科技部補助專題研究計畫成果報告
期末報告

國產本土諾麗果汁抑制肝纖維化之保健功效與機制探討

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 103-2320-B-040-004-
執 行 期 間 ： 103年08月01日至104年07月31日
執 行 單 位 ： 中山醫學大學醫學系微生物及免疫學科

計 畫 主 持 人 ： 張元衍

處 理 方 式 ：
1.公開資訊：本計畫涉及專利或其他智慧財產權，2年後可公開查詢
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3.「本報告」是否建議提供政府單位施政參考：否

中 華 民 國 104 年 10 月 27 日

中文摘要：諾麗(noni)，學名為Morinda citrifolia，含有豐富類黃酮(flavonoids)、配苷(glycosides)、維生素、及多元不飽和脂肪酸等，其中以抗氧化成份最為廣泛研究。近幾年本土栽種noni在台南市學甲區至少栽種約10公頃。

肝纖維化是一個動態、進行性的過程，在某些階段為可逆的，而肝臟星狀細胞的活化是肝纖維化的成因，其導致的肝硬化是肝癌的高風險因素之一。故如何阻延或逆轉肝纖維化為預防肝硬化、肝癌的重要課題。

分析本實驗所使用的諾麗果汁中主要酚系抗氧化物質及含量，同時也已利用液相層析儀(HPLC) 分析抗氧化物含量成分，其中我們發現多酚物質以gentisic acid (龍膽酸; 19.16 mg/100 mL),的含量最多，其次是p-Hydroxybenzoic acid (羥基苯甲酸; p-HBA; 14.12 mg/100 mL), 再來則是chlorogenic acid (氯原酸; 10.49 mg/100 mL)。之後以thioacetamide (TAA)誘發大鼠(Wistar rat)產生肝纖維化的症狀，實驗為期8週，共分成五組，分組如下：(1) Control group, (2) TAA, (3) TAA+NJ-L (2.51 mL NJ /Kg), (4) TAA+NJ-M (5.02 mL NJ /Kg), and (5) TAA+NJ-H (7.52 mL NJ /Kg)。結果發現TAA會誘使大鼠體重及相關血脂降低，但增加了肝臟重量及血清中ALT/AST的值，進而誘發肝纖維化。而餵食諾麗果的實驗組可藉由下降ER stress相關基因的表現、發炎相關的細胞激素及MMP-2/MMP-9的活性；並提升TIMP-1 與 TIMP-3的基因表現。同時也發現餵食諾麗果的實驗組可提升大鼠肝臟中的抗氧化能力、降低collagen的堆積而降低肝臟的損傷指數(HAI scores)。在本動物模式的探討下，提供諾麗果汁具有抑制TAA所誘發的肝纖維化的能力，未來可提供作為預防肝纖維化的保健食品。

中文關鍵詞：諾麗果汁、抗氧化、肝纖維化

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英文關鍵詞：noni juice, thioacetamide , liver fibrosis, inflammation,

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科技部補助專題研究計畫成果報告

（□期中進度報告/V期末報告）

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計畫主持人：張元衍
共同主持人：
計畫參與人員：林嘉緯、邱秉松、蔡宗憲、游凱婷

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：
□執行國際合作與移地研究心得報告
□出席國際學術會議心得報告

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中　華　民　國 104 年 10 月 30 日

附件二

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否有嚴重損及公共利益之發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   - V 達成目標
   - □ 未達成目標（請說明，以 100 字為限）
     - □ 實驗失敗
     - □ 因故實驗中斷
     - □ 其他原因

   說明：

2. 研究成果在學術期刊發表或申請專利等情形：

   論文：□已發表 □未發表之文稿 V 撰寫中 □無

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3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性），如已有嚴重損及公共利益之發現，請簡述可能損及之相關程度（以 500 字為限）

   明確了解國產諾麗果汁主要的酚系抗氧化物質/含量與礦物質成分，並藉由動物模式的探討下，提供諾麗果汁所具有的保健功能之依據。並建立一個篩選的模式，來尋找具抗氧化、發炎反應與抑制肝纖維化的天然物。

摘要

諾麗(noni)，學名為 *Morinda citrifolia*，含有豐富類黃酮 (flavonoids)、配苷(glycosides)、維生素、及多元不飽和脂肪酸等，其中以抗氧化成份最為廣泛研究。近幾年本土栽種 noni 在台南市學甲區至少栽種約 10 公頃。

肝纖維化是一個動態、進行性的過程，在某些階段為可逆的，而肝臟星狀細胞的活化是肝纖維化的成因，其導致的肝硬化是肝癌的高風險因素之一。故如何阻延或逆轉肝纖維化為預防肝硬化、肝癌的重要課題。

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關鍵詞：諾麗果汁、抗氧化、肝纖維化

ABSTRACT

The ameliorative effect of naturally fermented noni juice (NJ) which contains phenolic acids and polysaccharides on thioacetamide (TAA) induced liver fibrosis in rats was investigated. Five groups of 10 rats each were divided into (1) Control group, (2) TAA, (3) TAA+NJ-L (2.51 mL NJ /Kg), (4) TAA+NJ-M (5.02 mL NJ /Kg), and (5) TAA+NJ-H (7.52 mL NJ /Kg) for 8 weeks. TAA treatment resulted in lower ($p<0.05$) body weight and serum lipid levels, but increased ($p<0.05$) liver weight and collagen contents and serum ALT/AST values. Protective effects of NJ on TAA treatment were observed in decreased ($p<0.05$) ER stress-related gene expressions, inflammatory cytokines, and MMP-2/MMP-9 activities, as well as upregulating ($p<0.05$) TIMP-1 and TIMP-3 in livers. NJ also increased ($p<0.05$) hepatic antioxidant capacities, collagen accumulation, and HAI scores. It demonstrated that NJ retards the progression of liver fibrosis in TAA-treated rats and has a protective potential on liver fibrosis.

Keywoods*:* noni juice, thioacetamide , liver fibrosis, inflammation, antioxidant capacity

INTRODUCTION

Most chronic liver injuries including alcoholic disorder, viral hepatitis, biliary obstruction, or hemochromatosis consequently lead to hepatic fibrosis, a critical step deciding clinical outcome of chronic liver diseases.[1] Liver is a vital organ of metabolism and excretion in the body. It is involved in the biochemical conversions of varied administered substances which significantly increase the reactive oxygen species (ROS) generation.[2] The liberated radicals can be produced by hepatotoxins, such as thioacetamide (TAA). Several investigations have shown that a single dose of this hepatotoxic agent could produce centrilobular hepatic necrosis while a chronic administration led to cirrhosis.[3] TAA is a very effective, reliable, and satisfactory model for producing liver fibrosis in laboratory rodents. Various investigators have used different methods of TAA administration in experimental animals for producing fibrosis and cirrhosis, such as intraperitoneal or subcutaneous administration, mixing the toxin with the diet or in drinking water.[4, 5] It was assumed that oxidative stress contributes to the development of TAA-induced liver fibrosis. A previous study suggested that reactive oxygen species (ROS) are important factors in the cascade necessary for cytokine-induced liver fibrogenesis by TAA induction.[6] It has been reported that high levels of ROS can effectively induce apoptosis, likely through an activation of the endoplasmic reticulum (ER) stress-induced apoptotic pathway.[7] While transient and low grade ER stress can be overcome by the unfolded protein response (UPR), persistent and severe ER stress results in cell apoptosis and also causes inflammatory gene expressions.[8,9] Gebhardt (2002)[10] proposed that antioxidant supplements may emerge as potential anti-fibrotic agents by either protecting hepatocytes against ROS or inhibiting the activation of HSCs. Glutathione (GSH) is the major endogenous antioxidant in hepatocytes, whereas superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) are in charge of

counteracting ROS and hydrogen peroxide (H_2O_2) in biological systems. Recently, our previous reports have indicated that enhanced liver antioxidant capacities in high-cholesterol/fat dietary hamsters [4] or alcohol-diet fed mice [11] supplemented with NJ resulted from the polyphenolic contents in NJ.

Liver fibrosis is a chronic liver damage usually caused by alcohol, TAA, viruses or other toxins and is characterized by an excessive accumulation of extracellular matrix (ECM) proteins such as collagen.[12] The injured liver cells stimulate hepatic stellate cells (HSCs) to transform into myofibroblast-like cells which secrete large amounts of collagen, thereby producing liver fibrosis. Synthesis of collagen I is regulated by the ECM itself, and even though the basement membrane matrix preserves quiescence of HSCs, collagen I further enhances HSC activation in a paracrine manner. Increasingly, ROS are viewed as candidate drivers of HSC activation and collagen I up-regulation.[13,14] However, downstream mediators for the ROS effects on the activation of HSCs and the increase in collagen could be a potential way to alleviate liver fibrosis and inflammation.

Noni (*Morinda citrifolia*) belongs to the family Rubiaceae (coffee family), subfamily Rubioideae, and has been locally planted in southern Taiwan for decades. Polysaccharides, fatty acid esters, glycosides, iridoids, anthraquinones, flavonoids, phytosterols, carotenoids, vitamin A, antraquinones, potassium, etc. have been identified as putative active ingredients in noni juice. [15, 16,17] Gentisic, *p*-hydroxybenoic, and chlorogenic acids are the major phenolic acids in our fermented NJ [4] while the hepatic antioxidant and antiinflammation of NJ in a high-fat diet were partially due to its phenolic acids. Furthermore, the major mineral is potassium (K), followed by magnesium (Mg), and sodium (Na). Interestingly, some trace minerals, i.e. zinc (Zn), manganese (Mn), and selenium (Se) are also analyzed in NJ.[7] Based on our knowledge, macrominerals, that is, K, Mg, Na, and calcium (Ca),

are necessary and beneficial to human health, whereas trace minerals, Zn, Mn, and Se, are important as well. In addition, our previous study reported that our naturally fermented NJ contains polysaccharides (2141.52 mg/100 mL)[11] and antiinflammation of polysaccharides was also discussed. Astragalus polysaccharides downregulated the phosphorylation of ERK and JNK and then suppressed NFκB activation, which implicate decreased secretions of TNF-α and IL-1β.[11] Hence, we speculate that the bioactive compounds (polyphenols, polysaccharides, and minerals) in NJ may contribute to increased hepatic antioxidant capacities and anti-inflammatory responses in TAA treated rats.

Previous studies also indicated that these bioactive ingredients impart anti-inflammatory effects via regulating secretions of interleukin-1 beta (IL-1β), IL-6, and iNOS[18] as well as antitumorigenic effects via suppressing cell transformation and blocking of phosphorylation of c-Jun.[19] Although NJ showed hypolipidemic, antioxidative, and anti-inflammatory effects in high-fat/cholesteroldiet [4] and alcohol liquid diet [11] fed to hamsters and mice, respectively, the protective mechanism of NJ against TAA-induced rat liver fibrosis is still lacking. Therefore, by employing a TAA-induced liver fibrosis rat model, the present study addressed the protective effects of NJ via (1) increased antioxidative capacities, (2) down-regulation of inflammatory and ER stress, (3) inhibited collagen accumulation.

Materials and methods

Noni Juice (NJ) Preparation. Noni juice was prepared according to our previous methods. [4, 11] Noni fruits were purchased from a local fruit farm (Xuejia District, Tainan City, Taiwan), stored in a stainless steel bottle at room temperature for one year, and then separated through a wire mesh screen. The experimental NJ materials

were further centrifuged from collected NJ at 3000 g for 15 min, pasteurized at 80 °C for 60 s, and then stored at −20 °C until used to feed animals. On the basis of our previous studies,[4, 11] the major identified phenolic acids in NJ were gallic acid, gentisic acid, chlorogenic acid, p-hydroxybezoic acid, caffeic acid, ferulic acid, and p-anisic acid, whereas flavonoids in NJ included epicatechin, hesperidin, and naringin. Meanwhile, the amount of phenolic acids in NJ was almost 6 times higher than that of the flavonoids. Gentisic acid, *p*-hydroxybenzoic acid, and chlorogenic acid were the dominant phenolic acids in NJ. Hence, the total amounts of phenol, flavonoid, and condensed tannin contents were also measured in this study. Total phenolic contents were measured using FolinCiocalteu's phenol reagent and expressed as milligrams of gallic acid equivalent (GAE) per 100 mL of sample. [20] Flavonoid contents were determined with 10% $AlCl_3 \cdot H_2O$ solution and expressed as milligrams of catechin equivalent (CE) per 100 mL of sample. [21] Condensed tannin contents were surveyed through a modified method and expressed as milligrams of CE per 100 mL of sample.[20] Ascorbic acid was quantified according toa previous method .[22] The crude polysaccharides were precipitated with 95% ethanol at 4 °C for 12h.[23] The concentration of total polysaccharides in NJ was measured by the absorbance at 490 nm (UV−visible spectrophotometer, model T60, PG Instruments Ltd., Leicestershire, UK) against a standard curve (glucose as a standard) by using the phenol−sulfuric method.

Animals and experimental design. Sixty male Wistar rats (6-week old, 200–220 g) were purchased from BioLASCO Taiwan Co., Ltd., Taipei, Taiwan, and acclimated under an environmentally controlled room at 22±2℃ and 12/12-h light/dark cycle. After one week of acclimation, the 60 rats were randomly divided into five groups: (1) Control: saline+NDW (ddH_2O), (2) TAA, (3) TAA+NJ-L (rats were given 2.51 mL

NJ /Kg BW orally), (4) TAA+NJ-M (rats were given 5.02 mL NJ/Kg BW orally), and (5) TAA+NJ-H (rats were given 7.52 mL NJ /Kg BW orally). The dose and schedule of NJ were calculated in comparison with the dose from our previous report[11] between mice and rats .[24] During the experimental period, the saline or TAA (100 mg/kg) injections were administered on Monday, Wednesday, and Friday and the ddH_2O or NJ oral gavages on Tuesday, Thursday, and Saturday. Body weight, liver weight, serum biochemical values, and histopathological results were analyzed at the end of the 8week experiment.

The animals were killed under the ether anesthesia at the last experimental day. Rats were sacrificed by CO_2 asphyxiation. Blood was collected for biochemical analyses and other measurements, and livers were removed and weighed quickly. The liver tissues were fixed or stored in Bouin's solution or RNAlater (Ambion, TX, USA) for deep freezer (−70 °C) for further analyses. The animal use and protocol were reviewed and approved by the National Taiwan University Animal Care and Use Committee (IACUC No. 100-101).

Determination of serum biochemical values and hepatic lipids. The serum biochemical values, that is, triacylglycerol (TAG), cholesterol (TC), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined by using commercial enzymatic kits with the SPOTCHEM EZ SP-4430 automated analyzer (ARKRAY Inc., Kyoto, Japan). Hepatic triacylglycerol and total cholesterol were measured using commercial kits (Randox Laboratories Ltd., Antrim, UK) according to the previous method.[11]

Collagen Content in Livers. Collagen was determined by a dye-binding method (Sircol collagen assay; Biocolour Ltd, Carrickfergus, UK). Assay of each Liver

extracts was performed according to the manufacturer's instructions. In short, 1 ml of Sirius red dye reagent was added to each sample and mixed by shaking for 30 min to complete collagen-dye binding. After centrifugation at 10 000*g* for 10 min, unbound dye was decanted. The dye bound to the collagen pellet was then dissolved in 1 ml of alkali reagent. The dye concentration was measured by spectrophotometry at 550 nm. Hydroxyproline was measured according to the method by Reddy and Enwemeka. [25]

Preparation of Liver Homogenate. The liver homogenate (10%, w/v) was made with phosphate buffered saline (PBS, pH 7.0, containing 0.25 M sucrose), and the supernatant was collected by centrifugation at 12,000 g for 30 min. The protein content in the supernatant was measured according to the procedures of a Bio-Rad protein assay kit (catalog no. 500-0006, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Determination of liver lipid peroxidation level and antioxidant capacity. The liver thiobarbituric acid reactive substances (TBARS) level, an indicator, was used to determine liver lipid peroxidation, whereas reduced glutathione (GSH), trolox equivalent antioxidant capacity (TEAC), and activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were assayed as indices for liver antioxidant capacities. These assays were performed according to previously described procedures.[26] The measurement of hepatic TBARS value was based on the production of MDA. The liver TBARS value was calculated by taking the extinction coefficient of MDA to be 1.56×10^5 M^{-1} cm^{-1} and expressed as

nanomoles per milligram of protein. Due to the unique thiol compound in GSH, 2, 2-dithiobisnitrobenzoic acid (DTNB) is commonly used for thiol assay. The hepatic GSH content was calculated by taking the extinction coefficient of 2-nitro-5-thiobenzoic acid (NTB) to be 1.36×10^4 M^{-1} cm^{-1}at 412 nm and expressed as micromoles of MDA equivalents per milligram of protein. Hepatic GSH-Px activity was measured by taking the extinction coefficient of NADPH to be 6.22×10^6 nM^{-1} cm^{-1} at 340 nm and expressed as nanomoles of NADPH oxidized per minute per milligram of protein. Hepatic TEAC was measured by the scavenging 2,2′-azinobis(3-ethylbenzthiazoline-6- sulfonic acid) (ABTS+) capacity, was absorbed at 734 nm, and was expressed as millimoles per milligram of protein. Hepatic SOD was detected by the inhibitory effect of SOD on purpurogallin of pyrogallol oxidation product, was recorded at 420 nm, and was expressed as milliunits per milligram of protein. Hepatic CAT activity was calculated by taking the extinction coefficient of H_2O_2 to be 39.5 M^{-1} cm^{-1} at 240 nm and expressed as units per milligram of protein. Hepatic GSH-Px activity was measured according to the procedure as described by Hong and Lee (2009) with a slight modification.[27] Briefly, 2-μL liver homogenate was mixed with 988-μL reaction solution containing 100 mM PBS (pH 7.0), 10 mM EDTA, 10 mM NaN_3, 2 mM NAPDH, 10 mM GSH, 1 U/ml GSH reductase. After 5 min, 100-μL H_2O_2 (2.5 mM) was then added. The

difference of absorbance between 0 and 3 min was measured at 340 nm. Hepatic GSH-Px activity was calculated by taking the extinction coefficient of NADPH to be 6.22×10^6 $nM^{-1}cm^{-1}$, and expressed by nmole NADPH oxidized/min/mg protein.

Determination of Hepatic TNF-α and IL-1β levels. Hepatic TNF-α and IL-1β concentrations were assayed by using ELISA kits based on anti-mouse TNF-α and IL-1β monoclonal antibodies (BD Pharmingen, San Diego, CA, USA) and converted to the TNF-α and IL-1β levels expressed as picograms per milligramof protein by using standard curves.

Histopathological Analysis. The liver tissues were fixed in neutral-buffered formalin solution for no more than 24 h, dehydrated in graded alcohol, cleared in xylene and then embedded in paraffin. Histopathological examination was performed by haematoxylin and eosin (H&E) and Masson's Trichrome staining. The liver damage was measured by a double-blind test according to the histological activity index (HAI) scoring.[28]

Real-time PCR. Total RNA was isolated from the stored frozen liver tissues by using the protocol described by Rneasy Mini Kits (Qiagen, Valencia, CA, USA). Reverse transcription was carried out with 2 μg total RNA, 8 μL reaction buffer, 2 μL dNTPs, 4.8 μL $MgCl_2$, 4 μL Oligo-dT (10 pmole/L) and 200U RTase (Promega, Madison, WI, USA) with diethyl pyrocarbonate (DEPC) H_2O in a final volume of 40 μL at 42 ^oC for 1 h. After heat inactivation, 1μL cDNA product was used for a quantitative real-time PCR to determine the expression of tissue inhibitor of metalloproteinase 1 (TIMP-1), TIMP-2 and TIMP-3. Real-time PCR was carried out in a 15 μl reaction

volume using ABsolute Blue SYBR Green ROX (Thermo Scientific, Epson, Surrey, UK) and the following primers: iNOS (F :5’CCAACAATACAAGATGACCCTAAG3’ R: 5’GTTGATGAACTCAATGGCATGAG3’*;* NM_010927.3*),* Bip (F:5’CTATTCCTGCGTCGGTGTGTTCAA3’; R: 5’GGTTTGCCCACCTCCAATATCAA3’), XBP-1 (F :5’GAAAGCGCTGCGGAGGAAAC3’; R: 5’GAGGGGATCTCTAAAACTAGAGGC3’; NM_013842), ATF4 (F:5’GCCATCTCCCAGAAAGTTT3’; R: 5’AGGTGGGTCATAAGGTTTGG3’; NM_009716), Calr (F:5’AAGAGGACAAGAAGCGTAAA3’; R5’ATCAGAATCTACCCCAGATCT3’; GenBank No:??), IRE1 (F:5’GTGTCGTCAGCAGCAGTCTCT3’; R:5’GGGTCCCTGGTCATTGAG3’; GenBank No:??); TIMP-1 *(F: 5’GGCATCCTCTTGTTGCTATCACTTG3’; R:* GTCATCTTGATCTCATAACGCTGG3’; NM_009992.3), TIMP-2 (F: 5’CTCGCTGGAGGACGTTGGAGGAAAGAA3’; R: 5’AGCCCATCTGGTACCTGTGGTTCA3’; NM_011594), TIMP-3 (F: 5’CTTCTGCAACTCCGACATCGTGAT3’; R: 5’CAGCAGGTACTGGTACTTGTTGAC3’; NM_000362), GAPDH (F: 5’-GACCCCTTCATTGACCTCAAC-3’,R: 5’-GGAGATGATGACCCTTTTGGC-3’; NM_007393.2). To avoid amplification of genomic DNA, the primers were placed at the junction of two exons. Semiquantitative real-time PCR was done using GAPDH as an internal control to normalization for other gene expressions.

Activities of MMP-2 and MMP-9 in Liver Tissues. The activities of MMP-2 and MMP-9 in liver tissues were measured by gelatin zymography protease assays as described by Chou et al.[29] Briefly, liver homogenates were prepared with SDS

sample buffer without boiling or reduction and subjected to 0.1% gelatin-8% SDS-PAGE. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in a reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% NaN_3) at 37°C for 12 h. Gels were stained with Coomassie brilliant blue R-250, and gelatinolytic activities were detected as clear bands against the blue background.

Statistical Analysis. The values are expressed as the mean ± SEM. A significant difference was used at the 0.05 probability level. One-way analysis of variance and the least significant difference test were used to differentiate the differences between treatments. All statistical analyses of data were performed using SAS (SAS Institute Inc., Cary, NC, USA, 2002).

RESULTS

Effects of NJ on Body Weight and Liver Weight Changes of TAA-treated Rats

After 8 weeks of experiment, the body and liver weights of rats among groups are shown in Table 1. TAA treatment significantly decreased the body weight but increased the liver weight and sizes (LW/BW, %) compared to those of the Control group ($p < 0.05$). NJ cotreatment ameliorated the increased body weights compared to only TAA treated group (TAA+NJ-M and TAA+NJ-H group, $p < 0.05$). Although there were not significant differences on liver weight (g) among the TAA-treated rats ($p > 0.05$), increased liver sizes of rats treated with TAA were exhibited compared to that without TAA (Control group) [TAA group vs. Control group, 1:1.48] ($p < 0.05$) and meanwhile NJ cotreatment lowered the liver sizes in TAA-treated rats ($p < 0.05$).

Effects of NJ on Serum Biochemical Parameters, Liver Lipid and Collagen Contents, as well as Histophathological Analysis of TAA-treated Rats

In the present study, serum lipid and liver damage indices (Fig. 1), as well as liver lipid and collagen contents (Fig. 2) of rats treated with TAA were changed ($p < 0.05$) when compared to those of Control rats. TAA treatment reduced ($p < 0.05$) serum TAG and TC levels at the 4th and 8th week of observations (Fig. 1A & B), but increased ($p < 0.05$) AST and ALT values at the end of experiment (Fig. 1C & D). Meanwhile, liver TAG and TC levels (Fig. 2A), as well as collagen contents (Fig. 2B) were also increased when compared with the Control group ($p < 0.05$). However, those elevated values were significantly improved by NJ cotreatment compared to those of TAA group ($p<0.05$) (Fig. 1C&D & Fig. 2). NJ cotreatment increased ($p<0.05$) serum TAG levels in TAA-treated rats but the values were still lower ($p<0.05$) than that of Control rats while NJ cotreatment normalized ($p<0.05$) serum TC levels in TAA-treated rats as that of Control rats. As an observation in histopathological examination, H&E staining revealed that TAA treatment caused acute focal necrosis and vacuolization in liver cells with mild inflammatory cell infiltration (Fig. 3A). The HAI scores also indicated higher portal and lobular inflammation, as well as periportal necrosis in TAA treated rats than those of Control rats ($p<0.05$), but only the periportal necrosis levels of TAA-treated rats were ameliorated by NJ cotreatment ($p<0.05$) (Fig. 3B). Masson's trichrome staining showed the severe collagen accumulation (part of the blue staining) in the TAA treated groups while NJ cotreatment remarkably reduced the accumulation of those fibrotic tissues (Fig. 3C).

Effects of NJ on TBARS Level, GSH Contents, and Antioxidant Enzymatic Activities in Livers of TAA-treated Rats

Changes of lipid peroxidation and antioxidant capacities in livers are demonstrated in Table 2. TAA treatment resulted in an increased hepatic TBARS

levels and depleted hepatic reduced GSH contents as compared to those of Control group ($p < 0.05$), suggesting the impairment of hepatic antioxidant capabilities. Although NJ cotreatment did not ($p>0.05$) alter hepatic TBARS values in TAA-treated rats, there was a tendency toward higher hepatic reduced GSH contents in TAA-treated rats by NJ cotreatment while a significant difference was only observed in medium dosages of NJ (TAA+NJ_M group). Regarding antioxidant enzymatic activities, higher ($p < 0.05$) SOD and CAT activities were measured in the TAA-treated rats compared to those in Control group, but NJ cotreatment further enhanced ($p < 0.05$) those activities in TAA-treated rats. Besides, TAA group had the lower ($p < 0.05$) hepatic GSH-Px activity than Control group, but NJ cotreatment normalized ($p < 0.05$) it (TAA+NJ-L group) or even resulted in higher GSH-Px activities (TAA+NJ-M and TAA+NJ-H groups) than that of Control group.

Effect of NJ on mRNA Expressions of Proinflammatory Mediator iNOS, Endoplasmic Reticulum (ER) Stress , and Tissue Inhibitors of Metalloproteinase, as well as Cytokine Contents (TNF-α and IL-1β) and Metalloproteinase-9/2 Activities in Livers of TAA-treated Rats

After TAA treatment, the mRNA levels of iNOS in livers of rats were greatly ($p < 0.05$) upregulated by approximately 2.4-fold compared with that of Control group (Fig. 4A). However, NJ cotreatment retarded ($p < 0.05$) the upregulated iNOS expressions. These down-regulated changes represent about a 35% reduction for iNOS from the TAA group to the TAA groups treated NJ. Similarities were detected in gene expressions of BiP (immunoglobulin binding protein or glucose-regulated protein 78-kDa, GRP78), IRE1 (inositol-requiring enzyme 1), XBP1 (X-box binding protein 1), and ATF4 (activating transcription factor 4) while the lower ($p < 0.05$) ATF4 gene expression was only observed in low dosage of NJ supplementation

(TAA+NJ-L group) compared to that of TAA group. However, the increased Calre gene expressions in TAA treated rats compared to that of Control rats were not ($p > 0.05$) influenced by NJ cotreatment (Fig. 4A). TNF-α and IL-1β levels in the TAA-treated rats were significantly higher than those in Control rats, but only IL-1β levels in TAA-treated rats were decreased by NJ supplementation (Table 2). Among TIMP-1, -2, and -3 mRNA expressions, hepatic TIMP-1 and-3 gene expressions were increased ($p < 0.05$) after 8-week injury compared with those of Control group while TIMP-2 gene expressions were not ($p > 0.05$) different among groups (Fig. 4B). However, NJ cotreatment downregulated ($p < 0.05$) hepatic TIMP-1 and 3 expressions. For detecting the degrees of inhibition of MMP-2 and MMP-9 by TIMPs in this study, the zymography technique was applied (Fig. 4C). The MMP-2 and -9 activities in Control group were very low, but TAA treatment dramatically increased those activities . NJ cotreatment significant decreased ($p < 0.05$) activities of those two enzymes in TAA-treated rats where the decreased relative activities in MMP-9 are larger than that in MMP-2.

Discussion

Recently, growing understandings of the pathophysiology behind liver fibrosis has contributed to the development of agents that could potentially inhibit and reverse the fibrotic process in livers. TAA is considered a potent hepatotoxin which is frequently used to produce an experimental model on investigation of mechanisms involved in the progression of hepatic diseases and the impact of various drugs on this progression. Hepatic injury caused by TAA shows lesions similar to those seen in most cases of human liver diseases.[30] In this study, we tried to elaborate the ameliorative effects of NJ on TAA-induced hepatic fibrosis via a rat model.

In the present study, the damage and inflammation in hepatocytes were observed by serum biochemical values, liver cytokines, and H&E and Masson's Trichrome stainings. Hence, our results showed that 100 mg TAA/ kg BW applied three times weekly successfully induced chronic liver fibrosis in rats which also confirmed the results from a previous report. [31] According to the histopathological findings, cotreatment with NJ showed an amelioration of liver structures in TAA-induced liver fibrosis in rats. It has been reported that the hepatoprotection of silymarin on TAA-induced chronic liver damage is attributed to down-regulations of hepatic MMP-2, MMP-13, TIMP-1, TIMP-2, activator protein 1 (AP-1) and Kruppel-like factor 6 (KLF6),, transforming growth factor beta 1 (TGF-β1), alpha smooth muscle actin (α-SMA) and collagen alpha 1 (COL-α1).[5] Based on our results, there was a remarkable reduction in the extent of liver fibrotic scars in TAA-treated rats cotreated with NJ, which is valued to dig into the protective molecular mechanisms of NJ in TAA-treated rats. Besides, these indices of liver inflammation and fibrosis were significantly lower in TAA-treated rats administered with NJ. Serum liver biomarkers (ALT and AST values) are important criteria for the evaluation of liver injuries where TAA-induced hepatic damage resulted in significant increases in serum ALT and AST levels.[32] In the present study, the rats intoxicated with TAA experienced the hepatic injury evidenced by significant changes (Fig. 1) in serum liver biomarkers when compared to Control rats. However, cotreatments of low, medium, and high dosages of NJ in TAA-treated rats significantly lowered the ALT activity by 20.01, 29.2, and 35.7%, respectively, as well as AST activity by 45.72, 55.26, and 56.65%, respectively as compared with TAA-treated rats without NJ, indicating a potential hepatoprotection of NJ supplementation.

In addition, the TAA-treated groups showed a marked reduction in body weight with significantly increased liver weights and sizes compared to Control rats, but NJ

cotreatments reversed those observations (Table 1). The reduction in body weights could be attributed to the toxic effect of TAA throughout the period of the experiment.[6] Moreover, TAA altered fatty acid composition in tissues, thus decreasing fatty acid biosynthesis in the liver and lowering serum TAG.[33] It was reported that an improvement of NJ on serum lipids and oxidative status in high-cholesterol/fat dietary hamsters is highly related regulations of lipid homeostasis by the phytochemicals in NJ.[4] Pallottini *et al.*, also reported that TAA reduced 3-hydroxy-3-methylgltaryl coenzyme A reductase (HMG-CoA reductase) activity,[34] which may explain the lower serum TC level observed in rats treated with TAA (Fig. 1). Furthermore, TAA group strongly showed a significant elevation of liver TC and TAG levels in comparison to NJ-treated groups (Fig. 2). Decreased liver lipid accumulation in alcohol-diet fed mice with NJ partially result from increased daily fecal lipid and bile acid outputs.[11] The liver plays a major role in lipid metabolism, importing free fatty acid (FFAs) and manufacturing, storing, and exporting lipids; derangements in any of these processes can lead to the development of NAFLD.[35]As a previously study, fatty acid accumulation also leads to the induction of ER stress and ROS formation, which again promotes hepatic injury.[36] TAA is known to induce centrilobular hepatic necrosis, liver cirrhosis, hepatocellular carcinoma, and bile duct proliferation.[37]TAA-induced liver fibrosis is caused by free radical-mediated lipid peroxidation.[38] MDA is the main product of lipid peroxidation and its concentration usually reflects the total level of lipid peroxidation. Lipid peroxidation occurs during the processes of liver fibrosis and inflammation. Generations of reactive oxygen species (ROS), mitochondrial dysfunction, and antioxidant insufficiency have been reported to advance the development of liver cirrhosis.[39, 40] Thus, oxidative stress also triggers production of inflammatory cytokines, causing inflammation and a fibrogenic response, and however, known to be of major importance in the

progression of this disease. The current study demonstrated that TAA rats exhibited a significant increase in hepatic TBARS values (a marker of lipid peroxidation levels) and depletion of reduced GSH as compared to Control rats, suggesting the impairment of hepatic antioxidant capabilities.[38] Our results showed that NJ can decrease the liver TBARS values but increase liver reduced GSH content, and SOD, CAT, and GSH-Px activities in TAA-treated rats (Table.2). Besides, NJ cotreatment effectively reduced serum ALT and AST levels (Fig. 1), hepatic fibrosis scores (Fig. 3B), the gene expressions of iNOS, Bip, IRE1, XBP-1, and ATF4 in livers (Fig. 4A), hepatic IL-1β contents (Table 2) and collagen accumulation (Fig. 2B) in a rat model of hepatic fibrosis induced by TAA intoxication. Therefore, anti-inflammation/fibrosis of NJ treatment against an TAA induction could be corresponding to the reduction of liver oxidative levels. Oxidative stress, along with unresolved ER stress, contributes to the cell death. Oxidative stress and ER stress are closely related. Whereas oxidative stress triggers ER stress and UPR, excessive or prolonged ER stress can decrease mitochondrial membrane potential, limit bioenergetics changes, and foster the generation of ROS generation, and ultimately induce apoptosis.[41] The present study is the first evidence to show the hepatic oxidative stress and damage, along with lipid accumulation, inflammation, ER stress and its associated cell death, fibrotic response, and consequently hepatic dysfunction in TAA treated rat model. It was also demonstrated here that supplementing NJ to TAA-treated rats for 8 weeks alleviate these pathological changes, including ER stress (Fig. 4), lipid accumulation (Fig. 2), inflammation (Table 2), and fibrosis (Fig. 3).

Our previous study indicated that NJ naturally fermented for one year contains many polysaccharides, polyphenols, and some trace minerals (Zn, Mn, and Se).[11] Natural polysaccharides, largely found in fruits and vegetables, have been confirmed

to play a vital role as free radical scavengers (antioxidant activity),[42] and the discovery and assessment of them as new safe compounds in functional foods could attract much attention. Reports demonstrated that polysaccharides and polyphenols were synergistic in reduction of serum leptin levels and in anti-inflammatory activity.[43] It has been proposed that Mn and Se are cofactors for SOD and GSH-Px, respectively.[44] The contents of Se and Zn have been reported to be associated with the antioxidant and anticancer activities and help to reduce cardiovascular disease.[45]

Therefore we speculate that the bioactive compounds (polyphenol, polysaccharide, Zn, Mn, and Se) in NJ may contribute increased liver antioxidant capacities in TAA treated rats (Table 2). In addition to collagen I, activated HSC also expressed tissue TIMPs, leading to the hypothesis that matrix degradation is inhibited during progressive fibrosis.[46,47] Our results showed that TAA induces pro-inflammatory response as evidenced by increase in ROS production, the release of cytokines like TNF-α, IL-1β (Table 2), and matrix degrading enzymes such as MMP-9 and MMP-2 (Fig. 4C). Recently, the TIMP-1 inhibition may not be maximal and MMP-mediated degradation still occurs in remodeling during progressive fibrosis. [48,49] Our results showed that NJ cotreatment exerts hepatoprotective effects against TAA damage possibly via down-regulation of MMP-2 and MMP-9 activities and TIMP-1 and TIMP-3 (Fig. 4B&C). Taken together, these data strongly suggest that the protection of NJ in fibrotic tissues is potentially regulated by MMP-9-mediated degradation, with active MMP-9 being inhibited by increased interaction with TIMP-1 and TIMP-3 with worsening fibrosis *in vivo*. In summary, NJ manifested effective hepatocellular protective action and ameliorative effect against chronic liver damage and developing liver fibrosis induced by TAA induction.

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Table 1. Effects of NJ on body and liver weight/size changes of experimental rats.

Group	**Body weight, BW (g)**	**Liver weight, LW (g)**	**LW/BW (%)**
Control	**453.37±6.73a**	**10.46±0.71b**	**2.37±0.04c**
TAA	**335.55±6.59c**	**12.57±1.08a**	**3.51±0.06a**
TAA+NJ-L	**351.88±2.03bc**	**11.51±0.43a**	**3.15±0.06b**
TAA+NJ-M	**359.54±5.79b**	**11.50±0.48a**	**3.27±0.08b**
TAA+NJ-H	**363.32±4.27b**	**11.91±0.37a**	**3.23±0.06b**

* The data are given as mean ± SEM (n=10). Mean values with different letters are significantly different (p<0.05).

Table 2. Liver peroxidation, antioxidant capacities, cytokine of the experimental rats

	Control	TAA	TAA+NJ-L	TAA+NJ-M	TAA+NJ-H
			Liver lipid peroxidation		
TBARS (nmole MDA eq./mg protein)	0.11±0.00b	0.28±0.01a	0.26±0.01a	0.26±0.01a	0.25 ±0.01a
			Liver antioxidant capacities		
GSH (nmole/mg protein)	53.95±3.23a	40.77±2.73b	44.07±2.84b	55.52±3.57a	47.50±4.04ab
SOD (munit/mg protein)	17.29±1.12b	19.23±1.80b	25.00±0.92a	26.86±1.29a	28.70±2.83a
CAT (unit/mg protein)	16.59±1.76d	20.47±0.70c	25.24±1.31b	29.57±0.97a	28.73±0.90a
GSH-Px (nmole NADPH oxidized/min/mg protein)	66.08±3.32b	51.01±2.19c	70.85±3.08ab	73.71±2.83a	76.19±1.36a
			Cytokine		
TNF-α (pg/mg protein)	3.87±0.26b	5.02±0.14a	4.84±0.26a	4.88±0.29a	4.51±0.22ab
IL-β (pg/mg protein)	17.66±0.74c	26.05±0.95a	20.62±0.71b	20.58±1.34b	22.83±1.02b

* The data are given as mean ± SEM (n=10). Mean values with different letters are significantly different (p<0.05).

Figure captions

Figure 1. Serum triacylglycerol (TAG) (A), total cholesterol (TC) (B), AST (C) and ALT (D) of the experimental rats. The data are given as mean $\pm$ SEM (n=10). Mean values with different letters on data bars are significantly different ($p<0.05$).

Figure 2. Liver TAG and TC contents (A), and collagen contents (B) of the experimental rats. The data are given as mean $\pm$ SEM (n=10). Mean values with different letters on data bars are significantly different ($p<0.05$).

Figure 3. (A) The H&E staining of representative liver tissues in experimental rats, (B) The HAI scores of liver portal, lobular, and periportal necrosis, and (C) Masson's Trichrome staining for liver tissues in experimental rats. The data are given as mean $\pm$ SEM (n=10). Mean values with different letters on data bars are significantly different ($p<0.05$). The scale is shown in 200 μm. CV is the abbreviation for central vein.

Figure 4. The gene expressions of the endoplasmic reticulum stress pathway (A) and TIMPs (B), and MMP-2 and 9 activities (C) in livers of the experimental rats. The data are given as mean $\pm$ SEM (n=10). Mean values in each target gene with different letters were significantly different ($p<0.05$).

Fig.1

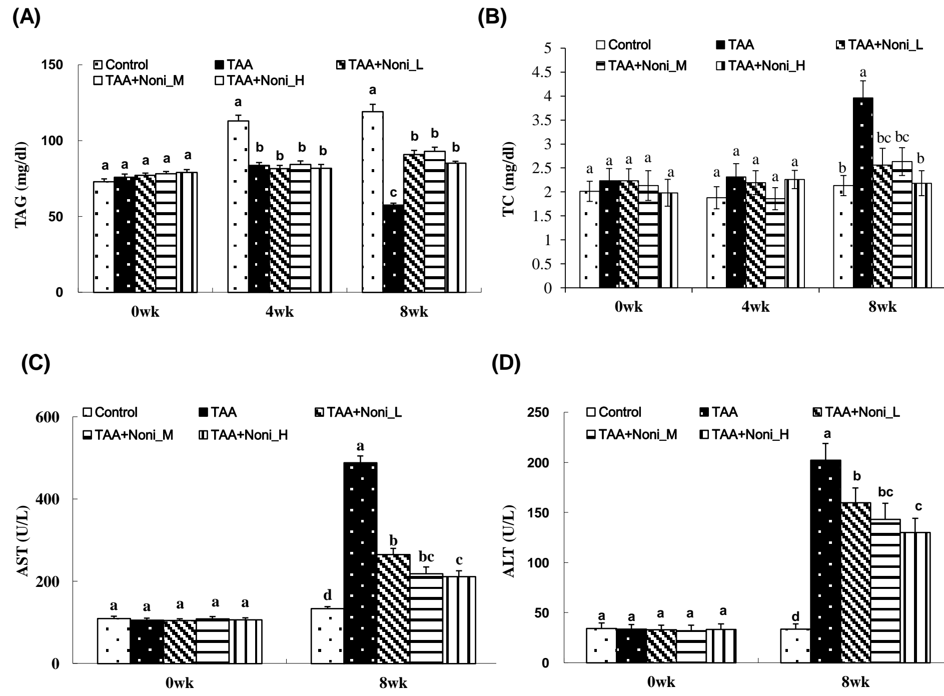
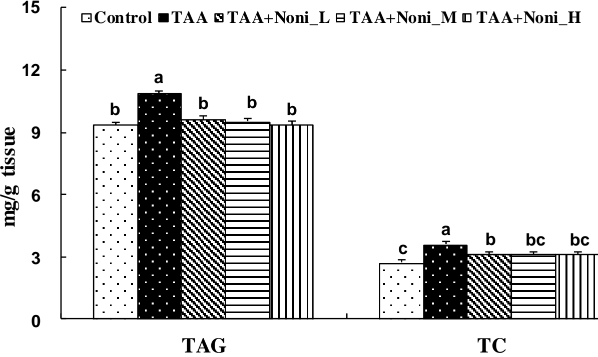


Fig. 2

(A)



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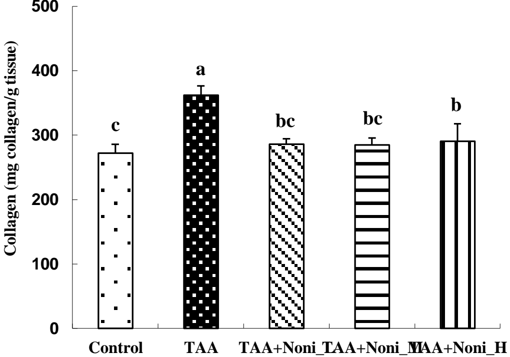
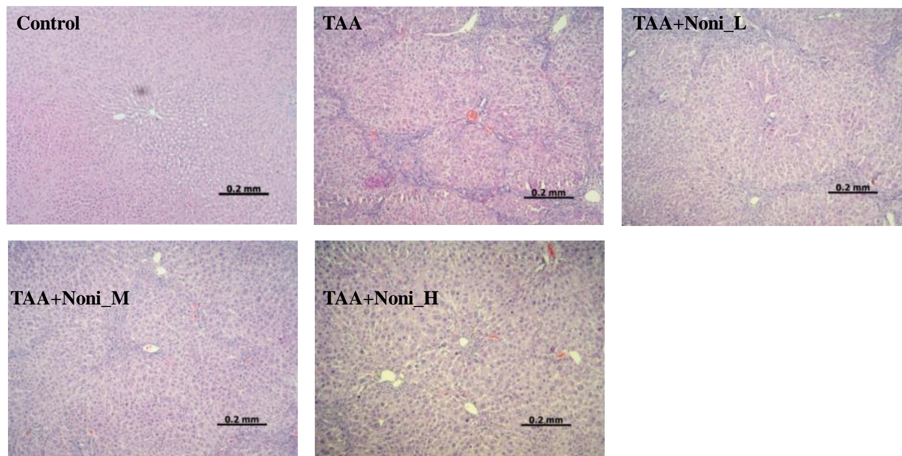


Fig. 3

(A)



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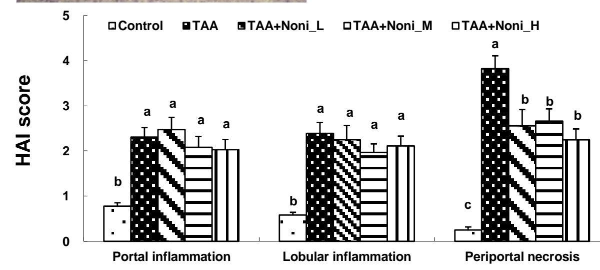


(C)

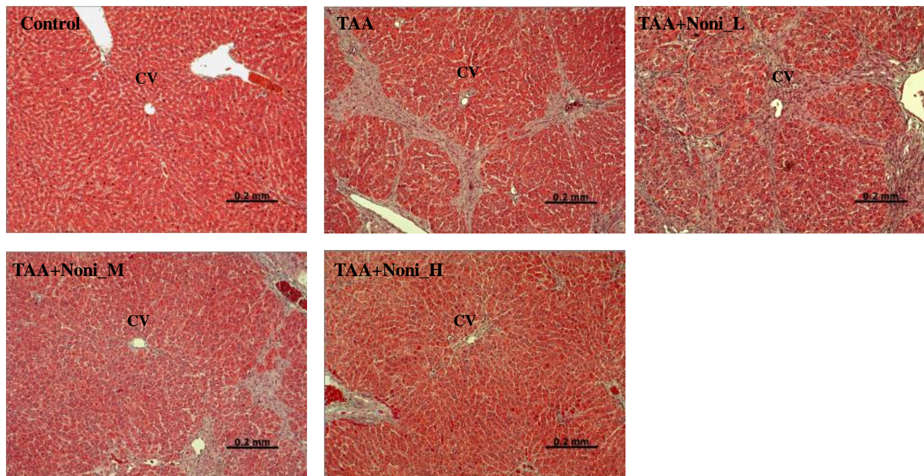
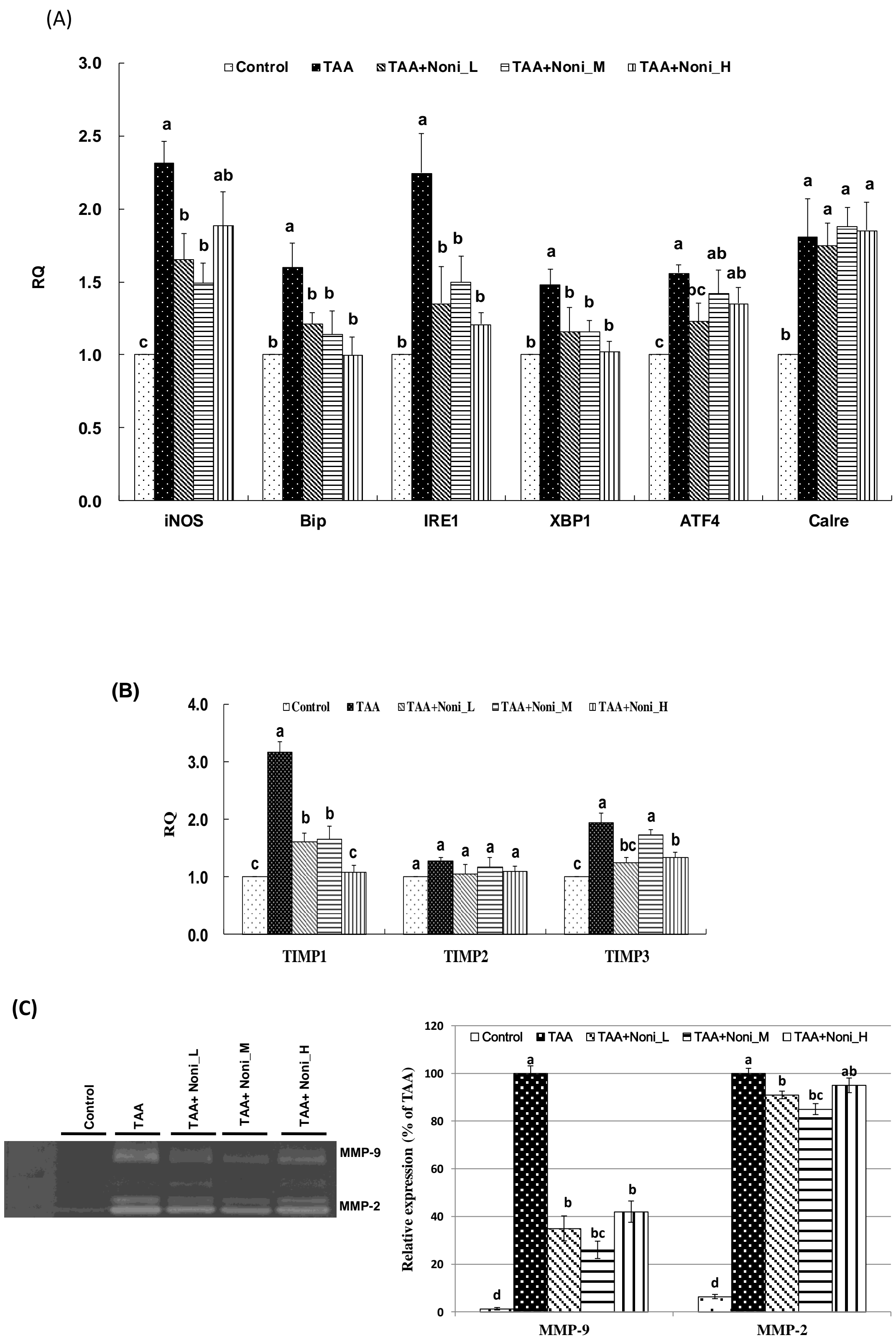


Fig. 4



科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/27

<table>
<tr><td rowspan="3">科技部補助計畫</td><td>計畫名稱: 國產本土諾麗果汁抑制肝纖維化之保健功效與機制探討</td></tr>
<tr><td>計畫主持人: 張元衍</td></tr>
<tr><td>計畫編號: 103-2320-B-040-004- 學門領域: 保健營養</td></tr>
<tr><td colspan="2">無研發成果推廣資料</td></tr>
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103年度專題研究計畫研究成果彙整表

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<tr><td colspan="3" rowspan="2">成果項目</td><td colspan="3">量化</td><td rowspan="2">單位</td><td rowspan="2">備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）</td></tr>
<tr><td>實際已達成數（被接受或已發表）</td><td>預期總達成數（含實際已達成數）</td><td>本計畫實際貢獻百分比</td></tr>
<tr><td rowspan="12">國內</td><td rowspan="4">論文著作</td><td>期刊論文</td><td>0</td><td>0</td><td>100%</td><td rowspan="3">篇</td><td></td></tr>
<tr><td>研究報告/技術報告</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>研討會論文</td><td>1</td><td>1</td><td>100%</td><td></td></tr>
<tr><td>專書</td><td>0</td><td>0</td><td>100%</td><td>章/本</td><td></td></tr>
<tr><td rowspan="2">專利</td><td>申請中件數</td><td>0</td><td>0</td><td>100%</td><td rowspan="2">件</td><td></td></tr>
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<tr><td>權利金</td><td>0</td><td>0</td><td>100%</td><td>千元</td><td></td></tr>
<tr><td rowspan="4">參與計畫人力（本國籍）</td><td>碩士生</td><td>0</td><td>0</td><td>100%</td><td rowspan="4">人次</td><td></td></tr>
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<tr><td>博士後研究員</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
<tr><td>專任助理</td><td>0</td><td>0</td><td>100%</td><td></td></tr>
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<tr><td colspan="2">其他成果（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）</td><td colspan="6">投稿中。</td></tr>
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	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與(閱聽)人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
      □實驗失敗
      □因故實驗中斷
      □其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形：
   論文：□已發表 □未發表之文稿 ■撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以100字為限）
   投稿中。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）
   明確了解國產諾麗果汁主要的酚系抗氧化物質/含量與礦物質成分，並藉由動物模式的探討下，提供諾麗果汁所具有的保健功能之依據。並建立一個篩選的模式，來尋找具抗氧化、發炎反應與抑制肝纖維化的天然物。未來可提供作為預防肝纖維化的保健食品。