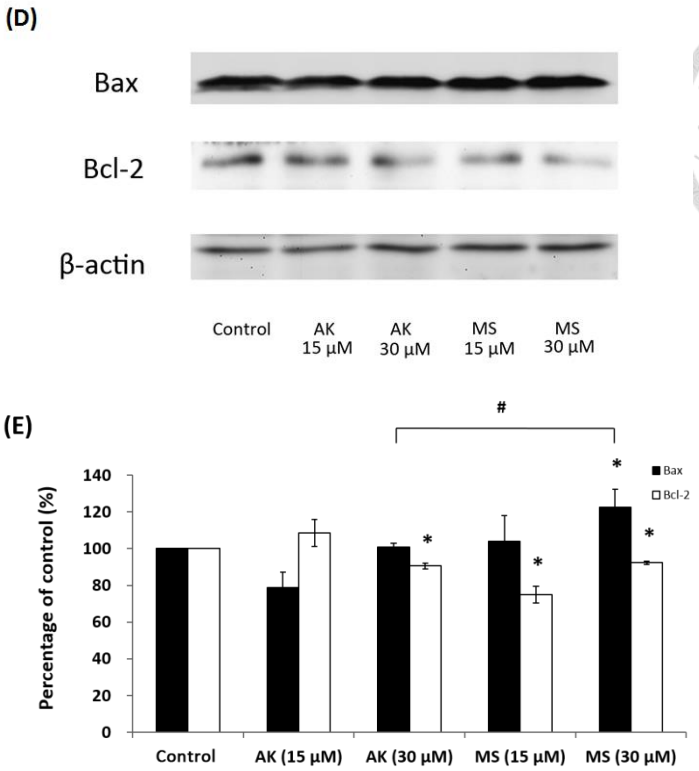

圖 4-16 AK 及 MS 誘導 HSC-T6 細胞凋亡分析 (A) 流式細胞儀分析 Annexin V-FITC 含量 (B) Annexin V-FITC 含量量化 (C) AK 或 MS (30 μM) 處理 24 小時後 caspase 3 活性測定 (D) 西方墨點法測定 Bax 和 Bcl-2 蛋白質含量 (E) 蛋白質含量量化圖。

Figure 4-16 AK and MS induce the apoptosis of HSC-T6. (A) Flow cytometry analysis of cells stained with Annexin V-FITC and propidium iodide (PI). (B) The percentage of annexin V positive cells, following treatments with AK or MS. (C) Caspase 3 activity was measured and represented by $OD_{treated}/OD_{control}$ ratio, after the exposure to AK or MS (30 μM) for 24 h. (D) Bax and Bcl-2 levels were analyzed by Western blotting, while β-actin was used as a loading control. (E) Densitometric quantification of band intensity. Data are expressed as the fold of control. All obtained results were normalized to β-actin levels. Results are expressed as mean ± standard deviation (SD; n = 3). *, $P < 0.05$, compared with the control group; #, $P < 0.05$, between the two indicated groups. AK, ankaflavin; MS, monascin

doi:10.6342/NTU201700799

(四) AK 和 MS 影響 HSC-T6 細胞 PI3K/Akt 分子路徑及 NF-κB 活性

由我們先前研究中得知，MS 會活化轉錄因子 PPAR-γ，並通過活化 PPAR-γ 影響 HSCs 的活化 (Hsu *et al.*, 2013b)，PPAR-γ 已被確定為許多蛋白質的重要因子，包括 Akt、NF-κB、p38 MAPK、ERK 和 JNK 分子路徑，並且為 HSC 活化與否的重要分子開關 (Zhang *et al.*, 2012)。PI3K/Akt 和 IκB/NF-κB 分子路徑在肝臟的纖維化形成具有關鍵作用。PI3K 的活化可刺激其關鍵下游 Akt 分子，並抑制細胞凋亡且刺激 HSC 活化增殖。PI3K/Akt 分子路徑的活化與否，為 HSC 增殖、存活、凋亡、代謝和膠原蛋白表現的關鍵因素。因此 PI3K/Akt 的持續活化與肝纖維化的發病機制相關 (Reif *et al.*, 2003; Liu *et al.*, 2006)。AK 和 MS 對 PI3K/Akt 分子路徑的影響，如圖 4-17A 和 4-17B 所示，利用西方墨點法測定磷酸化 Akt 及 Akt 含量，可發現經由 AK 和 MS (30 μM) 處理後，磷酸化 Akt 表現顯著下降而磷酸化 Akt/Akt 比例也顯著下降。此外，Bcl-2 蛋白家族的成員會受到 NF-κB 的影響 (Barkett and Gilmore, 1999)，並且調節 HSC-T6 細胞凋亡和細胞存活，經由細胞激素刺激 HSC 的活化反應，也會受到 NF-κB 基因調節的影響，這些因素與肝臟發炎反應相關，並且其表現在臨床診斷上也具有重大關係 (Hellerbrand *et al.*,1998)。當被細胞激素例如 TNF-α 刺激時，IκB 被 IKK 磷酸化並降解，釋放 NF-κB，其從細胞質進入細胞核中，並活化其目標基因的轉錄 (Szuster-Ciesielska *et al.*, 2011)，因此，抑制 NF-κB 活性被認為是抗纖維化治療的良好策略。在圖 4-14A 中，AK 和 MS 顯著降低 NF-κB 表現並增加 IκB 表現。而於 30 μM 濃度下 MS 與 AK 相比，可顯著提升 IκB 的表現 (圖 4-17C)。

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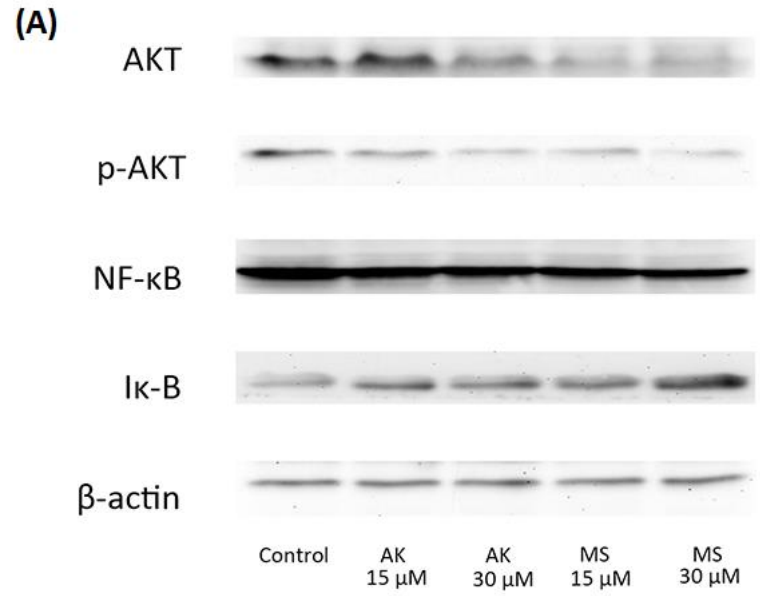

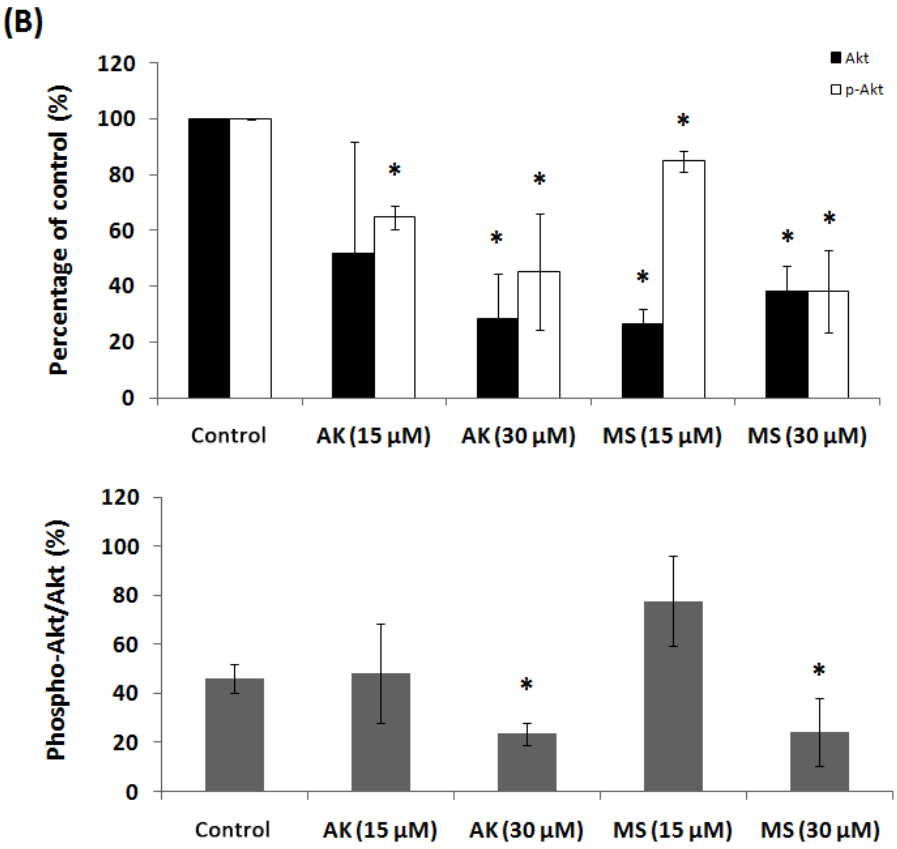

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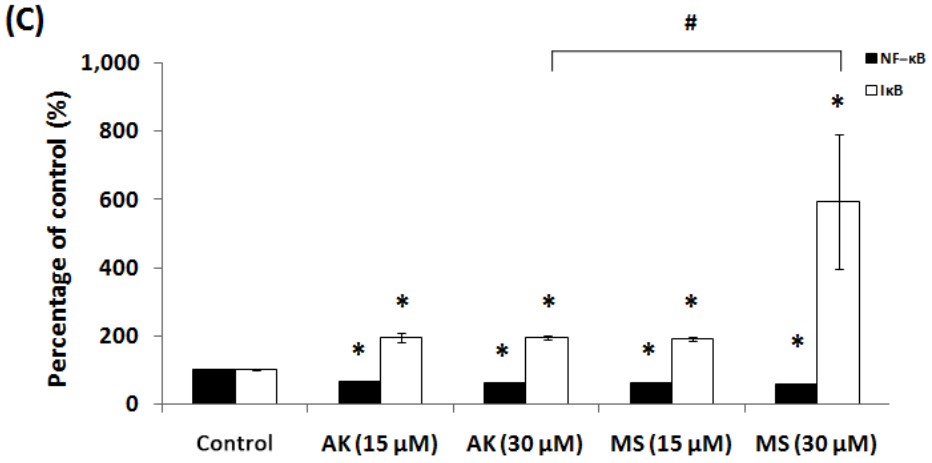

圖 4-17 AK 及 MS 影響 NF-κB 及 PI3K/Akt 分子路徑 (A) 西方墨點法測定磷酸化 Akt、Akt、NF-κB 及 IκB 蛋白質含量 (B) 磷酸化 Akt 及 Akt 蛋白質含量定量及磷酸化 Akt/Akt 比值 (C) NF-κB 及 IκB 蛋白質含量定量。

Figure 4-17 Effects of AK and MS treatments on NF-κB and PI3K/Akt pathway activation. (A) Phosphorylated-Akt, Akt, NF-κB, and IκB levels were analyzed in Western blots. (B) Quantification of the intensities of the bands corresponding to phosphorylated-Akt and Akt, using densitometry. The obtained results are presented as the relative phosphorylated-Akt/Akt ratio, and all levels were normalized to the level of β-actin. (C) NF-κB and IκB band intensities were quantified by densitometry, and all results were normalized to β-actin levels. Corresponding total proteins served as the loading controls. Results are expressed as mean ± standard deviation (SD; n = 3). *, $P < 0.05$, compared with the control; #, $P < 0.05$, between the two indicated groups. AK, ankaflavin; MS, monascin.

doi:10.6342/NTU201700799

(五) AK 和 MS 影響 HSC-T6 細胞 p38 MAPK 分子路徑

p38 MAPK 分子路徑與 HSC 分化相關 (Varela-Rey *et al*., 2002)，p38 的磷酸化活化可進一步促使 NF-κB 活化。p38 蛋白質中 histone H3 蛋白的磷酸化，可促使 NF-κB 活化由細胞質回到細胞核中進行基因調控 (Saccani *et al*., 2002)，因此我們研究 p38 MAPK 分子路徑，包含了 ERK1/2、p38 和 JNK 的活化。細胞經由 AK 和 MS 處理後，顯著減低磷酸化 p38/p38 比例（圖 4-18A 及 4-18B），但不影響 p38、ERK、JNK、磷酸化 ERK 及磷酸化 JNK 的表現。

(六) AK 和 MS 經由 PI3K/Akt 與 p38 MAPK 分子路徑促使 HSC-T6 細胞凋亡

為了確定 NF-κB、Akt 磷酸化和 p38 磷酸化 與 AK 和 MS 誘導的 HSC-T6 細胞凋亡有關，先將細胞用 DMSO、PI3K 抑制劑 （LY294002） 30 μM、NF-κB 抑制劑（Bay117082） 1 μM 或 p38 MAPK 抑制劑 （SB203580） 30 μM 預處理 1 小時，然後用 30 μM 濃度的 AK 或 MS 處理 24 小時，測定細胞存活率。與控制組相比，預先處理抑制劑能有效抑制 HSC-T6 細胞增殖。但是，不論單獨使用抑制劑或是抑制劑與 AK 或 MS 同時處理，其細胞存活率與 AK 或 MS 單獨處理組別相比，沒有顯著下降的情況 (圖 4-19A)。依照以上實驗研究結果顯示，AK 或 MS 誘導細胞凋亡，是經由 Akt/NF-κB 和 p38 MAPK 分子路徑誘導（圖 4-19B）。

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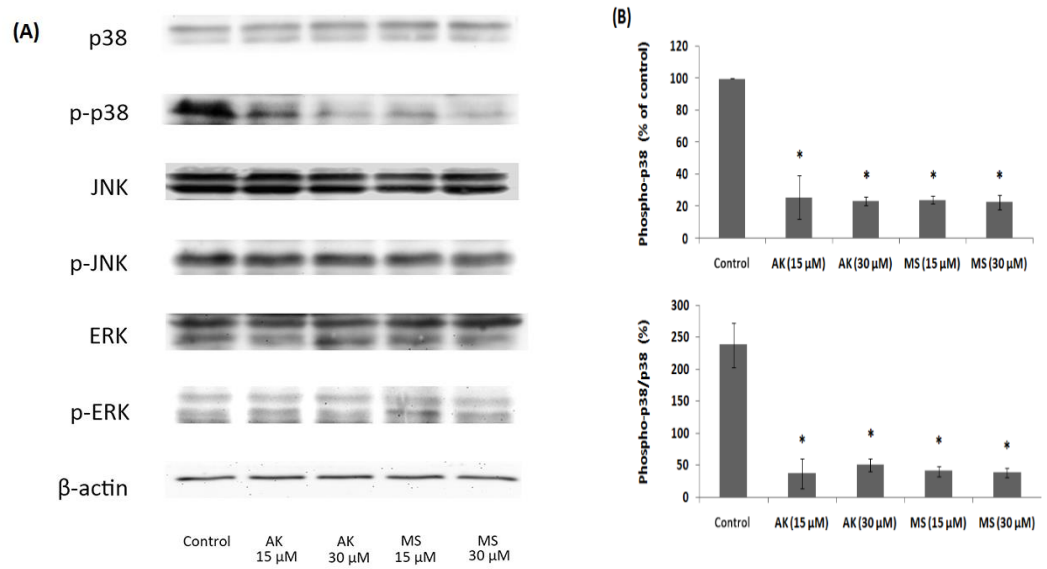

圖 4-18 AK 及 MS 影響 p38 MAPK 分子路徑 (A) 西方墨點法測定 p38、JNK、ERK、磷酸化 p38、磷酸化 JNK 及磷酸化 ERK (B) 磷酸化 p38 及 p38 蛋白質含量定量及磷酸化 p38/p38 比值。

Figure 4-18 AK and MS effect on p38 MAPK pathway proteins. (A) p38, JNK, ERK, phosphorylated-p38, phosphorylated-JNK, and phosphorylated-ERK expression levels were analyzed in Western blots. (B) Quantification of the intensity of the band corresponding to phosphorylated-p38, using densitometry. The obtained results are presented as the relative phospho-p38/p38 ratio, and all levels were normalized to the level of β-actin. Total proteins served as the loading control. Results are expressed as mean ± standard deviation (SD; n = 3). *, $P < 0.05$, compared with the control. AK, ankaflavin; MS, monascin.

doi:10.6342/NTU201700799

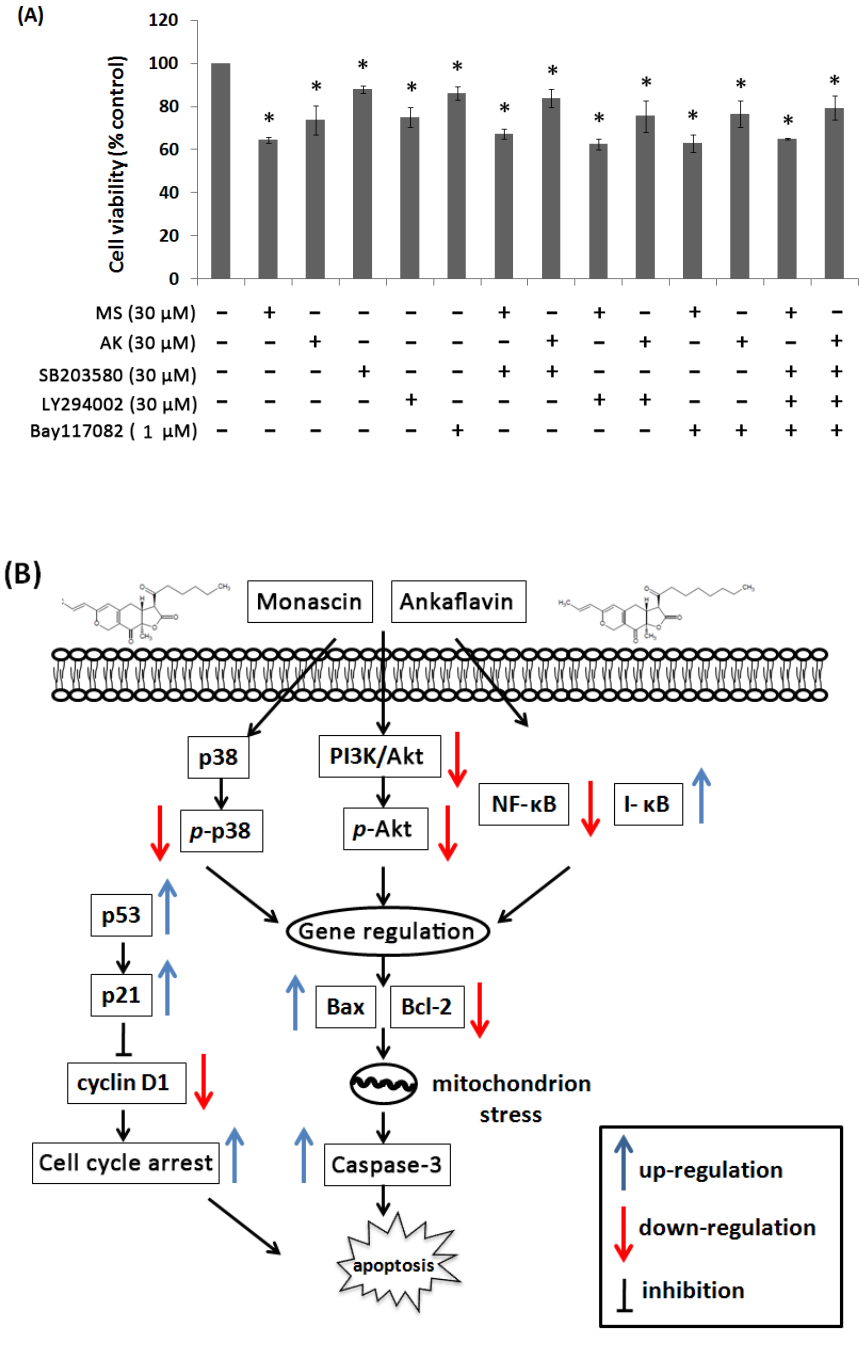

圖 4-19 AK 及 MS 經由 Akt/NF-κB 及 p38 MAPK 分子路徑促使 HSC-T6 細胞凋亡 (A) HSC-T6 細胞預先處理 DMSO、p38 MAPK 抑制劑 SB203580 (30 μM)、PI3K 抑制劑 LY294002 (30 μM) 或 NF-κB 抑制劑 Bay117082 (1 μM) 一小時後，與 30 μM 濃度的 AK 或 MS 培養 24 小時，並測定細胞存活率 (B) AK 及 MS 誘導 HSC-T6 細胞凋亡分子路徑圖。

Figure 4-19 AK- and MS-induced apoptotic signaling pathway is mediated through the

doi:10.6342/NTU201700799

inactivation of Akt/NF-κB and p38 MAPK pathways in activated HSCs. (A) HSC-T6 was pretreated with DMSO or p38 MAPK inhibitor SB203580 (30 μM), PI3K inhibitor LY294002 (30 μM), or NF-κB inhibitor Bay117082 (1 μM) for 1 h and then incubated with 30 μM AK or MS for 24 h, and the cell viability was determined using MTT assay. (B) A proposed AK- and MS-induced apoptotic signaling pathway in activated HSC-T6 cells. AK and MS inhibit Akt, NF-κB, and Bcl-2 expression and Akt and p38 phosphorylation and, in turn, lead to an increase in IκB, p53, and p21 expression, inducing the apoptosis of HSC-T6 cells. Moreover, MS increase the Bax level but not AK. The obtained results are expressed as mean ± standard deviation (SD; n = 3). *, $P < 0.05$, compared with the control group. AK, ankaflavin; MS, monascin.

doi:10.6342/NTU201700799

第伍章 結論

綜合以上結果，動物試驗部分，經由長期酒精誘導肝損傷小鼠模式，我們發現紅麴米及紅麴山藥具有肝臟保護的功效，可以提升因為酒精造成的體重下降，減少肝臟中 TG 和 TC 的含量，減緩肝臟脂肪變性的程度，也增加抗氧化酶 SOD、CAT、GR 及 GPx 之活性，進而降低肝臟中氧化壓力，避免肝臟細胞死亡而維持正常代謝功能，同時降低促發炎因子 IL-1β、IL-6、TNF-α 及 TGF-β 的表現，減少持續性的發炎反應避免肝臟受損，同時 TGF-β 的表現下降也可以避免肝臟星狀細胞活化，減低肝臟纖維化的發生機會，然而紅麴山藥在本研究結果中發現其保護能力較紅麴米顯著，經由化學分析可知紅麴山藥代謝產物中黃色素較紅麴米多，因此將黃色素分離純化後進行細胞實驗，經過 300 mM 乙醇處理 HepG2 細胞後，會使得細胞核內 PPAR-γ 下降並且顯著提升細胞核內 SREBP-1 含量，造成酒精性肝損傷及三酸甘油酯累積，而活性成分 AK 和 MS 可以顯著提升細胞核內 PPAR-γ 含量並且顯著降低細胞核內 SREBP-1 及降低細胞內三酸甘油酯的含量，顯示對乙醇誘導之細胞毒性及三酸甘油酯的累積具有預防作用，同時 PPAR-γ 對於肝臟星狀細胞相當重要，若是促進其活性可以導致肝臟星狀細胞的 NF-κB 被抑制，使得肝臟星狀細胞被抑制降低肝臟纖維化的發生機率，因此接下來也利用此兩種活性成分進行抑制肝臟星狀細胞的分子路徑分析。

AK 和 MS 經由改變 p53 和 Bcl-2 家族成員的蛋白表現，造成專一性抑制 HSC 的增生，但不影響正常肝臟細胞的生長及功能，因此具有治療肝纖維化的潛力。我們的研究結果顯示，其分子路徑可能與抑制 Akt、NF-κB、磷酸化 Akt 和 p38 的表現和增加 IκB 的表現相關，其導致 PI3K/Akt、IκB/NF-κB 和 p38 MAPK 分子路徑被抑制，並經由增加 p53 和 p21 促細胞凋亡蛋白的表現，而阻斷 cyclin D1 蛋白的功能造成細胞週期停滯，同時誘導增加 Bax 及減少 Bcl-2 蛋白質表現量來活化 caspase-3 路徑，最終誘導活化的 HSC 產生細胞凋亡。此外，我們發現 MS 對細胞凋亡的誘導、細胞增殖的抑制和 IkB 蛋白表現量增加比 AK 具有更好的效果。由以上結論可知，紅麴發酵產物可透過抗氧化及抗發炎相關機制達到改善酒精性肝損傷之功效，並且抑制肝臟纖維化的發生。而純物質 AK 及 MS 有效劑量則是在 10 μM 到 30 μM 具有顯著的功效，在日後的研究中，可以進行最佳條件研究進行黃色素的大量生產，本研究發現紅麴發酵產物具有潛力開發為預防酒精性肝臟疾病保健品，並對肝纖維化提供新的治療策略。

doi:10.6342/NTU201700799

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第柒章 附錄

發表論文 (poster)

1. **Chih-Fu Cheng**, Tzu-Ming Pan, 2016, Protective effect of *Monascus*-fermented red mold rice against alcoholic liver disease by attenuating oxidative stress and inflammatory response. 台灣保健食品學會與台灣抗老化保健學會聯合年會。台北，台灣。

發表論文 (journal paper)

1. **Cheng, C. F.**; Pan, T. M. Protective effect of *Monascus*-fermented red mold rice against alcoholic liver disease by attenuating oxidative stress and inflammatory response. J. Agr. Food Chem. 2011, 59, 9950-9957.
2. **Cheng, C. F.**; Pan, T. M. Ankaflavin and monascin induce apoptosis in activated hepatic stellate cells through suppression of the Akt/NF-κB/p38 signalling pathway. J. Agr. Food Chem. 2016, 64, 9326-9334.
3. **Cheng, C. F.**; Pan, T. M. *Monascus*-fermented red mold dioscorea protects mice against alcohol-induced liver injury, with ankaflavin and monascin regulates ethanol induced PPAR-γ and SREBP-1 expression in HepG2 cells. J. Sci. Food Agr. manuscript ID: JSFA-17-1180. (已投稿審稿中)

doi:10.6342/NTU201700799

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Protective Effect of *Monascus*-Fermented Red Mold Rice against Alcoholic Liver Disease by Attenuating Oxidative Stress and Inflammatory Response

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ABSTRACT: *Monascus purpureus* NTU 568 fermented rice is reported to exhibit a wide variety of biological effects, including antitumor, antihypertriglyceridemia, antioxidant, and anti-inflammatory activities. However, its role in the pathogenesis of alcoholic liver disease remains obscure. In this study, the hepatoprotective effects of *Monascus*-fermented red mold rice (RMR) was evaluated in vivo using chronic alcohol-induced mice as an experimental model. The alcohol-induced mice were orally treated with RMR at 307.5 mg/kg (1-fold), 615 mg/kg (2-fold), and 1537.5 mg/kg (5-fold) for 5 weeks, whereas controls received vehicle only. Treatment with RMR significantly attenuated the increased level of serum transaminases (aspartate aminotransferase and alanine aminotransferase) and hepatic triglyceride and total cholesterol accumulation. Furthermore, RMR elevates hepatic antioxidant ability that reduced hepatic cell damage (steatosis) and decreased tissue inflammatory cytokine levels. These findings suggest that *Monascus*-fermented RMR may represent a novel, protective strategy against alcoholic liver disease by attenuating oxidative stress, inflammatory response, and steatosis.

KEYWORDS: *Monascus purpureus*, hepatoprotective, alcoholic liver disease, oxidative stress, steatosis, inflammation

INTRODUCTION

In the World Health Organization (WHO) global status report on alcohol and health (2011), the hazardous and harmful use of alcohol is a major global contributing factor to death, disease, and injury. Almost 4% of all deaths worldwide are attributed to alcohol, greater than deaths caused by HIV/AIDS, violence, or tuberculosis. Alcohol drinking is a major etiologic factor in causing fatty liver, alcoholic hepatitis, cirrhosis, and/or hepatocellular carcinoma.[1] Alcoholic liver disease (ALD) results in a fatty liver (or steatosis), a disorder in which hepatocytes contain macrovesicular droplets of triglycerides. Steatosis predisposes people who continue to drink to hepatic fibrosis and cirrhosis.[2] The important progress in this field has been the appreciation of the role of oxidative stress and inflammatory responses in the pathogenesis of alcohol liver injury.[3] These mechanisms include the activation of Kupffer cells expressing pro-inflammatory cytokines, chemokines, and reactive oxygen species (ROS) and interactions between alcohol metabolism, various hepatic cells, multiple cytokines, and the immune system.[4,5]

Monascus-fermented rice, known as red mold rice (RMR), has been proven to produce many functional secondary metabolites. In the current study, *Monascus*-fermented products such as monacolin K (antihypercholesterolemic agents), γ-aminobutyric acid (GABA) (antihypertension and antidepression agents), and dimerumic acid (antioxidant) were found.[6−8] In addition, the *Monascus*-fermented rice contains various pigments (yellow pigments, ankaflavin and monascin; orange pigments, monascorubrin and rubropunctanin; red pigments, monascorubramine and rubropuctamine) that may have biological activity.[9,10]

This research focused on the effects of oral administration of a RMR fermented by *Monascus purpureus* NTU 568 for alcohol-induced mice on liver antioxidant enzyme, pro-inflammatory cytokine, triglyceride (TG), and total cholesterol (TC) accumulation. This study examined the kidney index and kidney histopathology to investigate the safety of *Monascus*-fermented rice.

MATERIALS AND METHODS

Chemicals and Reagents. Glutathione (GSH), glutathione reductase (GRd), glutathione disulfide (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH), 5,5-dithiobis[2-nitrobenzoic acid] (DTNB), ethylenediaminetetraacetic acid (EDTA), NaN_3, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), and $MgCl_2 \cdot 6H_2O$ were purchased from Sigma Chemical Co. (St. Louis, MO). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from BioQuant (San Diego, CA). Superoxide dismutase (SOD) assay kits were purchased from Cayman (Ann Arbor, MI). TC assay kit was purchased from Randox (Crumlin, Co. Antrim, U.K.). TG assay kit was purchased from Fortress (Fortress Diagnostics Limited, Antrim, U.K.). Interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α) immunoassay kit were purchased from eBioscience (San Diego, CA). Transforming growth factor β1 (TGF-β1) immunoassay kit was purchased from R&D Systems (Minneapolis, MN).

Sample Preparation. RMR is obtained from *M. purpureus* NTU 568 fermented rice. *M. purpureus* NTU 568 strain was maintained on potato dextrose agar (PDA) slants at 10 °C and transferred monthly. The preparation of RMR was carried out under the substrate of long-grain rice (*Oryza sativa*) purchased from a local supermarket in Taiwan and using the method of solid-state culture.[11] Briefly, 500 g of rice was soaked in water for 8 h. After that, excess water was removed with a sieve.

Received: June 28, 2011
Revised: August 23, 2011
Accepted: August 23, 2011
Published: August 23, 2011

dx.doi.org/10.1021/jf202577t | J. Agric. Food Chem. 2011, 59, 9950–9957

doi:10.6342/NTU201700799

The rice was autoclaved (HL-341 model, Gemmy Corp., Taipei, Taiwan) for 20 min at 121 °C in a "koji-dish" (the koji-dish was made of wood with dimensions of 30 × 20 × 5 cm), which is a fermented instrument tray of RMR during the fermentation process. After having been cooled, the rice was inoculated with a 5% (v/w) spore suspension. The inoculated rice was cultivated at 30 °C for 10 days. During the culturing stage, 100 mL of water was daily added to the rice from the second day to the fifth day. At the end of cultivation, the crushed and dried product with the mold was used for the experiments.

Animals and Treatments. Male C57BL/6J mice were purchased from National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) at 8−10 weeks of age and housed in a 25 °C controlled room under a 12 h light/dark cycle with free access to food and water. After acclimatization, mice were divided into five groups (eight mice pre group) and fed either control Lieber−DeCarli liquid diet or a Lieber−DeCarli alcohol-containing liquid diet (Dyets Inc., Bethlehem, PA). The alcohol-fed group was allowed free access to an alcohol-containing diet with increasing content by 1% (v/v) every day until the mice were consuming diets containing 6.2% (v/v) ethanol for 5 weeks.[12] Five groups of mice were separated as follows: (1) control liquid diet fed untreated mice; (2) model alcohol-feeding mice; (3) model mice treated with different doses of RMR (1× RMR, 2× RMR, and 5× RMR, respectively). In the treatment groups, different test materials were administered orally by stomach tube every day around 2:00 p.m. for 5 weeks. The mice in normal and model control groups were given the same volume of vehicle only.

Doses and Groups. Mice were divided at random into five treatment groups, including normal, alcohol-containing diet, 1× RMR, 2× RMR, and 5× RMR groups. The dose of RMR powder was calculated in accordance with Body's formula of body surface area as recommended by the U.S. Food and Drug Administration (FDA).[13] The daily dietary dosage of RMR is usually recommended at 1.0−2.0 g for adults.[14] Therefore, 1.5 g of RMR was used as the 1-fold dosage for an adult with a body weight (bw) of 60 kg and a height of 170 cm; 307.5 mg/kg of bw (1-fold dosage) of RMR was used as a frame of reference for conversion of the dosage into a mice model, 615 mg/kg bw of RMR was used as 2-fold dosage, and 1537.5 mg/kg bw of RMR was used as 5-fold dosage relatively.

Determination of Monsacin and Ankaflavin Concentration. RMR (1 g) was extracted respectively with 10 mL of ethanol at 60 °C for 30 min. The extracts (10%, w/v) were further filtered with 0.45 μm pore size filter and analyzed by high-performance liquid chromatography (HPLC). HPLC was performed in triplicate according to the method described previously. Monascin and ankaflavin were detected using a UV detector UV2075 plus (Jasco Co., Tokyo, Japan) set at 231 nm.[15]

Determination of Citrinin Levels. A 1 g portion of dried RMR powder was extracted with 10 mL of ethanol at 65 °C for 30 min. The extracts (10% w/v) were further filtered with a 0.45 μm filter and were analyzed by HPLC. HPLC analysis was performed according to the method previously described[16] and was carried out on an HPLC system PU2089 plus (Jasco Co.) in this study. Citrinin is determined by HPLC on a C_{18} column (25 cm × 4.6 mm inner diameter, 5 μm, Luna, Phenomenex, Inc., Torrance, CA) using the mobile phase with the composition of water, acetonitrile, and trifluoroacetate (450:550:0.5). The flow rate is set at 1.0 mL/min, and the detector used is a fluorescence detector (FL-1, Rainin, Woburn, MA). The excitation and emission wavelengths were set at 330 and 500 nm, respectively.[17]

Pigment Estimation. One gram of RMR was extracted with 95% ethanol at 37 °C for 90 min. Pigment concentrations were estimated using a spectrophotometer set at 500, 470, and 400 nm for red, orange, and yellow pigments, respectively. The pigment level was expressed as optical density units per gram of dried medium multiplied by the dilution factor.[18]

Liver and Kidney Histology. Liver or kidney histology was performed by fixing a sample of tissue in 10% (v/v) neutral phosphate-buffered formalin and embedding it in paraffin. Slide-mounted tissue sections (3−4 mm) were histochemically stained with hematoylin and eosin (H&E).

Plasma Liver and Kidney Analysis. Blood samples were allowed to clot, and the sera were isolated by centrifugation at 1000*g* for 10 min and then kept at −20 °C before the following assay. The enzymatic activityies of AST and ALT were measured using commercial kits BQ042-CR and BQ006A-CR from BioQuant (San Diego, CA). Blood urea nitrogen (BUN) was measured in triplicate using an automatic biochemical analyzer (Beckman-700, Fullerton, CA).

Determination of GSH/GSSG in Liver Tissues. The concentration of reduced GSH in liver tissues was measured using GSH (0−100 μM) as the standard. The diluted sample solution or standard (10 μL) was mixed with 95 μL of the reagent (2 U/mL GRd, 200 μM NADPH, and 2 mM EDTA in 50 mM phosphate buffer, pH 7.2), followed by the addition of 100 μL of the reagent (10 mM DTNB in 50 mM phosphate buffer, pH 7.2). The reaction mixture was then incubated at room temperature, and the absorbance at 405 nm was determined every 1 min up to 5 min. The concentration was expressed as GSH (μM) in liver tissues.

The concentration of reduced GSSG in liver tissues was measured using GSSG (0−100 μM) as the standard. The diluted sample solution or standard (70 μL) was mixed with 4 μL of M2VP. The mixture was incubated for 1 h at room temperature. The reaction mixture (10 μL) was mixed with 95 μL of the reagent (2 U/mL GRd, 200 μM NADPH, and 2 mM EDTA in 50 mM phosphate buffer, pH 7.2), followed by the addition of 100 μL of the reagent (10 mM DTNB in 50 mM phosphate buffer, pH 7.2). The reaction mixture was then incubated at room temperature, and the absorbance at 405 nm was determined every 1 min up to 5 min. The concentration was expressed as GSSG (μM) in liver tissues.

The GSH/GSSH ratio is then calculated by dividing the difference between the total GSH and GSSG concentrations (reduced GSH). GSH/GSSG = (total GSH − 2 × GSSG)/GSSG.

Antioxidant Enzyme Activities. Liver tissues were homogenized with ice-cold 20 mM Tris-HCl buffer (pH 7.4; 1:10, w/v). After centrifugation at 12000*g* for 30 min at 4 °C, the supernatant was collected for the measurement of antioxidant enzyme activities. The activity of GRd was determined using a 0.1 mL mixture of liver homogenate and 0.1 M phosphate buffer (1 mM $MgCl_2 \cdot 6H_2O$, 5 mM GSSG, and 0.1 mM NADPH, pH 7.0). The decreased absorbance at 340 nm was measured for 3 min.[19] The activity of glutathione peroxidase (GPx) was determined using 0.1 mL of liver homogenate mixed with 100 mM potassium phosphate buffer (1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GR, and 1 mM GSH, pH 7.0) and incubated for 5 min at room temperature; the reaction was initiated after the addition of 0.1 mL of 2.5 mM hydrogen peroxide (H_2O_2). GPx activity was calculated by the change in absorbance at 340 nm for 5 min.[20] The activity of SOD was determined using a commercially available kit (Cayman, Ann Arbor, MI). To determine the activity of catalase (CAT), 50 μL of homogenate and 950 μL of 0.02 M H_2O_2 were incubated at room temperature for 2 min. CAT activity was calculated by the change in absorbance at 240 nm for 3 min.[21]

Serum and Liver Lipid Analysis. Serum TC and TG levels were measured in triplicate using commercial enzymatic kits. These kits were as follows: TC assay kit (CH 200, Randox Laboratories Ltd.) and TG assay kit (BXC0272C, Fortress Diagnostics).

Liver tissue (0.5 g) was ground in 10 mL of ice-cold Folch solution (chloroform/methanol, 2:1, v/v) and incubated for 30 min at room temperature. The aqueous layer was aspirated and discarded, and the fixed volume of the organic layer was then evaporated to dryness. The dried lipid layer was dissolved with an equal volume of DMSO and then

doi:10.6342/NTU201700799

Table 1. Production of Various Secondary Metabolites by *M. purpureus* NTU 568[a]

monascin (mg/kg)	ankaflavin (mg/kg)	citrinin (mg/kg)	red pigment (A_{500}/g)	orange pigment (A_{470}/g)	yellow pigment (AWg)
1385 ± 242	2166 ± 177	ND	168.5 + 6.6	109.2 + 14.0	224.1 ± 31.6

[a] Results are expressed as the mean + SD (n = 3).

Table 2. Mouse Body Weight after Various Weeks of Alcohol Diet Feeding

	body weight[a] (g)			
group[b]	initial weight	2 weeks	4 weeks	final weight
control	20.0 ± 1.7	21.9 ± 1.7	24.5 ± 1.7	26.1 ± 1.3
ALD	20.0 ± 1.3	20.9 ± 0.5	22.5 ± 0.9*	22.7 ± 0.5*
1× RMR	20.0 ± 1.1	21.5 ± 0.6	22.8 ± 1.4	23.3 ± 1.1
2× RMR	20.0 ± 0.8	21.1 ± 0.9	23.3 ± 1.5	23.8 ± 1.1
5× RMR	20.0 ± 0.7	21.6 ± 0.5	23.0 ± 1.1	23.3 ± 1.5

[a] Results are expressed as the mean ± SD (n = 8). *, $p < 0.05$ with control group. [b] ALD, alcohol diet treatment; RMR, red mold rice treatment.

used to determine the TC and TG levels using commercial enzymatic kits.

Hepatic Cytokine Levels. The levels of the inflammatory cytokine TNF-α, IL-1β, and IL-6 in liver were determined using a commercially available ELISA kit (eBioscience). The hepatic TGF-β was determined using a commercially available ELISA kit (R&D Systems), according to the manufacturer's protocol.

Statistical Analysis. All values are expressed as the mean ± SD. Statistical differences between groups were compared by two-way analysis of ANOVA software for statistical analysis. $p < 0.05$ was considered to be statistically significant.

RESULTS

HPLC Assay for Monascin, Ankaflavin, and Pigment Content of Red Mold Rice. The amounts of monascin and ankaflavin in RMR, which possess antioxidative abilities and inhibitory effects on tetradecanoylphorbol acetate (TPA)-induced inflammation in mice,[22] were 1385 and 2166 mg/kg, respectively (Table 1).

Effects of Red Mold Rice on Body and Organ Weight Change. Body weight was monitored every 2 weeks, as shown in Table 2. Animals fed an alcohol diet for 5 weeks gained weight at a slower rate than the control mice did. However, the mean of the differences between the weights of alcohol-induced disease (ALD) mice fed different doses of RMR and those not fed RMR were not statistically significant.

The liver weight per body weight in the ALD group was higher than that of the normal control group, but ALD mice fed 5× RMR recovered normal liver weight per body weight (Figure 1A). The difference between kidney weight per body weight in all alcohol-induced groups and the control group was not statistically significant (Figure 1B).

Effects of Red Mold Rice on Liver and Kidney Histopathology of ALD Mice. Histology of liver sections from the control animals exhibited normal liver architecture with well-preserved cytoplasm and prominent nucleus and nucleolus (Figure 2A), whereas extensive macrovesicular steatosis was observed in the livers of the ALD mice (Figure 2B). However, there was an obvious improvement in liver morphology compared to that of the ALD group that had not been fed RMR when the ALD mice were

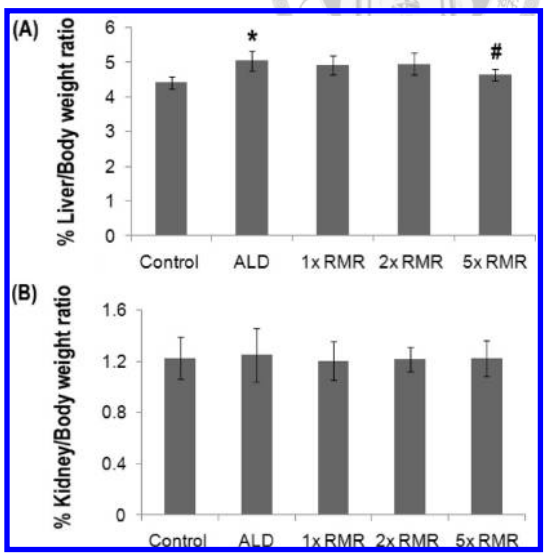

Figure 1. Effects of RMR on liver and kidney: (A) liver and body weight ratio; (B) kidney and body weight ratio. Results are expressed as the mean ± SD (n = 8). *, $P < 0.05$ with control group; #, $P < 0.05$ compared with ALD group. ALD, alcoholic liver disease; RMR, red mold rice treatment.

simultaneously fed 1× or 2× RMR (Figure 2C,D). Particularly, the hepatocytes of the 2× RMR group demonstrated mild steatotic change, but there was no visible change in the histopathology of the 5× RMR group (Figure 2E).

In histology of the kidney, there were no macroscopic changes at necropsy in sections obtained from the control or ALD mice (Figure 3A,B). Similarly, no significant differences in microscopic pathological changes were observed between the kidneys of the treated and control groups, as shown in Figure 3C−E.

Red Mold Rice Improved Alcohol-Induced Hepatic Injury. Table 3 illustrates the protective effect of RMR on the ALD mice as assessed by serum levels of ALT and AST and the AST/ALT ratio. Chronic daily feeding of alcohol caused liver injury as determined by serum markers for liver damage and hepatic histopathological changes. In the control group, serum ALT and AST levels were 55.6 ± 4.9 and 81.3 ± 11.0 U/L, respectively. After 5 weeks, administration of alcohol significantly increased the levels of serum AST and the AST/ALT ratio compared to that of the control group, but there was no significant change in ALT levels. Serum AST levels were significantly reduced with 2× RMR supplementation, whereas supplements of 5× RMR in mice on alcohol treatment significantly blunted the alcohol-induced increase in AST levels (from 104.0 ± 11.3 to 75.1 ± 9.4 U/L) and the AST/ALT ratio (from 1.9 ± 0.3 to 1.5 ± 0.2 U/L). In addition, there was no difference in BUN (kidney index) between the control and ALD mice.

Red Mold Rice Increased Antioxidant Enzyme Activities. In states of oxidative stress, GSH is converted into GSSG and becomes depleted, leading to lipid peroxidation. Table 4 shows the

dx.doi.org/10.1021/jf202577t |J. Agric. Food Chem. 2011, 59, 9950–9957
doi:10.6342/NTU201700799

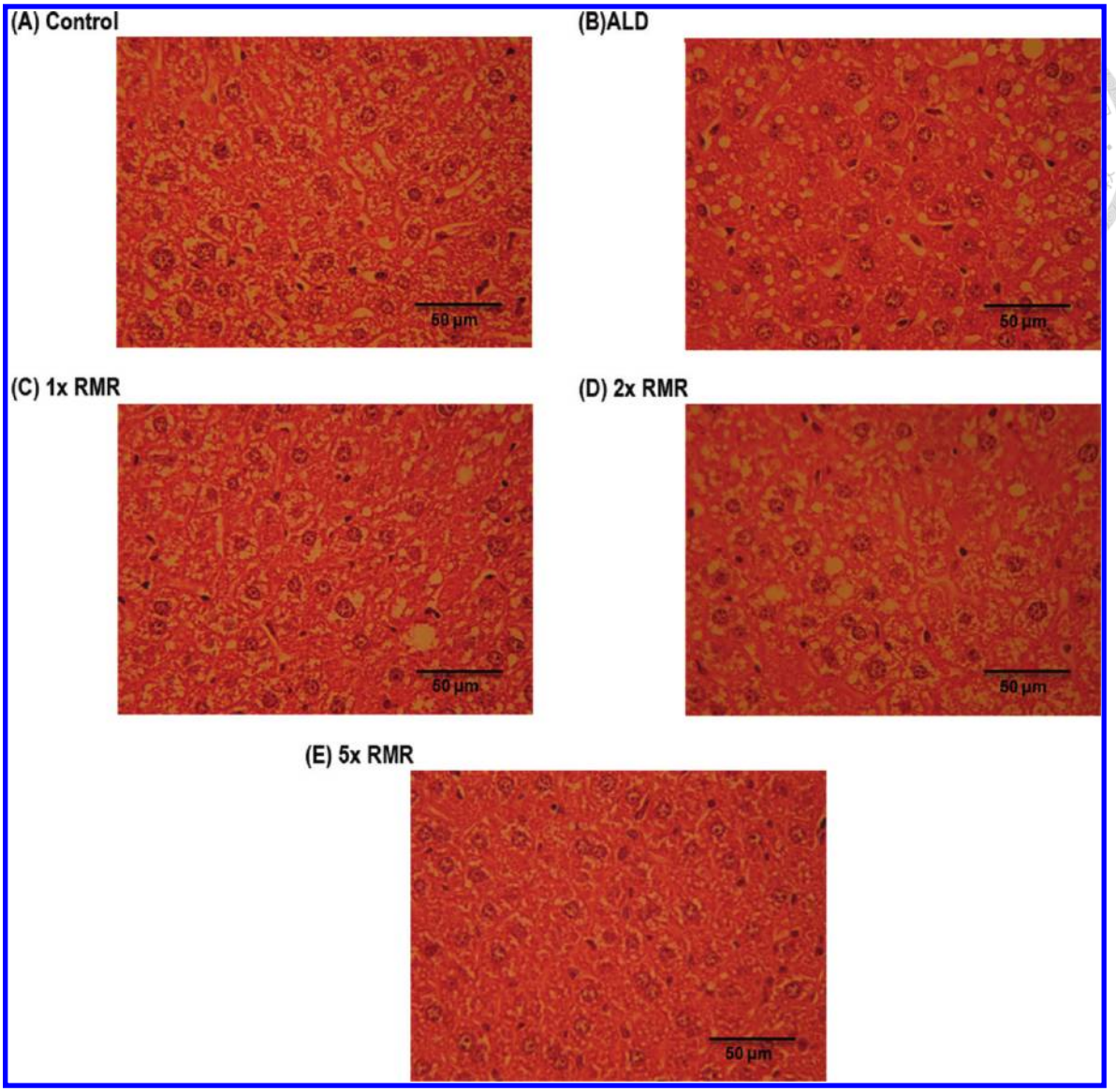

Figure 2. Effects of RMR on liver histopathology of ALD mice using H&E staining: (A) liver tissue of control mice; (B) ALD group; (C) 1× RMR group; (D) 2× RMR group; (E) 5× RMR group (bar = 50 μm).

effects of RMR on GSH/GSSG levels in ALD mice. Administration of 5× RMR to the mice resulted in significant elevation of GSH/GSSG in the liver, whereas low doses did not result in elevation of liver GSH/GSSG. Activities of liver CAT, SOD, GRd, and GPx in the ALD mice were significantly lower compared to those of the control mice. Activities of the antioxidant enzymes CAT, SOD, GRd, and GPx in the liver were increased in all treatment groups compared to those of the ALD mice. Notably, significant elevations in liver CAT, SOD, GRd, and GPx activities were observed in the 5× RMR group, which demonstrated increases of 1.59-fold, 56.6%, 30.5%, and 35.3%, respectively.

Red Mold Rice Attenuated Alcohol-Induced TG and TC Accumulation. Panels A and B of Figure 4 illustrate the effect of chronic alcohol ingestion on hepatic triglycerides and total cholesterol in ALD mice. Hepatic TG and TC levels in the ALD mice increased to match those of the control mice. Hepatic TG accumulation in all treatment groups was significantly attenuated, but there was significant attenuation in hepatic TC accumulation in the 2× and 5× RMR mice.

Panels C and D of Figure 4 illustrate the serum TG and TC contents in chronic alcohol-fed mice. Serum TG content in the ALD mice was significantly elevated compared to that of the control mice, but there was no significant elevation of serum TC. Similarly, the serum TC content in all treatment groups showed no significant change after 5 weeks of alcohol feeding, whereas there was significant attenuation in serum TG accumulation in all treatment groups.

Red Mold Rice Attenuated Alcohol-Induced Pro-inflammatory Cytokines in the Liver. Measurement of the role of RMR in hepatic IL-6, IL-1β, TNF-α, and TGF-β production under chronic alcohol exposure is illustrated by the hepatic cytokine protein values shown in Figure 5.

After 5 weeks, chronic alcohol administration significantly increased hepatic IL-6, IL-1β, and TNF-α production, and this increase was attenuated by RMR supplementation. In addition, hepatic TGF-β production increase was attenuated by RMR supplementation, but the 1× RMR group did not demonstrate significantly attenuated TGF-β production.

dx.doi.org/10.1021/jf202577t |*J. Agric. Food Chem.* 2011, 59, 9950–9957
doi:10.6342/NTU201700799

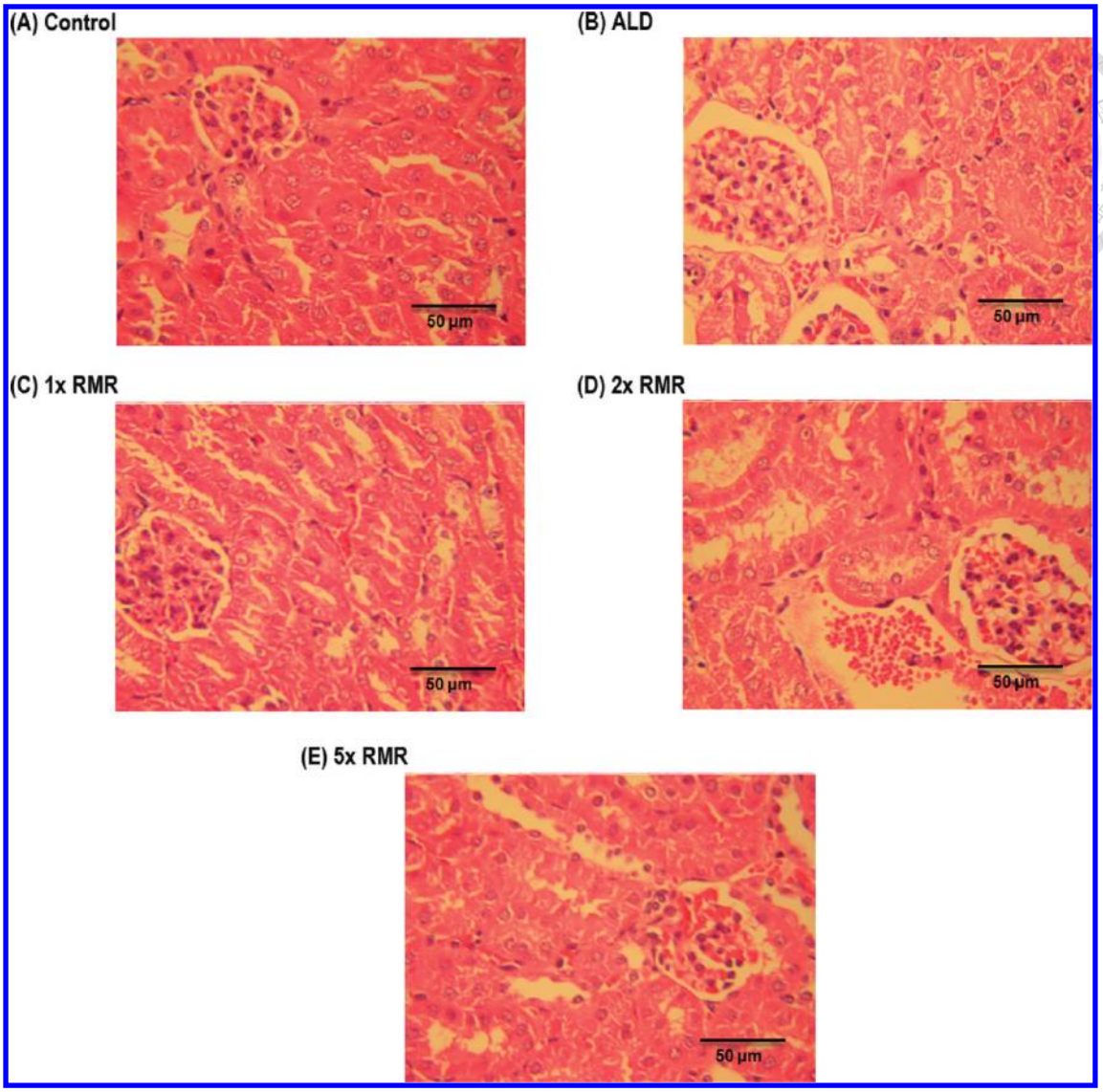

Figure 3. Effects of RMR on kidney histopathology of ALD mice using H&E staining: (A) liver tissue of control mice; (B) ALD group; (C) 1× RMR group; (D) 2× RMR group; (E) 5× RMR group (bar = 50 μm).

Table 3. Effect of RMR on Serum Enzymes and BUN in Alcohol-Induced Liver Damage in Mouse[a]

group[b]	AST (U/L)	ALT (U/L)	AST/ALT ratio	BUN (mg/dL)
control	81.3 ± 11.0	55.6 ± 4.9	1.5 ± 0.2	20.4 ± 2.6
ALD	104.0 ± 11.3*	57.8 ± 8.6	1.9 ± 0.3*	20.5 ± 4.4
1× RMR	96.7 ± 12.2	53.8 ± 4.6	2.0 ± 0.3	21.2 ± 3.1
2× RMR	82.7 ± 10.3#	52.2 ± 3.5	1.7 ± 0.2	23.8 ± 3.9
5× RMR	75.1 ± 9.4#	51.4 ± 1.7	1.5 ± 0.2#	19.5 ± 3.7

[a] Results are expressed as the mean ± SD (n = 8). *, $p < 0.05$ with control group; #, $p < 0.05$ compared with ALD group. AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen. [b] ALD, alcohol diet treatment; RMR, red mold rice treatment.

DISCUSSION

Alcohol misuse is one of the most common public health problems in the world. Alcohol misuse has resulted in high socioeconomic waste and serious individual health damage. In Western societies, >10% of the general adult population may be classified as harmful users. In China, the problems caused by alcohol misuse have become salient following the Westernization of lifestyles. Alcohol misuse is a problem now and is the second highest cause of liver disease in the country after the hepatitis virus.[23]

For many years, investigators have studied the impact of ethanol feeding on various liver mechanisms in attempts to describe the chronic alcohol animal model response leading to alcoholic liver injury. Of all the mechanisms studied, oxidative stress plays a very important role in the pathogenesis of alcohol-induced cellular injury.[5,24,25] It has been suggested that ROS are generated during alcohol metabolism mainly by the catalytic action of alcohol-inducible cytochrome P450 2E1 in microsomes. In addition, chronic alcohol consumption permeabilizes the gut, leading to the accumulation of endotoxins in the liver. These endotoxins activate Kupffer cells, producing ROS and a variety of soluble factors and cytokines (e.g., TNF-α) and precipitating liver injury.[26]

Therefore, ALD progresses from ROS generation during alcohol metabolism; at the same time, hepatic endotoxins and

dx.doi.org/10.1021/jf202577t |J. Agric. Food Chem. 2011, 59, 9950–9957
doi:10.6342/NTU201700799

Table 4. Antioxidase and GSH Activities of Liver in Control and Experimental Animals[a]

group[b]	CAT (nmol H_2O_2/min/mg protein)	GPx (nmol NADPH/min/mg protein)	GRd (nmol NADPH/min/mg protein)	SOD (U/mg protein)	GSH/GSSG ratio
control	317.0 ± 90.8	19.2 ± 6.1	17.1 ± 6.2	23.0 ± 3.3	33.7 ± 6.5
ALD	97.0 ± 35.7*	13.3 ± 3.4*	11.8 ± 3.2*	19.6 ± 2.2*	13.7 ± 8.6*
1× RMR	207.4 ± 68.0 #	16.9 ± 0.7 #	14.1 ± 3.8 #	23.5 ± 4.1 #	19.3 ± 15.7
2× RMR	218.9 ± 83.8 #	18.2 ± 1.8 #	15.3 ± 3.7 #	24.4 ± 7.2 #	22.3 ± 8.2
5× RMR	251.5 ± 59.3 #	18.0 ± 4.2 #	15.4 ± 4.7 #	30.7 ± 12.8 #	23.8 ± 8.7#

[a] Results are expressed as the mean ± SD (n = 8). *, $p < 0.05$ with control group; #, $p < 0.05$ compared with ALD group. CAT, catalase; GPx, glutathione peroxidase; GRd, glutathione reductase; SOD: superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione. [b] RMR, red mold rice treatment; ALD, alcohol diet treatment.

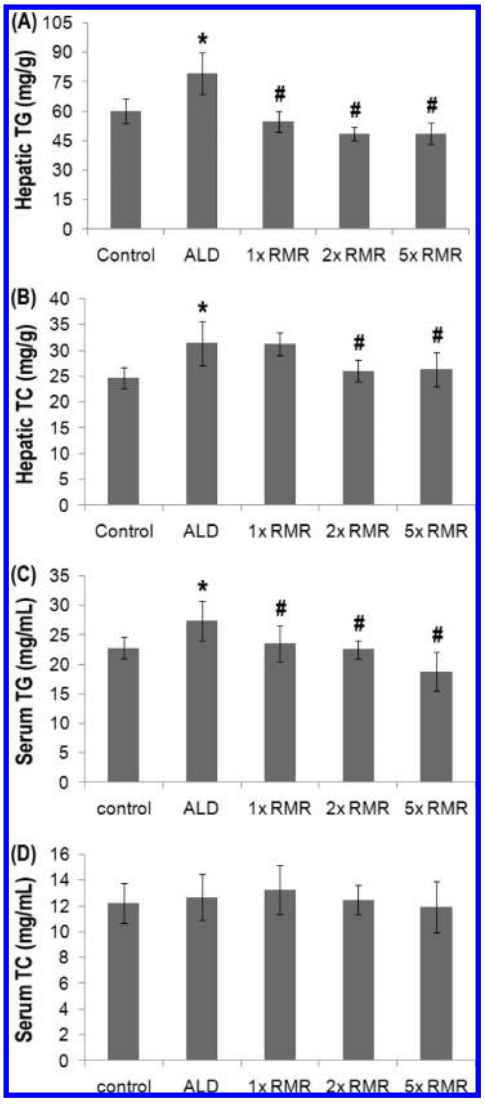

Figure 4. RMR alleviates alcohol-induced liver fatty level: (A) hepatic triglyceride content; (B) hepatic total cholesterol content; (C) serum triglyceride content; (D) serum total cholesterol content. Results are expressed as the mean ± SD (n = 8). *, $p < 0.05$ with control group; #, $p < 0.05$ compared with ALD group. TG, triglyceride; TC, total cholesterol.

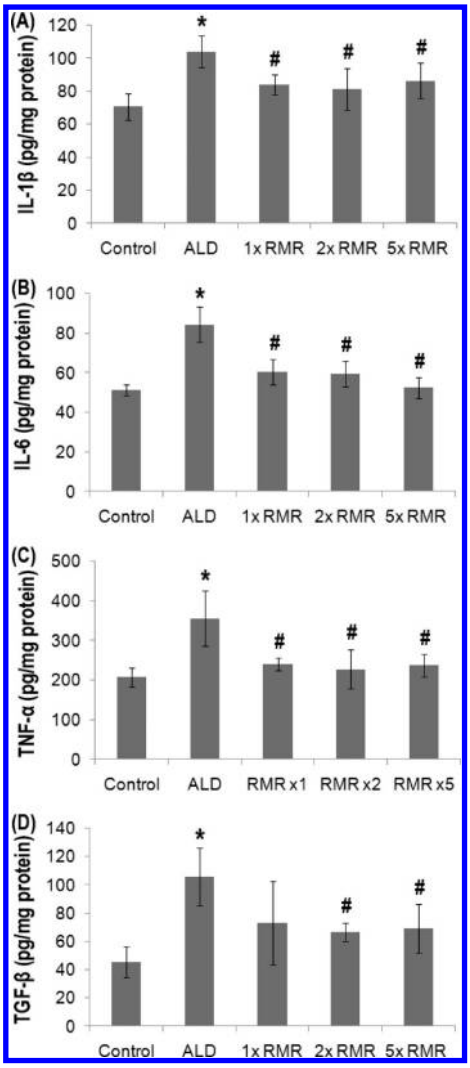

Figure 5. Effects of RMR on inflammatory cytokine expression in the liver from experimental animals. RMR attenuates (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) TGF-β in experimental animals. Results are expressed as the mean ± SD (n = 8). *, $p < 0.05$ with control group; #, $p < 0.05$ compared with ALD group.

fatty levels increase, following a harmful course of inflammation that leads to cellular injury. Although the histologic abnormalities of ALD, that is, steatosis, hepatitis, fibrosis, and cirrhosis, have been well documented,[27] the exact mechanisms of pathogenesis of this devastating disease are still unknown, and useful treatments are still lacking at present.

To investigate the possible protective qualities of RMR, we established the chronic alcohol-fed mouse model under experimental

dx.doi.org/10.1021/jf202577t |J. Agric. Food Chem. 2011, 59, 9950–9957
doi:10.6342/NTU201700799

conditions through a 5 week alcohol diet, which can also lead to steatosis, inflammation, and necrosis.

An increased AST level and AST/ALT ratio are conventional indicators of liver injury. In the present study, alcohol also caused histopathological damages to the mouse liver (Figure 2A) and increased serum levels of hepatic enzymes, that is, AST and the AST/ALT ratio (Table 3). The liver is the primary organ of oxidative drug metabolism, so it is particularly susceptible to oxidative damage caused by increased ROS and GSH depletion. Moreover, previous studies indicated that GSH depletion of ≥20% in the liver results in impaired cell defense and tissue injury.[28] There is substantial evidence that ethanol toxicity in the liver is directly linked to the depletion of GSH.[29,30] In addition, CAT, SOD, GPx, and GRd (Table 4) play crucial roles in maintaining the redox balance in cells, and alcohol toxicity is related to the depletion of important enzymes possessing antioxidative actions.[31] In this study, RMR treatment markedly prevented ethanol-induced elevation of serum AST levels and the AST/ALT ratio compared to that of the ALD mice. These findings can be further corroborated with histopathological studies. The histopathological examination clearly revealed that macrovesicular steatosis was attenuated and hepatic cells in the RMR groups were almost normal compared to those of the alcohol-treated group. In addition, alcohol-induced lipogenesis and subsequent fatty liver are among the earliest stages of ALD, characterized by predominant TG and TC accumulation in hepatocytes as a consequence of alcohol-mediated metabolic disturbances.[32−34] The hepatic TG and TC contents in the RMR treatment groups were attenuated compared to that of the ALD mice (Figure 4A,B), but there was no significant difference in serum TC content compared to that of the controls (Figure 4C,D).

Currently, most researchers favor the hypothesis suggesting that the inflammatory reactions associated with alcoholic liver injury are a consequence of an alcohol-induced increase in the absorption of gut-derived endotoxins. The subsequent activation of Kupffer cells releases pro-inflammatory cytokines and chemokines (TNF-α and IL-1β), ROS, and NO. In human beings, levels of TNF-α and IL-6 tend to be increased in alcohol-related hepatitis and cirrhosis, whereas levels of IFN-α tend to be reduced.[35] Furthermore, activated HSC also releases a large number of cytokines during liver injury, among which TGF-β is considered to be the main fibrogenic cytokine. De novo expression of pro-inflammatory cytokines and chemokines further enhances hepatic fibrogenesis by recruiting leukocytes and perpetuating the inflammatory response.[36,37] Finally, it is also generally assumed that soluble mediators secreted by hepatocytes contribute to the stimulation and activation of HSC in chronic liver diseases.[38] These cytokines contribute to the intrahepatic recruitment and activation of granulocytes that are characteristically found in acute severe alcoholic hepatic inflammation.[39] In the RMR treatment groups, hepatic cytokine levels, including IL-1β, IL-6, TNF-α, and TGF-β, were significantly attenuated compared to those of the ALD mice (Figure 5).

In this chronic alcohol-induced liver injury model, we found that RMR possesses a hepatoprotective ability that not only decreased pro-inflammatory cytokine, TG, and TC contents but also increased antioxidative enzyme levels. Collectively, our results indicate that RMR alleviates alcoholic liver injury by inhibiting oxidative stress and inflammation. Therefore, RMR may represent a novel functional food to prevent alcoholic liver disease.

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ABBREVIATIONS USED

IL , interleukin; TNF , tumor necrosis factor; TGF, transforming growth factor.

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Ankaflavin and Monascin Induce Apoptosis in Activated Hepatic Stellate Cells through Suppression of the Akt/NF-κB/p38 Signaling Pathway

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ABSTRACT: The increased proliferation of activated hepatic stellate cells (HSCs) is associated with hepatic fibrosis and excessive extracellular matrix (ECM)-protein production. We examined the inhibitory effects of the *Monascus purpureus*-fermented metabolites, ankaflavin and monascin (15 and 30 μM), on the Akt/nuclear factor (NF)-κB and p38 mitogen-activated protein kinase (MAPK) signaling pathways in HSC-T6 (activated hepatic stellate cell line). Ankaflavin and monascin (30 μM) induced apoptosis and significantly inhibited cell growth (cell viabilities: 80.2 ± 5.43% and 62.8 ± 8.20%, respectively, versus control cells; $P < 0.05$). Apoptosis and G1 phase arrest (G1 phase percentages: 76.1 ± 2.85% and 79.9 ± 1.80%, respectively, versus control cells 65.9 ± 4.94%; $P < 0.05$) correlated with increased p53 and p21 levels and caspase 3 activity and decreased cyclin D1 and Bcl-2-family protein levels ($P < 0.05$, all cases). The apoptotic effects of ankaflavin and monascin were HSC-T6-specific, suggesting their potential in treating liver fibrosis.

KEYWORDS: *Monascus purpureus, ankaflavin, monascin, liver fibrosis, apoptosis*

1. INTRODUCTION

Hepatic fibrosis represents a wound-healing process in the liver and a response to acute or chronic hepatic injuries observed in diseases with different etiologies such as autoimmune diseases (e.g., primary biliary cirrhosis) or infections, metabolic disorder, or exposure to chemicals (e.g., alcohol).[1] Cirrhosis is a worldwide health problem that is also associated with substantial economic burden.[2] Therefore, the prevention of fibrosis progression, which may lead to cirrhosis, can help improve health-associated quality of life in patients. Hepatic stellate cells (HSCs) are located within the perisinusoidal space and they interact with liver sinusoidal endothelial cells and hepatocytes,[3] which are mainly responsible for the development of hepatofibrosis. Fibrogenic cytokines, such as tumor necrosis factor α (TNF-α), platelet-derived growth factor (PDGF), and transforming growth factor β1 (TGF-β1), lead to the transformation of HSCs from their quiescent phenotype to a myofibroblast-like phenotype. The activated HSCs overexpress pro-inflammatory growth factors and extracellular matrix (ECM) proteins during the progression of liver fibrosis.[4] Therefore, the inhibition of activated HSCs represents an important area in clinical research. Current therapeutic approaches include the following: removal of the injurious agent (hepatitis B virus eradication), use of anti-inflammatory agents (corticosteroids in autoimmune hepatitis), antioxidants (polyenylphosphatidylcholine in alcoholic hepatitis), or cyto-protective agents (ursodeoxycholic acid), and the inhibition of HSC activation.[5] Furthermore, an increasing amount of evidence indicates that the inhibition of the growth of activated HSCs or the induction of their apoptosis may represent a potential strategy for the treatment of hepatofibrosis.[6]

Monascus species produce numerous functional secondary metabolites. In recent studies, dimerumic acid (antioxidant), γ-aminobutyric acid (GABA; antihypertensive agent and antidepressant), and monacolin K (antihypercholesterolemic agent) were isolated from *Monascus purpureus* NTU 568.[7−9] Additionally, various pigments with potential biological activities can be found among the metabolites.[10,11] Ankaflavin and monascin are two yellow pigments produced by *M. purpureus*. They possess cytotoxic/cytostatic,[12] anti-inflammatory,[13] and antiobesity[14] properties. We previously demonstrated that monascin shows antioxidative and anti-inflammatory effects via the upregulation of nuclear factor-erythroid 2-related factor 2 (Nrf2).[15] Recently, the inhibitory effects of monascin on carboxymethyllysine (CML)-induced RAGE (receptor for advanced glycation end products) signaling in HSCs were investigated.[16] In this study, we investigated the effects of ankaflavin and monascin on the induction of apoptosis and molecular pathways in the activated rat HSC-T6 cells.

2. MATERIALS AND METHODS

2.1. Chemicals. Ankaflavin and monascin were isolated from *Monascus*-fermented rice (red mold rice).[17] Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Triton-X 100, propidium iodide (PI), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), deoxyribonuclease 1 (DNase 1), trypsin, SB203580 (inhibitor of p38 MAPK), LY2940022 (inhibitor of phosphoinositide 3-kinase; inhibitor of PI3K), and Bay117082 (inhibitor of NF-κB) were purchased from Sigma Co. (St. Louis, MO, USA). Annexin-V was purchased from eBioscience (San Diego, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, antibiotics (penicillin/streptomycin), and sodium pyruvate were purchased from Gibco (Grand Island, NY,

Received: August 17, 2016
Revised: November 24, 2016
Accepted: November 25, 2016
Published: November 25, 2016

DOI: 10.1021/acs.jafc.6b03700
J. Agric. Food Chem. XXXX, XXX, XXX−XXX
doi:10.6342/NTU201700799

USA). Caspase 3 activity assay kit was purchased from BioVision (Mountain View, CA, USA). Anti-p53 antibody, anti-21 antibody, anticyclin D1 antibody, antiextracellular signal-regulated kinases (ERK) antibody, anti-c-Jun amino-terminal kinases (JNK) antibody, anti-p-JNK antibody, anti-NF-κB antibody, and anti-NF-κB inhibitor of kappa B (IκB) antibody were purchased from Abcam Ltd. (Cambridge, UK). Anti-p-ERK antibody, anti-Akt antibody, anti-p-Akt antibody, anti-p38 antibody, anti-p-p38 antibody, anti-Bax antibody, and anti-Bcl-2 antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-β-actin antibodies were purchased from Proteintech Group, Inc. (Chicago, IL, USA).

2.2. Ankaflavin and Monascin Purification. Ankaflavin and monascin were purified via high-performance liquid chromatography (HPLC) from *Monascus purpureus* NTU 568-fermented products (>95% purity),[18] and all extracts were dissolved in DMSO; however, the concentration of DMSO was maintained below 0.1%. These compounds were identified by electrospray ionization-mass spectrometry (ESI-MS, FinniganMAT LCQ, Thermo Electron Co., Waltham, MA, USA) and nuclear magnetic resonance (NMR, Varian Gemini, 200 MHz, FT-NMR, Varian Inc., Palo Alto, CA, USA).[17]

2.3. Primary Hepatocytes Isolation. Male Sprague−Dawley (SD) rats, 6−7 weeks old, were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and housed at room temperature (25 °C) and under a 12 h light/dark cycle, with free access to standard pellet diet and water. Primary hepatocytes were isolated from rat livers as described previously.[19] Briefly, the liver was perfused and the tissue was digested by collagenase. The samples were further digested with DNase I and collagenase, which resulted in the removal of the stromal tissue by successive filtrations and centrifugation. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 0.15% insulin, 100 mg/L streptomycin, and 70 mg/L penicillin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.4. Cell Culture. HSC-T6 cell line is a rat hepatic stellate cell line purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The obtained cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.5. Cell Proliferation Analysis. Cell proliferation rate was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Hepatocytes and HSC-T6 cells (1 mL, 5 × 10^4 cells/mL) were cultured in 24-well plates. After stabilization for 24 h, hepatocytes and HSC-T6 were cocultured without or with a series of ankaflavin or monascin (6, 15, and 30 μM) for 24 h. The vehicle-treated (DMSO) cells represented a control group. The medium was removed from the treated cells, and it was replaced with 1 mL of fresh culture medium supplemented with 0.75 mg/mL MTT. The samples were further incubated at 37 °C for 3 h. Thereafter, the medium was discarded, and 500 μL of DMSO was added to each well. Each sample was mixed using a pipet, and the absorbance was measured at 570 nm.

2.6. Cell Cycle and Sub-G1 Phase Analysis. HSC-T6 cells (1 mL, 5 × 10^5 cells/mL), grown in a 6 cm dish, were serum-starved (DMEM with 0.5% FBS) for 24 h and then released by the addition of 10% FBS and ankaflavin or monascin (15 and 30 μM, respectively) and further incubated for 24 h. Cells were harvested via trypsinization at 0, 6, 12, and 24 h, after the treatment, and fixed in ice-cold 75% ethanol (1 mL) for 13 h at 4 °C. Cell cycle and sub-G1 phase analyses were performed using BD FACSCanto (BD Biosciences, San Jose, CA, USA), and the obtained results were analyzed using ModTit LT3.0 software.

2.7. Annexin V Assay. Cells were trypsinized and analyzed with Annexin V-FITC apoptosis assay kit. HSC-T6 cells (1 mL, 5 × 10^5 cells/mL) were grown in a 6 cm dish and treated with different concentrations of ankaflavin or monascin (15 and 30 μM). The treated cells and the controls were analyzed using BD FACSCanto (BD Biosciences, San Jose, CA, USA).

2.8. Caspase 3 Activity Assay. HSC-T6 cells (1 mL, 5 × 10^5 cells/mL), grown in a 6 cm dish and treated with different concentrations of ankaflavin or monascin (30 μM). Cells were harvested by trypsinization after washing them in PBS, and caspase 3 activity was tested by using a colorimetric assay kit.

2.9. Western Immunoblot Analysis. HSC-T6 cells (1 mL, 5 × 10^5 cells/mL) were grown in a 6 cm dish and treated with the investigated compounds for 24 h, together with the controls. Thereafter, cell lysates were collected and centrifuged (10000*g*, 10 min). The cell supernatant was collected, and the protein concentration was determined by a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). β-Actin levels served as an internal control. Protein expression levels were determined using UVP Autochemi Image System (UVP Inc., Upland, CA, USA).

2.10. Statistical Analysis. Two-way analysis of ANOVA was used to compare the different groups. Data are expressed as mean ± standard deviation (SD). P values <0.05 were considered statistically significant.

3. RESULTS

3.1. Ankaflavin and Monascin Induce HSC-T6 Cell Death but Do Not Affect the Survival of Primary Hepatocytes. The fibrogenic function of HSCs is based on their activation and proliferation. To explore the antifibrotic effects of ankaflavin and monascin, we investigated ankaflavin- and monascin-mediated cell death of HSC-T6 cells. HSC-T6 cells were treated with 6, 15, or 30 μM of ankaflavin or monascin for 24 h. Ankaflavin and monascin at the concentration of 30 μM significantly inhibited HSC-T6 cell proliferation, with the maximum decrease in proliferation observed after 24 h of 30 μM monascin treatment (Figure 1A). Additionally, cytotoxic effects of ankaflavin and monascin on primary hepatocytes were investigated by treating the cells with these compounds (at 6, 15, and 30 μM concentrations), and the results obtained are presented in Figure 1B. We demonstrated that these treatments have no effects on hepatocyte survival in 24 h treatment. Taken together, our results show that ankaflavin and monascin are able to reduce the proliferation of activated HSCs, and because we found that the concentrations of 15 and 30 μM of the investigated compounds do not have cytotoxic effects on primary hepatocytes, we used these concentrations in the following experiments.

3.2. Ankaflavin and Monascin Induce G1 Phase Arrest of HSC-T6 Cells. To understand the potential mechanism underlying the antiproliferative activities of ankaflavin and monascin, cells were synchronized by starvation with a medium containing 0.5% FBS and cell cycle progression was determined by flow cytometry. The results presented in Table 1 show that incubation with 15 or 30 μM of ankaflavin or monascin for 0, 6, 12, or 24 h, promotes both time- and dose-dependent increase in the percentage of HSC-T6 cells in G1 phase of cell cycle. DNA content at G1 phase was determined to be 76.1% and 79.9%, after the treatment with 30 μM of ankaflavin or monascin, respectively, and 65.9% in the sample incubated with DMSO for 24 h, indicating that these treatments lead to a G1 phase arrest (Figure 2A,B) and a corresponding decrease in the percentage of HSC-T6 cells in S and G2/M phases of cell cycle. Additionally, ankaflavin and monascin (30 μM) treatments led to an increase in the number of HSC-T6 cells in sub-G1 phase, indicating the induction of apoptosis (Figure 2C). Next, we determined the effects of the investigated compounds on cell cycle regulatory proteins by using Western blot (Figure 2D). The key modulator of the cell progression from G1 phase to

DOI: 10.1021/acs.jafc.6b03700
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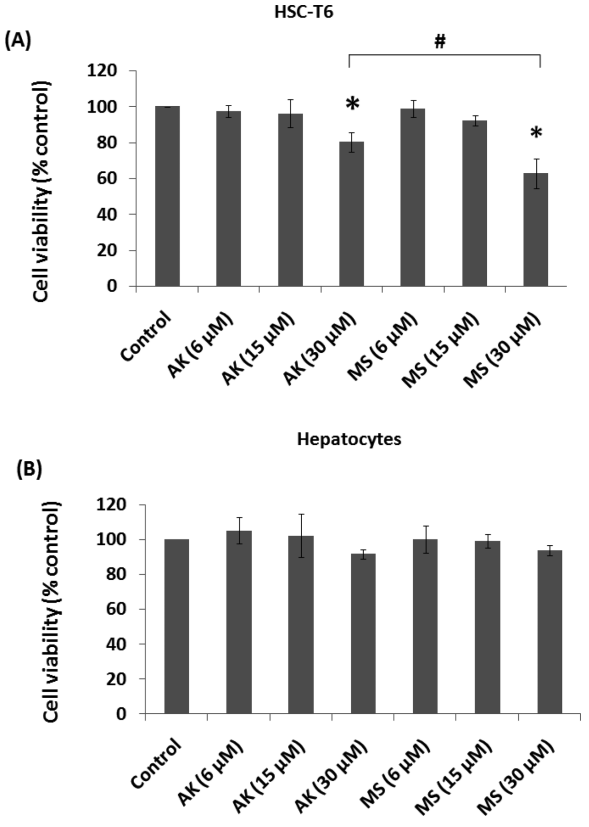

Figure 1. Ankaflavin and monascin-induced HSC-T6 and rat primary hepatocyte cell death. (A) HSC-T6 and (B) hepatocytes were treated with 6, 15, or 30 μM concentrations of ankaflavin and monascin for 24 h, and the cell viability was tested using MTT assay. The obtained results are expressed as mean ± standard deviation (SD; $n = 3$). *, $P < 0.05$, compared with the control group; #, $P < 0.05$, between the two indicated groups. AK, ankaflavin; MS, monascin.

mitosis is cyclin D1, which is regulated by upstream molecules, p53 and p21. Cyclin D1 levels decreased after 24 h of 30 μM ankaflavin and monascin treatment. The levels of p53 were significantly upregulated after the incubation with both investigated compounds. The expression levels of one of the main downstream targets of p53, cell cycle arrest inducer-p21, were significantly upregulated after ankaflavin and monascin treatment in a dose-dependent manner (Figure 2E).

3.3. Ankaflavin and Monascin Promote HSC-T6 Cell Apoptosis. We performed flow cytometric assay in order to determine whether ankaflavin and monascin induce apoptosis in the target cells. These cells were incubated with Annexin V-FITC/PI, and a significant, dose-dependent increase in the percentage of apoptotic cells, in comparison with the control, was detected after 24 h of treatment (Figure 3A,B). The possible mechanisms underlying this process were examined by determining the expression of apoptotic proteins. As shown in Figure 3C, the activity of caspase 3 significantly increased after the 24 h treatment with 30 μM of ankaflavin and monascin. Additionally, the Bcl-2 family members play crucial roles in HSCs survival, and therefore we analyzed Bcl-2 and Bax protein expression levels. As shown in parts D and E of Figure 3, Bcl-2 expression levels were significantly reduced after the treatment with 30 μM of ankaflavin or monascin, while Bax was significantly upregulated after the treatment with monascin but not with ankaflavin.

3.4. Ankaflavin and Monascin Repress the Phosphatidylinositol-4,5-bisphosphate 3-Kinase (PI3K)/Akt Pathway and NF-κB Activation in HSC-T6 Cells. The effects of ankaflavin and monascin on PI3K/Akt signaling were determined. As shown in parts A and B of Figure 4, the decrease in phospho-Akt/Akt ratio was observed following the treatments with ankaflavin and monascin (30 μM). Furthermore, the levels of phosphorylated Akt decreased significantly after these treatments. The members of the Bcl-2 protein family are the major targets of NF-κB,[20] which represents a central regulator of apoptosis and cell survival. We investigated the effects of ankaflavin and monascin on NF-κB expression in HSC-T6 cells. In Figure 4A, it is shown that ankaflavin and monascin significantly decrease NF-κB expression while increasing IκB expression levels. The increase in IκB levels was greater following the treatment with monascin, in comparison with the increase observed after ankaflavin treatment (Figure 4C).

Table 1. Ankaflavin and Monascin Induce HSC-T6 Cell Cycle Arrest[a]

	control					
time (h)	G0/G1 (%)	S (%)	G2/M (%)			
0	89.6 ± 2.31	6.51 ± 1.99	2.92 ± 1.17			
6	83.8 ± 3.21	12.5 ± 3.36	3.73 ± 1.08			
12	67.2 ± 3.87	18.6 ± 1.92	14.1 ± 2.65			
24	65.9 ± 4.94	20.7 ± 3.74	13.4 ± 3.55			
	ankaflavin (30 μM)			ankaflavin (15 μM)		
time (h)	G0/G1 (%)	S (%)	G2/M (%)	G0/G1 (%)	S (%)	G2/M (%)
6	83.0 ± 1.57	13.8 ± 1.06	3.23 ± 0.65	82.6 ± 1.22	14.0 ± 2.59	3.33 ± 0.91
12	75.6 ± 5.34	15.0 ± 2.53	9.37 ± 3.46	65.1 ± 4.49	21.5 ± 2.59	13.4 ± 2.98
24	76.1 ± 2.85	15.7 ± 2.47	8.17 ± 0.45	64.8 ± 1.25	22.3 ± 2.80	12.9 ± 4.57
	monascin (30 μM)			monascin (15 μM)		
time (h)	G0/G1 (%)	S (%)	G2/M (%)	G0/G1 (%)	S (%)	G2/M (%)
6	83.4 ± 124	13.4 ± 0.36	3.03 ± 0.17	82.5 ± 2.69	14.1 ± 1.69	3.43 ± 0.20
12	76.1 ± 3.81	14.6 ± 123	9.27 ± 2.86	67.2 ± 2.91	20.2 ± 2.32	12.6 ± 2.11
24	79.9 ± 1.80	15.8 ± 3.12	4.33 ± 0.34	72.3 ± 1.58	16.9 ± 2.23	10.8 ± 1.65

[a]. Monascin induces G1 phase cell cycle arrest of HSC-T6 cells. Cells were stained with propidium iodide and analyzed using flow cytometry. Results are expressed as mean ± standard deviation (SD; $n = 3$).

DOI: 10.1021/acs.jafc.6b03700
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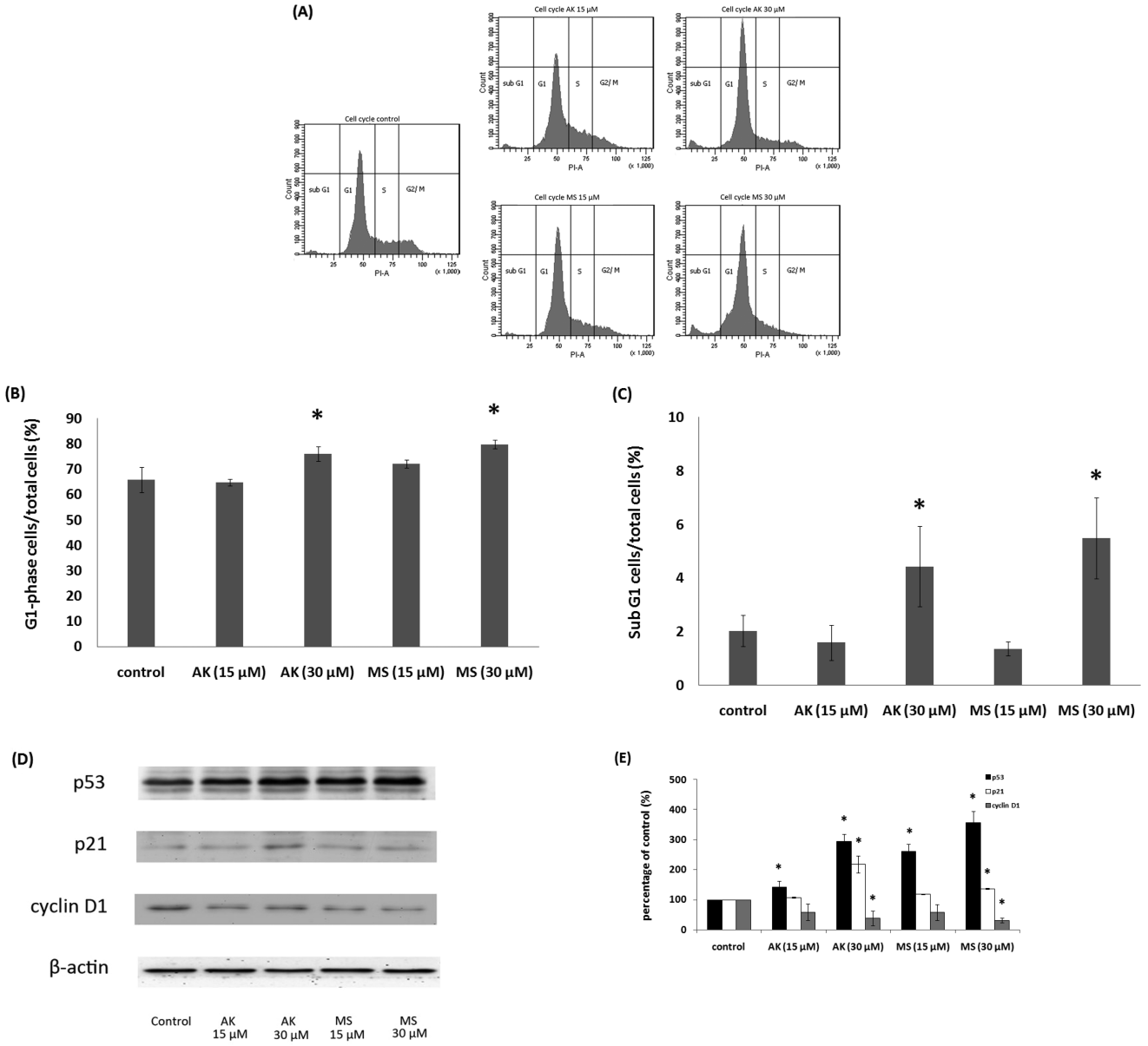

Figure 2. Ankaflavin and monascin induce HSC-T6 cell cycle arrest. (A) HSC-T6 cells, stained with propidium iodide (PI), sorted and analyzed using flow cytometry, following their treatment with ankaflavin or monascin (15 and 30 μM) for 24 h. (B) The changes in the percentage of cells in G1 phase, following the treatment with ankaflavin or monascin. (C) Dose-dependent changes in the population of cells in sub-G1 phase. (D) Western blot analysis of p53, p21, and cyclin D1 levels, after the treatments. (E) Densitometric analysis of band intensities. Data are expressed as the fold of control. All obtained results were normalized to β-actin levels. Results are expressed as mean ± standard deviation (SD; $n = 3$). *, $P < 0.05$, compared with the control. AK, ankaflavin; MS, monascin.

3.5. Ankaflavin and Monascin Decrease the Expression of Phosphorylated p38 MAPK. To determine a potential apoptotic signaling pathways affected by ankaflavin and monascin treatments, we determined the levels of phosphorylated-Akt and phosphorylated-p38 MAPK, which belong to the signaling pathways associated with oxidative stress. Western blot analysis showed that ankaflavin and monascin treatments lead to a significant decrease in phospho-p38/p38 ratio (Figures 5A,B), but no significant differences in the levels of p38, ERK, JNK, phospho-ERK, and phospho-JNK were observed after the treatments.

3.6. Akt/NF-κB and p38 MAPK Pathways Are Involved in Ankaflavin and Monascin-Induced HSC-T6 Cell Death. To determine whether NF-κB downregulation, Akt phosphorylation, and p38 phosphorylation are associated with the ankaflavin- and monascin-induced HSC-T6 cell apoptosis, these cells were pretreated with DMSO, PI3K inhibitor (LY294002, 30 μM), NF-κB inhibitor (Bay117082, 1 μM), or p38 MAPK inhibitor (SB203580, 30 μM) for 1 h and then treated with 30 μM ankaflavin or monascin for 24 h. Compared with the control, HSC pretreatment with either of these inhibitors effectively suppressed HSC-T6 cell proliferation. However, no significant differences in cell viability between the cells treated with ankaflavin alone or the ankaflavin−inhibitor combinations and cells treated with monascin alone or the monascin−inhibitor combinations (Figure 6A). Together, our results suggest that in activated HSCs, ankaflavin or monascin-induced cell death is mediated by Akt/NF-κB and p38 MAPK pathways (Figure 6B).

DOI: 10.1021/acs.jafc.6b03700
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

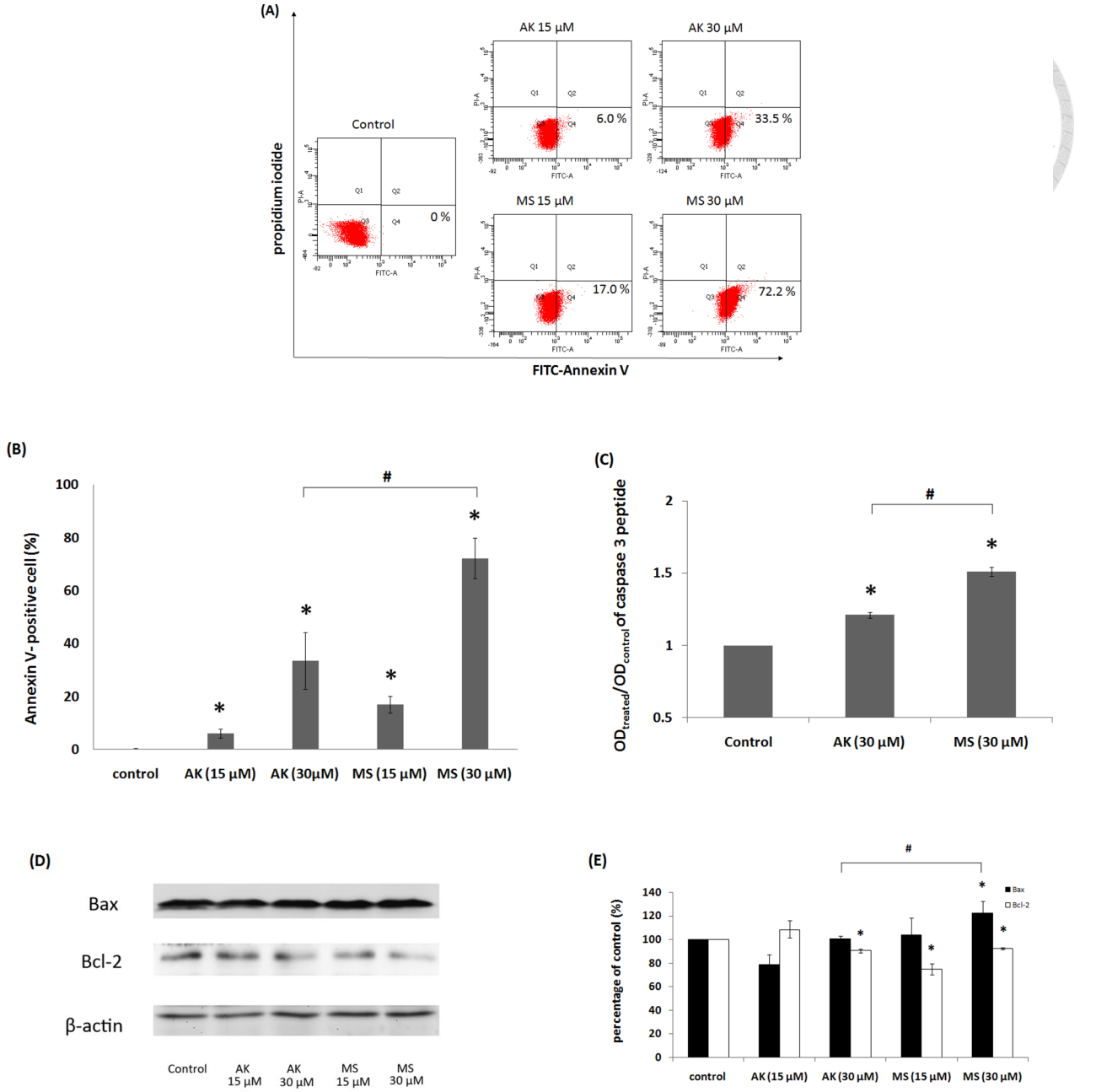

Figure 3. Ankaflavin and monascin induce the apoptosis of HSC-T6. (A) Flow cytometry analysis of cells stained with Annexin V-FITC and propidium iodide (PI). (B) The percentage of annexin V positive cells, following treatments with ankaflavin or monascin. (C) Caspase 3 activity was measured and represented by $OD_{treated}/OD_{control}$ ratio, after the exposure to ankaflavin or monascin (30 μM) for 24 h. (D) Bax and Bcl-2 levels were analyzed by Western blotting, while β-actin was used as a loading control. (E) Densitometric quantification of band intensity. Data are expressed as the fold of control. All obtained results were normalized to β-actin levels. Results are expressed as mean ± standard deviation (SD; $n = 3$). *, $P < 0.05$, compared with the control group; #, $P < 0.05$, between the two indicated groups. AK, ankaflavin; MS, monascin.

4. DISCUSSION

Many functional secondary metabolites were identified in the *Monascus*-fermented products, including pigments (red, monascorubramine and rubropuctamine; yellow, ankaflavin and monascin; orange, monascorubrin and rubropunctanin), and the hepatoprotective effects of these products in the alcoholic liver disease were investigated.[21] In our study, we found that both ankaflavin and monascin demonstrate significant anti-fibrotic properties. Chronic liver damage results from prolonged wound healing and continuous acute liver injury cycles. This liver damage occurs in conjunction with the ECM proteins production that represents a characteristic of many chronic liver diseases.[22] The major causes of hepatic fibrosis include alcohol abuse, nonalcoholic steatohepatitis, and HCV infection. Liver lesions are repaired through the fibrotic ECM proteins. Over time, this process can result in liver cirrhosis, where hepatocellular dysfunction is induced and blood flow through the liver is disrupted, which may result in portal hypertension and hepatic insufficiency, respectively.[23] The therapeutic targets in liver fibrosis include: (1) attenuation of inflammation and tissue damage, (2) inhibition of HSC proliferation and activation, (3) attenuation of fibrogenesis,

DOI: 10.1021/acs.jafc.6b03700
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

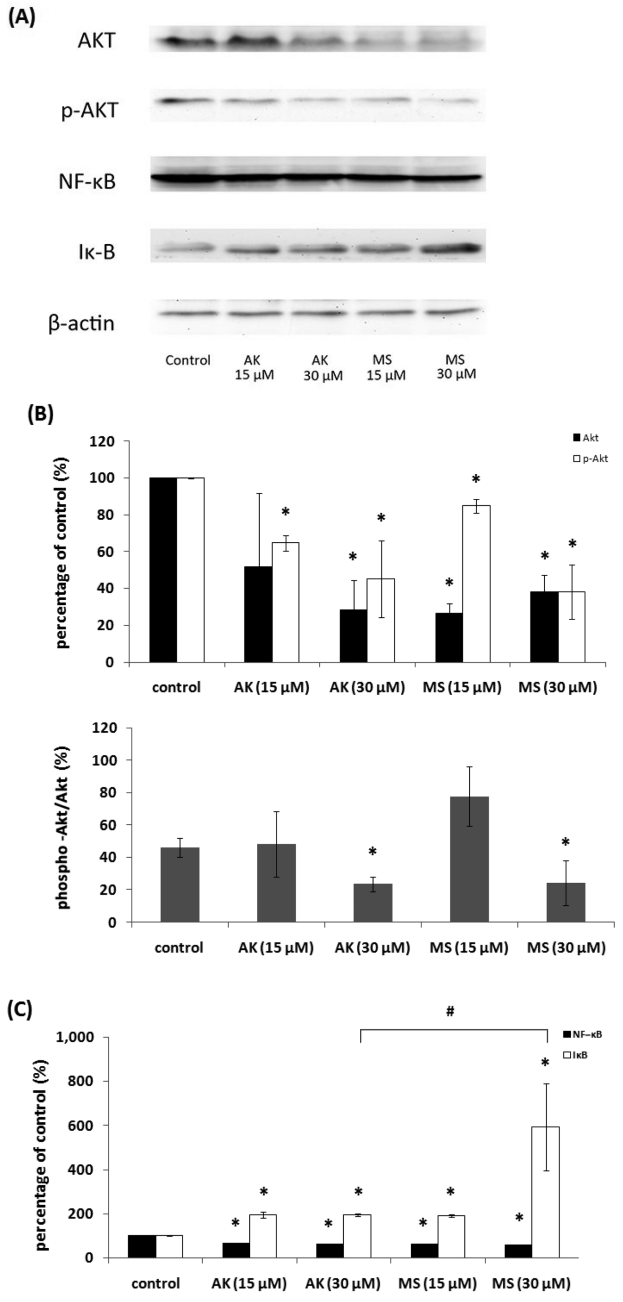

Figure 4. Effects of ankaflavin and monascin treatments on NF-κB and PI3K/Akt pathway activation. (A) Phosphorylated-Akt, Akt, NF-κB, and IκB levels were analyzed in Western blots. (B) Quantification of the intensities of the bands corresponding to phosphorylated-Akt and Akt, using densitometry. The obtained results are presented as the relative phosphorylated-Akt/Akt ratio, and all levels were normalized to the level of β-actin. (C) NF-κB and IκB band intensities were quantified by densitometry, and all results were normalized to β-actin levels. Corresponding total proteins served as the loading controls. Results are expressed as mean ± standard deviation (SD; $n = 3$). *, $P < 0.05$, compared with the control; #, $P < 0.05$, between the two indicated groups. AK, ankaflavin; MS, monascin.

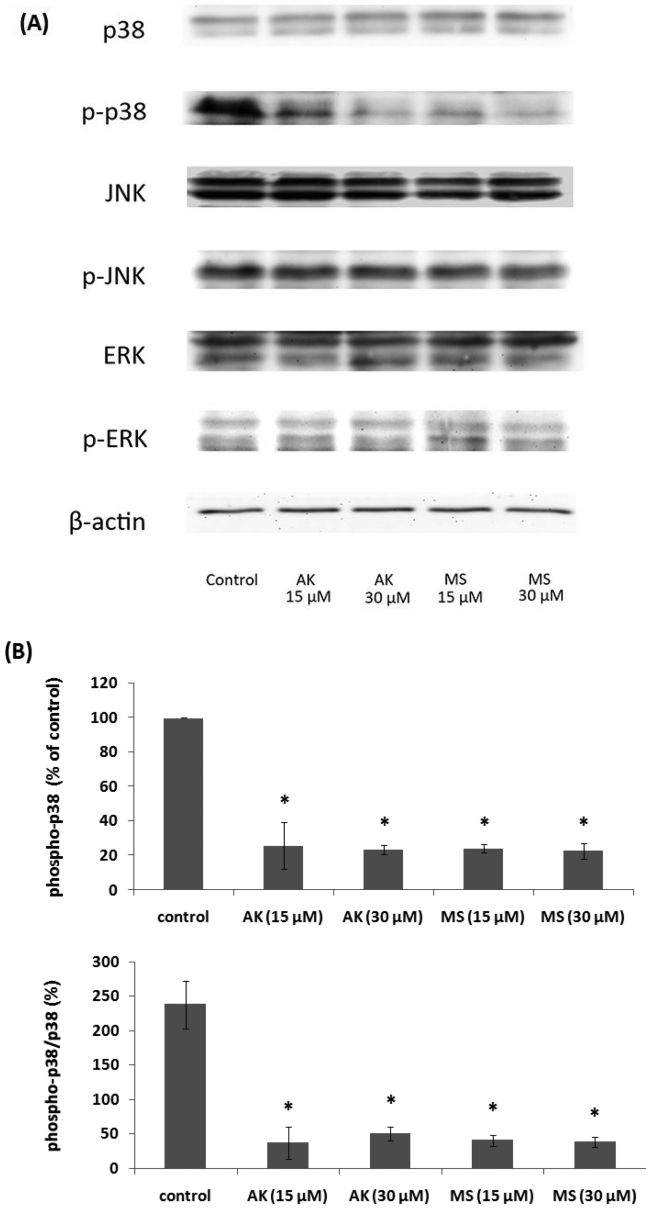

Figure 5. Ankaflavin and monascin effect on p38 MAPK pathway proteins. (A) p38, JNK, ERK, phosphorylated-p38, phosphorylated-JNK, and phosphorylated-ERK expression levels were analyzed in Western blots. (B) Quantification of the intensity of the band corresponding to phosphorylated-p38, using densitometry. The obtained results are presented as the relative phospho-p38/p38 ratio, and all levels were normalized to the level of β-actin. Total proteins served as the loading control. Results are expressed as mean ± standard deviation (SD; $n = 3$). *, $P < 0.05$, compared with the control. AK, ankaflavin; MS, monascin.

(4) induction of HSC apoptosis, and (5) the promotion of ECM degradation.[6] Liver fibrosis is always preceded by inflammation and oxidative stress.

In previous studies, ankaflavin and monascin were investigated, and it was demonstrated that they attenuate inflammatory responses.[24,25] Here, we demonstrated that ankaflavin and monascin attenuate HSC proliferation and activation and induce apoptosis. Additionally, apoptosis was found to be induced in activated HSCs, but not in hepatocytes, which may provide the safety strategy in antifibrotic therapy.[26] In the healthy liver, HSCs are responsible for the production of normal liver matrix, and these quiescent cells function as vitamin A storage. The transition of quiescent HSCs into activated myofibroblasts is the central pathogenic process during hepatic fibrosis. The production of fibrotic ECM proteins is increased, which contributes to the hepatic fibrosis as well.[27] Therefore, the potential inhibition of the activated

DOI: 10.1021/acs.jafc.6b03700
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

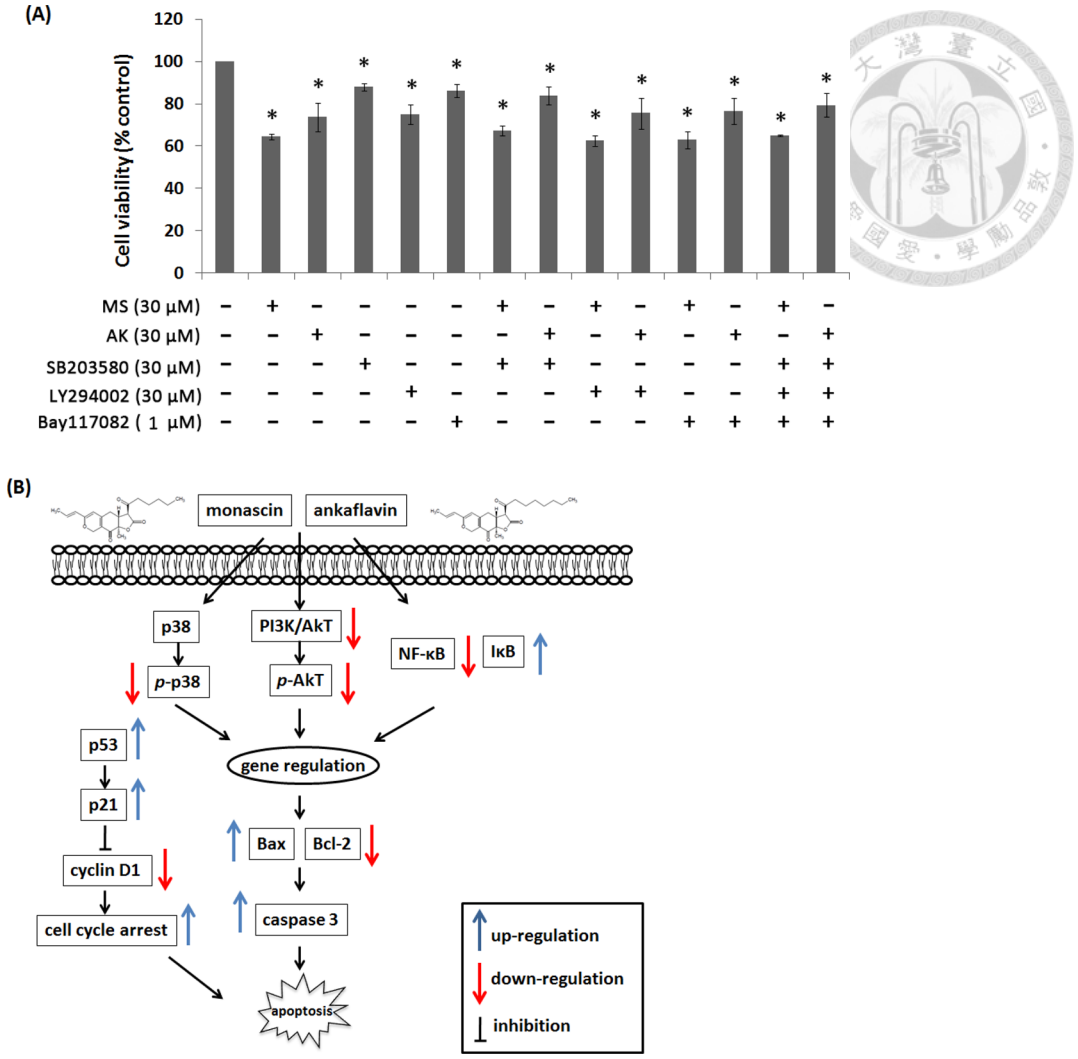

Figure 6. Ankaflavin- and monascin-induced apoptotic signaling pathway is mediated through the inactivation of Akt/NF-κB and p38 MAPK pathways in activated HSCs. (A) HSC-T6 was pretreated with DMSO or p38 MAPK inhibitor SB203580 (30 μM), PI3K inhibitor LY294002 (30 μM), or NF-κB inhibitor Bay117082 (1 μM) for 1 h and then incubated with 30 μM ankaflavin or monascin for 24 h, and the cell viability was determined using MTT assay. (B) A proposed ankaflavin- and monascin-induced apoptotic signaling pathway in activated HSC-T6 cells. Ankaflavin and monascin inhibit Akt, NF-κB, and Bcl-2 expression and Akt and p38 phosphorylation and, in turn, lead to an increase in IκB, p53, and p21 expression, inducing the apoptosis of HSC-T6 cells. Moreover, monascin increase the Bax level but not ankaflavin. The obtained results are expressed as mean ± standard deviation (SD; $n = 3$). *, $P < 0.05$, compared with the control group. AK, ankaflavin; MS, monascin.

HSCs is considered an important antifibrosis strategy.[28,29] HSC-T6 cell line, an immortalized rat liver stellate cell line, is often used as an in vitro model of hepatic fibrosis.[30] Here, HSC-T6 cells were maintained in the culture medium (DMEM with 10% FBS), in order to mimic the activated HSCs in vivo. In our study, ankaflavin and monascin show protective effects against in vitro hepatic fibrosis, inducing HSC-T6 cells apoptosis, inhibiting cell cycle progression, and proliferation. Notably, ankaflavin and monascin significantly decreased the proliferation of the HSC-T6 cells but showed no effects on primary hepatocytes. Monascin-induced inhibition of this proliferation was shown to be greater than that of ankaflavin (Figure 1A). We investigated the effects of ankaflavin and monascin on the expression of different proteins, including p53, p21, Bax, and Bcl-2, which can modulate apoptosis. Following the treatment with ankaflavin or monascin, p53 and p21 levels in HSC-T6 cells were found to be increased, together with the activity of caspase 3 (Figures 2 and 3). Additionally, p21 and p27 were previously shown to play a crucial role in the cell cycle regulation.[31] Notably, our findings demonstrate that the increased levels of p53 and p21 lead to the inhibition of cyclin D1 protein expression (Figure 2D,E), inducing G1 phase cell cycle arrest (Figure 2A,B) and the proliferative suppression of HSC-T6 cells. Bcl-2, Bax, and Bcl-XL are involved in the regulation of mitochondrial apoptotic pathway.[32] The activated HSCs were shown to resist pro-apoptotic stimuli, by over-expressing Bcl-2 for example, and this may play an important role in the development of chronic liver fibrosis.[33] Additionally, the levels of the proapoptotic protein, Bax, are low in these cells. Here, we showed that the treatment of HSC-T6 cell with ankaflavin or monascin led to the alterations in Bcl-2/Bax ratio, demonstrated by the inhibition of Bcl-2 expression and increased levels of Bax in the cells treated with 30 μM of monascin but not with ankaflavin (Figure 3D,E). These results indicate that the mechanism underlying ankaflavin- and monascin-induced apoptosis may be associated with p53/p21 pathway.

DOI: 10.1021/acs.jafc.6b03700
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In our previous study, monascin was shown to activate transcriptional factor PPAR-γ and affect the activation of HSCs through the activation of PPAR-γ.[16] PPAR-γ has been identified as a central regulator of many signaling pathways including the Akt, NF-κB, p38 MAPK, ERK, and JNK pathways, and it functions as a molecular switch in HSCs phenotype alteration and activation.[34] In our study, we demonstrated that both ankaflavin and monascin treatments lead to a considerable downregulation of Akt, NF-κB, phosphorylated-Akt, and p38 expression in HSC-T6 cells (Figures 4 and 5). PI3K/Akt and IκB/NF-κB pathways were reported to have a crucial role in the fibrogenic responses in liver. PI3K activation can activate its key downstream kinase, Akt, which leads to the inhibition of apoptosis and stimulates HSC proliferation. Growth factors may lead to the activation of PI3K/Akt signaling pathway, and it represents a crucial regulator of proliferation, survival, apoptosis, metabolism, and collagen expression in HSCs. A continuous activation of PI3K/Akt has been associated with the pathogenesis of liver fibrosis.[35,36] Furthermore, cytokine stimulation of HSC activation was shown to stimulate an increase in the expression of several NF-κB-regulated genes. These factors may be associated with hepatic inflammation, and the regulation of their expression may be clinically beneficial.[37] When stimulated by inflammatory cytokines, such as TNF-α, IκB is phosphorylated by IKK and degraded, releasing NF-κB, which translocates from the cytosol into the nucleus and activates the transcription of its target genes.[38] Therefore, the inhibition of NF-κB activity is considered a good strategy for antifibrosis treatment. In this study, we demonstrated that ankaflavin and monascin did not only decrease NF-κB expression but were also able to increase IκB levels. Additionally, monascin treatment was shown to lead to a significant upregulation of IκB protein expression compared with ankaflavin treatment (Figure 4C). The p38 MAPK signaling pathway was shown to be associated with HSC differentiation,[39] and the phosphorylation of p38 activates this pathway, which further induces NF-κB activation, such as histone H3 protein phosphoacetylation, while p38-dependent H3 phosphorylation leads to the upregulation of NF-κB recruitment.[40] We investigated the signaling pathways associated with hepatic fibrosis and determined that p38 MAPK signaling, involving ERK1/2, p38, and JNK, may be closely associated with liver fibrosis. We showed that ankaflavin and monascin repress the phosphorylation of p38 but do not affect JNK and ERK expression levels. We investigated the effects of the combination of ankaflavin or monascin with SB203580, LY294002, or Bay117082 inhibitors on HSC-T6 proliferation. Here, we found no significant differences between the effects of ankaflavin or monascin treatment alone and the combination of these inhibitors, or the combination of ankaflavin or monascin with the inhibitors, implying that the inhibitory effect of ankaflavin and monascin may be attributed mainly to their ability to suppress Akt, NF-κB, and p38 MAPK activation.

In conclusion, ankaflavin and monascin showed potential for the treatment of hepatic fibrosis by specific inhibition of HSC proliferation by altering protein expression levels of p53 and Bcl-2 family members. Our results showed that ankaflavin and monascin treatment significantly increases the levels of p53, p21, and Bcl-2 family apoptotic factors and inhibited the activation of HSC-T6 cell proliferation. Furthermore, the underlying mechanisms may be related to a significant downregulation of Akt, NF-κB, phosphorylated-Akt, and p38 expression and an increase in IκB expression, which leads to the inactivation of PI3K/Akt, IκB/NF-κB and p38 MAPK signaling pathways, ultimately inducing the apoptosis of activated HSCs. Additionally, we demonstrated that monascin has better effects on the induction of apoptosis, suppression of cell proliferation, and increase in IκB protein expression than ankaflavin. In our unpublished pharmacokinetic study we showed that, after the oral administration of ankaflavin or monascin (150 mg/kg of body weight) in SD rats, the C_{max} values of ankaflavin and monascin were 2.46 $\pm$ 0.72 and 1.73 $\pm$ 0.22 μmol/L, respectively (unpublished data). The distribution study showed that ankaflavin and monascin were distributed to the liver. Therefore, our study may help provide new insights for the development of therapeutic strategies against liver fibrosis.

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Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Notes

The authors declare no competing financial interest.

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DOI: 10.1021/acs.jafc.6b03700
J. Agric. Food Chem. doi: 10.6342/NTU201700799