DOI:10.1021/jf9017788

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Solanum nigrum L. Extract Inhibits 2-Acetylaminofluorene-Induced Hepatocarcinogenesis through Overexpression of Glutathione *S*-Transferase and Antioxidant Enzymes

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Solanum nigrum L. (SN) is a widespread plant and is regarded as a common relish in the east and the south of Taiwan. Our previous study has found that SN water extract (SNWE) alleviated carbon tetrachloride-induced liver damage in rats. However, the effects of SNWE on chemical-induced hepatic injury and hepatocarcinogenesis remain unclear. Therefore, this study aims to investigate the effects of SNWE on hepatic injury and hepatocarcinogenesis by using 2-acetylaminofluorene (AAF) and AAF/$NaNO_2$ treatment. The serum biomarkers for hepatic injury, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, and γ-glutamyl transferase, and for hepatocarcinogenesis, α-fetoprotein, were determined. Our results showed that AAF treatment led to a significant decrease of body weight and an increase of liver/body weight and serum biomarkers for hepatic injury and hepatocarcinogenesis. Interestingly, the SNWE supplement significantly lowered the liver/body weight and the biomarkers but did not affect the body weight. Further investigation revealed that a SNWE supplement increased the expression of glutathione *S*-transferase-α and -μ, the level of transcription factor for protection from oxidative stress, Nrf2, and the level of downstream targets regulated by Nrf2, including glutathione peroxidase, superoxide dismutase-1, and catalase. Moreover, the effects of SNWE on AAF/$NaNO_2$-induced hepatoma were also investigated, and the findings revealed that SNWE suppressed the progression of the hepatoma and resulted in a great increase of the survival rate. Our findings indicate that the SNWE supplement significantly alleviated the AAF-induced hepatic injury and early hepatocarcinogenesis as well as the AAF/$NaNO_2$-induced lethal hepatoma, which may result from the overexpression of glutathione *S*-transferases, Nrf2, and antioxidant enzymes.

KEYWORDS: *Solanum nigrum*; 2-acetyl aminofluorene; hepatic injury; hepatocellular carcingenesis; glutathione *S*-transferase; Nrf2

INTRODUCTION

Hepatocellular carcinoma (HCC) is a lethal disease with a poor prognosis and a 5 year survival rate of about 5%. It is one of the most common human malignancies in sub-Saharan Africa, Southeast Asia, and China. Although the incidence of HCC is generally lower in developed countries such as the United States, France, the United Kingdom, and Japan, it has increased significantly over the past decade (*1*, *2*). Today, the molecular mechanisms for the initiation and progression of HCC are not well-known, and most HCC patients are diagnosed at an advanced stage. Although chemotherapy is widely applied for preventing metastasis and recurrence, surgical resection remains a common treatment for HCC (*3*, *4*). Therefore, the development of new agents for hepatocellular cancer is important to reduce the mortality caused by this disease.

Hepatocarcinogenesis induced by carcinogens is a multistep and complex process (*5*) and is a favorite model in rat that facilitates the study of the mechanism of chemical carcinogenesis starting from a normal cell to a malignant transformation. Among the carcinogens, 2-acetylaminofluorene (AAF), belonging to an aromatic amine, is a genotoxic carcinogen that induces tumors in various species and tissues (*6*). In male rats, AAF is a powerful hepatic carcinogen leading to tumor formation (*7*). It is believed that the covalent interaction of metabolic derivatives of AAF with DNA is a critical step in the initiation of tumorigenesis (*6*). Although the presence of DNA adducts is a necessary prerequisite for tumor initiation, it may not be sufficient for tumor formation (*7*, *8*). AAF reacts in acidic conditions with nitrous fume yielding *N*-nitroso-AAF, which exerts more toxic and mutagenic effects than its parental compound. Our previous

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 Published on Web 08/24/2009

study also demonstrated that sodium nitrite ($NaNO_2$) significantly strengthened AAF-induced hepatocarcinogenesis (*9*). Accordingly, combining AAF with $NaNO_2$ is more useful and effective to induce malignant hepatoma than AAF alone.

Numerous medicinal herbs and their formulations are used for liver disorders in ethnomedical practice as well as traditional medicine in China. *Solanum nigrum* L. (SN), one of the medicinal herbs, has been used to treat inflammation, edema, mastitis, and hepatic cancer for a long time in Oriental medicine (*10*). SN grows wildly and abundantly in open fields in Southeast Asia. Traditionally, freshly prepared SN extract was directly used to treat cirrhosis of the liver and to alleviate pain in kidney and bladder inflammation. Previous studies have shown that SN extracts suppressed oxidant-mediated DNA-sugar damage (*10*), and the plant exerted cytoprotection against gentamicin-induced toxicity on Vero cells (*11*) and antineoplastic activity against Sarcoma 180 in mice (*12*). More recent studies revealed an inhibitory effect of SN extracts on 12-*O*-tetradecanoylphorbol 13-acetate-induced tumor promotion in HCT-116 cells (*13*) and a remarkable hepatoprotective effect of the ethanol extract of dried fruits of SN against carbon tetrachloride-induced liver damage (*14*, *15*). These studies suggest that SN possesses a beneficial activity as an antioxidant, antitumor-promoting and hepatoprotective agent; however, the mechanism for the activity remains to be elucidated.

Recently, we have demonstrated that the SN water extract (SNWE) contains several antioxidants, such as gallic acid, PCA, catechin, caffeic acid, epicatechin, rutin, and narigenin, and possesses strong antioxidative activities in vitro (*14*, *15*). In the present study, we further investigated the effects of SNWE on AAF-induced hepatic injury by quantitating body weight, liver weight, and the level of serum biomarkers for hepatic injury. To investigate the protective mechanisms induced by SNWE, the expression levels of glutathione *S*-transferases, antioxidant proteins, and their upstream transcription factors in liver were determined. Moreover, the effects of SNWE on AAF/$NaNO_2$-induced late stage lethal hepatoma and the resulting mortality were also investigated.

MATERIALS AND METHODS

Materials. Whole plants of SN were collected from a mountain region in Miaoli county, central Taiwan. Male Wistar rats were purchased from the National Laboratory Animal Center (Taipei, Taiwan). AAF, bovine serum albumin, dithiothreitol, ethylenediaminetetraacetic acid (EDTA), formaldehyde, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), Igepal CA-630, phenylmethylsulfonyl fluoride (PMSF), sodium chloride (NaCl), potassium chloride (KCl), sodium citrate, $NaNO_2$, and sodium phosphate were purchased from Sigma (St. Louis, MO). Antibodies against glutathione *S*-transferase (GST)-α and GST-μ were purchased from Oxford Biomedical Research Inc. (Metamora, MI). Antibodies against tubulin, Nrf2, GPx, SOD-1, and catalase were purchased from Cell Signaling Technology (Beverly, MA). Antibody against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated antibodies against mouse IgG or rabbit IgG were purchased from Sigma.

Preparation of SNWE and Composition Analysis. The whole plant of SN was collected from the mountain in Miaoli, Taiwan. The plants were washed with distilled water, cut into small pieces, shade-dried for 3 days, and then dried overnight in an oven. The dried SN plant (800 g) was cut into small pieces, mixed with water (5 L) for 30 min, and subjected to continuous hot extraction (100 °C, 40 min). The resulting extract was filtered and subsequently concentrated with a water bath (90 °C) until it became creamy and was dried in an oven (70 °C) that finally gave 185 g (23.125% of initial amount) of powder (SNWE). The SNWE supplement was prepared by mixing the SNWE powder with normal diet in 1 or 2% (w/w).

The content of polyphenol and polysaccharide in SNWE has been established in our previous study, and the analysis reveals that SNWE contains 20.4 ± 0.97% total phenolics using gallic acid as the standard, 14.9 ± 1.3% polysaccharide and 4.8 ± 0.4% protein. The further analysis by using high-performance liquid chromatography (HPLC) shows that SNWE consists of 2.90% gallic acid, 1.98% procatechuic acid, 2.53% catechin, 1.99% caffeic acid, 0.39 epicatechin, 0.84% rutin, and 5.11% naringenin (*14*).

Animals and Treatment. Male Wistar rats, age 4−5 weeks and weighing 140−160 g, were purchased from BioLASCO Taiwan Co., Ltd., kept at constant temperature at 22−24 °C, and illuminated for 12 h daily (lights on from 06:00 to 18:00). All procedures involving laboratory animal experiment were in accordance with the guidelines of the Instituted Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMU) for the care and the use of laboratory animals. After 1 week maintenance for adaptation to the environment, the rats were randomly grouped by body weight. Normal diet (Laboratory Rodent Diet 5001) was purchased from PMI Nutrition International, which consisted of 23.0% crude protein, 4.5% crude fat, 6.0% crude fiber, and 8.0% ash as described in the manufacturer's instructions.

For AAF-induced hepatic damage, the rats were divided into four groups, and each group contained 10 rats, which were fed on a unique diet for 6 months and weighed monthly. Diets for the four groups were (1) control, the normal diet; (2) AAF, the normal diet containing 0.03% AAF (w/w); (3) AAF + 1% SNWE, the diet of (2) containing 1% SNWE (w/w); and (4) AAF + 2% SNWE, the diet of (2) containing 2% SNWE (w/w). After 6 months of application of different diets, the blood and the whole liver were collected from rats that had been fasted 12−14 h and then were sacrificed. The whole livers were photographed, weighed, and then homogenized for protein extraction.

For AAF/$NaNO_2$-induced malignant hepatoma, the rats were divided into four groups, and each group contained 10 rats that were fed on a unique diet for 6 months. Diets for the four groups were (A) control, the normal diet; (B) AAF/$NaNO_2$, the normal diet containing 0.02% AAF (w/w) and 0.2% $NaNO_2$ (w/w); (C) AAF/$NaNO_2$ + 1% SNWE, the diet of (2) containing 1% SNWE (w/w); and (D) AAF/$NaNO_2$ + 2% SNWE, the diet of (2) containing 2% SNWE (w/w). The survival rate of each group was determined monthly. After 6 months, all of the surviving rats were sacrificed, and their livers were obtained for histopathological examination.

Determination of Serum Biomarkers for Liver Injury and Hepatocarcinogenesis. Blood samples were placed at room temperature for 1 h and then centrifuged at 1000*g* for 10 min to obtain serum. Serum biomarkers, including glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), γ-glutamyl transpeptidase (γ-GT), and fetoprotein (AFP), were assayed by the Boehinger Mannheim reagents [International Federation of Clinical Chemistry (IFCC) Scientific Committee recommended] (*16*, *17*).

Protein Extraction. Liver samples (0.1 g) were homogenized in 1 mL of ice-cold lysis buffer (10 mM HEPES, pH 7.2, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 1 mM PMSF). Eighty microliters of 10% (v/v) Igepal CA-630 was added into the homogenate and gently mixed well. After 10 min of incubation on ice, the homogenate was centrifuged at 14000*g* for 5 min, and then, the supernatant (cytosolic fraction) was transferred into a new 1.5 mL eppendorf and stored at −70 °C. The pellet was resuspended with the ice-cold lysis buffer containing 0.8% (v/v) Igepal CA-630 and incubated on ice for 10 min. After centrifugation, the pellet was resuspended with nuclear extraction buffer (50 mM HEPES, pH 7.2, 50 mM KCl, 300 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM PMSF, and 20% v/v glycerol) and incubated on ice for 10 min. After centrifuging at 14000*g* for 5 min, the supernatant (nuclear fraction) was transferred into a new 1.5 mL eppendorf and stored at −70 °C for analysis within 2 weeks.

Immunoblot Analysis. After feeding on indicated diets for 6 months, livers were obtained and homogenized for protein extraction. The crude proteins of liver were separated in a 12.5% sodium dodecyl sulfate−polyacrylamide gel and transferred onto a nitrocellulose membrane as previously described (*14*). The blot was subsequently incubated with 5% nonfat milk in phosphate-buffered saline (PBS) for 1 h, probed with a primary antibody against GST-α, GST-μ, tubulin, Nrf2, GPx, SOD-1, catalase, or β-actin for 2 h, and then reacted with an appropriate peroxidase-conjugated secondary antibody for 1 h. All incubations were

carried out at 30 °C, and intensive PBS washing was performed between each incubation. After the final PBS wash, the signal was developed by ECL chemiluminescence, and the relative photographic density was quantitated by image analysis system (Alpha Imager 2000, Alpha Innotech Corp., San Leandro, CA).

Assay of GST Activity. The total GST activity and the activities for specific GST isoform were determined according to the method of Habig et al. (*18*) using 4-chloro-7-nitrobenzofurazan (NBD-Cl) for GST-α and 1,2-dichloro-4-nitrobenzene (DCNB) for GST-μ. The enzyme activity was expressed as nanomoles of substrate-GSH conjugate produced per minute per milligram of cytosolic protein. The change in absorbance of GST-α and GST-μ was obtained at 419 and 345 nm, respectively, and the enzyme activity was calculated as nmol of NBD-Cl and DCNB conjugate formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}, respectively. The protein concentration was determined by Protein assay kit (Bio-Rad Laboratory, Watford, England) with bovine serum albumin as a standard.

Histopathological Examination for Malignant Hepatoma. The livers were collected, cut into small pieces, fixed in 10% buffered neutral formalin, and embedded in paraffin as described (*19*). Sections were cut at a thickness of 3−5 μm and stained with hematoxylin and eosin. The histopathological changes including cell morphology and cellular lipid vesicles were examined by light microscopy (400×).

Statistical Analysis. The experimental results were expressed as means ± standard deviations (SDs). Student's t test was used in the two-group comparison. A p value <0.05 was considered statistically significant (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA).

RESULTS AND DISSCUSION

AAF-Induced Loss of Body Weight Was Not Prominently Affected by SNWE Supplement. The variation of body weight during the 6 month experiment is shown in **Figure 1A**. The ratios of average body weight from month 6 to month 0 for each group were 1.385 (control), 1.172 (AAF), 1.260 (AAF + 1% SNWE), and 1.304 (AAF + 2% SNWE). During the 6 month experiment, AAF treatment significantly suppressed the increase of body weight as compared to the control, the findings of which indicated that the growth of treated rats was prominently suppressed. Although 1 or 2% SNWE supplement alleviated the AAF-induced interference on increase of body weight, the improvement was not significant in statistics.

Aromatic amines with highly hepatic toxicity usually lead to enlargement of the liver. Therefore, the effects of SNWE on AAF-induced enlargement of liver are investigated. At the end of the 6 month experiment, all of the rats were sacrificed, and the livers were obtained. The ratio of liver weight/body weight for each group is shown in **Figure 1B**. These findings indicated that AAF treatment significantly increased the ratio up to 4.6-fold of control, which was suggested resulting from the toxicity and the carcinogenicity of AAF. Interestingly, the ratio was significantly decreased in 1 and 2% SNWE-supplemented rats as compared to AAF treatment. The average increases of the ratio were 3.1- and 2.9-fold of control for 1 and 2% SNWE supplement, respectively.

Actually, the effects of SNWE itself on growth and liver function of rats were also examined. Our findings revealed that after treatment of up to 3% SNWE alone for 4 weeks, the body weight of the treated rats showed no significant changes as compared to normal control (data not shown). Moreover, the possible glycoalkaloid toxin solanine was undetectable in the SNWE by the HPLC analysis (data not shown). Accordingly, it is suggested that 1 and 2% SNWE alone should not affect the growth and liver function of the rats used in this study. Together, these findings suggest that SNWE supplement alleviates both the interference of growth and the abnormal enlargement of the liver induced by AAF treatment.

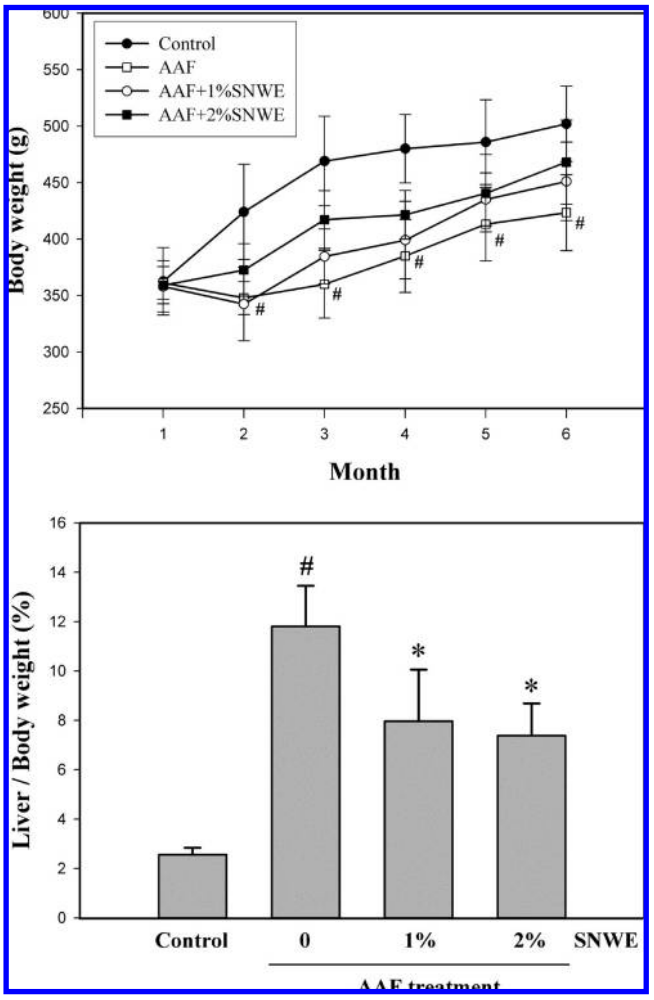

Figure 1. Changes of body weight and liver weight in Wistar rats fed on AAF and AAF supplemented with SNWE. (**A**) Changes of body weight in the rats fed on normal diet (control), normal diet containing AAF (AAF), normal diet containing AAF and 1% SNWE (AAF + 1% SNWE), and normal diet containing AAF and 2% SNWE (AAF + 2% SNWE). (**B**) Changes of liver weight in the rats fed with the four diets. Data are shown as the means ± SDs; #, $p < 0.05$ as compared with the control group; and *, $p < 0.05$ as compared with the AAF group.

SNWE Supplement Lowered the Levels of Serum Biomarkers for Hepatic Injury and Hepatocarcinogenesis Induced by AAF Treatment. After the 6 month experiments, four groups of rats were sacrificed, and the changes of serum markers for hepatic injury induced by AAF treatment examined. As shown in **Figure 2A−C**, AAF treatment significantly increased the levels of GOT and GPT in serum to 1.74- and 1.67-fold as compared to control. Although the level of γ-GT increased to 2.24-fold as compared to control, the increase was not statistically significant (**Figure 2C**). Moreover, AAF treatment also significantly increased the level of serum AFP, a well-known biomarker widely used for the diagnosis of HCC (*20*, *21*), to 1.84-fold as compared to control (**Figure 2D**). Notably, 2% SNWE supplement significantly decreased the levels of serum GOT, GPT, γ-GT, and AFP to 34.4, 39.7, 61.9, and 41.6%, respectively, as compared to AAF treatment (**Figure 2A−D**). The lower SNWE supplement also significantly decreased the levels of serum GOT and GPT to 32.7 and 23.5%, respectively, as compared to AAF treatment (**Figure 2A**,**B**).

The effects of SNWE alone on the serum biomarkers, including GPT, GOT, alkaline phosphatase, and γ-GT, in rats were also

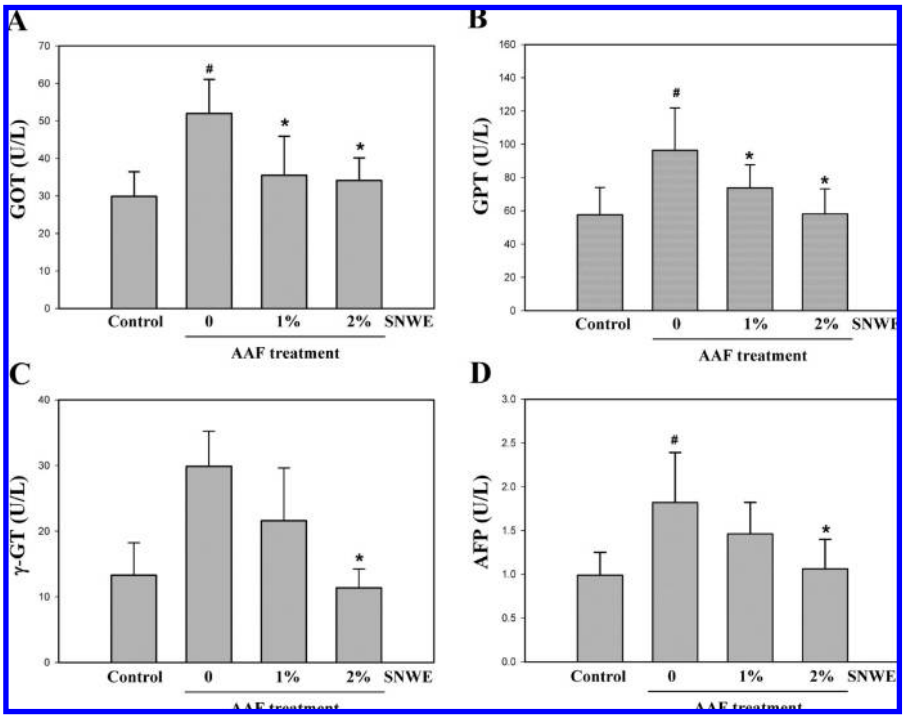

Figure 2. SNWE supplement lowered hepatic injury and hepatocarcinogenesis biomarkers. The biomarkers being quantitated were (**A**) GOT activity, (**B**) GPT activity, (**C**) γ-GT level, and (**D**) AFP level in plasma. Control, normal diet; AAF, normal diet containing AAF; 1%, normal diet containing AAF and 1% SNWE; and 2%, normal diet containing AAF and 2% SNWE. Data are shown as the means $\pm$ SDs; $^{\#}$, $p < 0.05$ as compared with the control group; and *, $p < 0.05$ as compared with the AAF group.

investigated. The findings revealed that higher than 2% SNWE decreased the level of GOT in female rats. Although the changes of serum biomarker GOT appeared to be significant in statistics, they did not exceed the corresponding standard range for normal physiological condition of rats (data not shown). Even so, only the male rats were used in this study. Taken together, the decrease of the serum biomarkers indicates that SNWE may not only improve the hepatic injury caused by AAF but also alleviate the early hepatic carcinogenesis induced by AAF.

SNWE Supplement Increased the Expression and the Activity of GSTs in AAF-Treated Rats. GSTs are a family of dimeric enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens. As shown in **Figure 3**, AAF inhibited the expression of both GST-α and GST-μ. Moreover, AAF significantly caused a decrease in the activity of GST-α and GST-μ to 38.6 and 37.2% of control level, respectively. Two percent SNWE supplement significantly increased the activity of GST-α and GST-μ to 3.5- and 3.3-fold, respectively, as compared to AAF treatment and increased to 1.2- and 1.3-fold, respectively, as compared to control. These results indicated that SNWE supplement induced the expression and the activation of both GST-α and GST-μ in AAF-treated rats.

GSTs are dimeric enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens (*22*). Previous studies have shown that the regulation of different GST isozymes was associated with various pathological processes; for example, the absent expression of GST-μ and GST-θ is related to cancer susceptibility in human (*23*). In this study, AAF treatment decreased the activity and expression of GST-α and GST-μ, indicating that the hepatic GST system had been shifted to a direction that may lead to hepatocarcinogenesis during chronic hepatic injury. The ability of SNWE supplement to increase hepatic GSTs expression under the treatment suggests that it is effective in protecting liver from AAF-induced hepatic injury and preventing further progression of carcinogenesis.

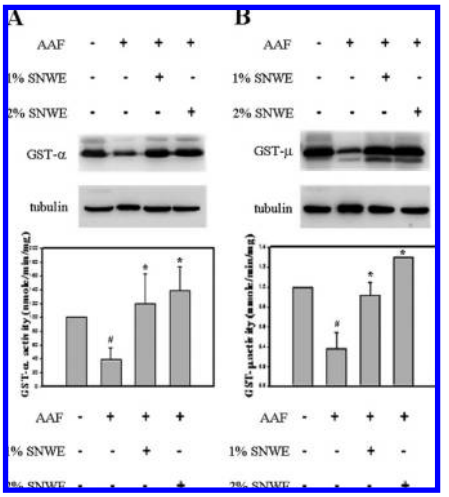

Figure 3. Effects of SNWE supplement on GSTs expression in liver. The crude extracts of liver obtained from Wistar rats fed on normal diet, normal diet containing AAF, normal diet containing AAF and 1% SNWE, and normal diet containing AAF and 2% SNWE were investigated. The levels of (**A**) GST-α and (**B**) GST-μ were determined by immunoblot, and the enzymatic activity was quantitated by spectrophotomic analysis. Data are shown as the means $\pm$ SDs; $^{\#}$, $p < 0.05$ as compared with the normal group; and *, $p < 0.05$ as compared with the AAF group.

SNWE Supplement Increased the Expression of Nrf2 in AAF-Treated Rats. Nrf2, a transcription factor belonging to the basic leucine zipper family, is associated with the expression of essential detoxification enzymes and antioxidant proteins (*24*, *25*). Thus, the effects of SNWE supplement on expression of Nrf2 were investigated. As shown in **Figure 4**, AAF prominently inhibited the expression of Nrf2. The quantitated levels of Nrf2 revealed that AAF treatment diminished the expression of Nrf2 to 18.2%

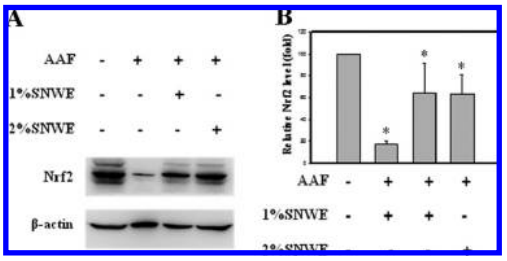

Figure 4. Effects of SNWE supplement on Nrf2 expression. The crude extracts of liver obtained from Wistar rats fed on normal diet, normal diet containing AAF, normal diet containing AAF and 1% SNWE, and normal diet containing AAF and 2% SNWE were investigated. The levels of Nrf2 were determined by (**A**) immunoblot and (**B**) relatively quantitated by densitometric analysis. Data are shown as the means ± SDs; #, $p < 0.05$ as compared with the normal group; and *, $p < 0.05$ as compared with the AAF group.

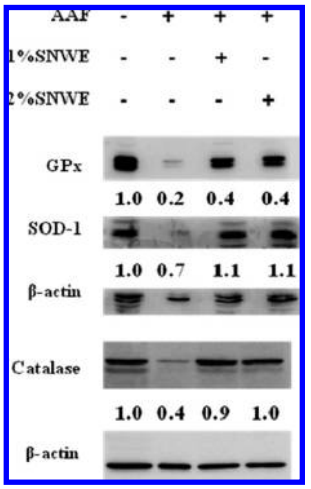

Figure 5. Effects of SNWE supplement on GPx, SOD-1, and catalase expression in liver. The crude extracts of liver obtained from Wistar rats fed on normal diet, normal diet containing AAF, normal diet containing AAF and 1% SNWE, and normal diet containing AAF and 2% SNWE were investigated. The levels of GPx, SOD-1, catalase, and β-actin were determined by (**A**) immunoblot and (**B**) relatively quantitated by densitometric analysis.

of control level. One percent and 2% SNWE supplement significantly increased the levels of Nrf2 to 3.4- and 3.6-fold as compared to AAF treatment alone. As compared to control, the 2% SNWE supplement recovered the level of Nrf2 to 65.6% of normal level. The results indicated that SNWE supplement effectively increased the expression of Nrf2 to recover the level of Nrf2 in AAF-treated rats.

Although AAF is generally known as a genotoxic carcinogen, recently, epigenetic (nongenetic) alterations, including an increase of DNA methyltransferase and proliferating cell nuclear antigen, are also reported to be involved in the AAF-induced hepatocarcinogenesis in rats (*26*). Recently, oxidative stress and Nrf2 have been reported to play a pivotal role in promoting and suppressing hepatocarcinogenesis (*27*, *28*). Our findings revealed that AAF treatment significantly diminished the level of Nrf2, suggesting that AAF may also enhance the hepatocarcinogenesis through increasing oxidative stress and decreasing levels of Nrf2. Taken together, these findings indicate that SNWE supplement increases the level of Nrf2 and may not only enhance the protection from oxidative stress but also inhibit the hepatocarcinogenesis epigenetically.

SNWE Supplement Increased the Expression of Antioxidant Proteins in AAF-Treated Rats. The expression level of Nrf2 was found increased by SNWE; therefore, the levels of downstream antioxidant enzymes regulated by Nrf2, including GPx, SOD, and catalases, were determined. As shown in **Figure 5**, AAF treatment reduced the levels of GPx, SOD-1, and catalase to 20, 70, and 40% of control, respectively. One percent SNWE supplement increased the levels of GPx, SOD-1, and catalase to 2.0-, 1.6-, and 2.3-fold as compared to AAF treatment, respectively. Interestingly, the level of SOD-1 in SNWE supplemented rats was higher than in control rats.

To maintain redox balance and alleviate oxidative damage, aerobic organisms possess an efficient molecular defense system, including GST, SOD, and reducing agents. However, these protective molecules could not provide complete protection against the severe oxidative stress (*29*). Therefore, bioactives possessing cytoprotective ability against cellular oxidative damage and enhancing ability on antioxidant enzymes activities have been investigated throughout (*30*, *31*). Our findings also indicate that SNWE supplement may induce the overexpression of GPx, SOD-1, and catalase involved in antioxidation mechanisms.

Chemopreventive compounds may prevent carcinogenesis through increasing the expression of phase II detoxification enzymes, including GSTs and quinone reductase, which can prevent the interaction of carcinogen with cellular DNA. Previous studies have demonstrated that polyphenols, including protocatechuic acid, naringenin, and epigallocatechin gallate, are able to induce and activate the phase II detoxification enzymes and antioxidant proteins (*32*−*34*). According to the previous composition analysis of SNWE, it consisted of approximate 20% total phenolics, 15% polysaccharide, and 5% protein. Because polysaccharides and proteins have been rarely reported to induce or activate the phase II enzymes and antioxidant proteins, we suggest that the polyphenols in SNWE should play important roles in the increase of GSTs, Nrf2, SOD, and catalase in this study.

Effect of SNWE on AAF/$NaNO_2$-Induced Liver Morphological Change and Fibrosis in Rats. To further investigate whether SNWE ameliorated the late stage lethal hepatoma, the AAF/$NaNO_2$ treatment was performed. The general liver morphological changes, fibrosis and tumorigenesis, induced by AAF/$NaNO_2$ treatment were evidenced by both qualitative and quantitative histopathological examination. As compared to normal rat liver (**Figure 6A**), the tissue section in **Figure 6B** showed a lot of abnormal hepatocytes with a high ratio of nucleoplasm/cytoplasm, a typical morphology of HCC, in the AAF/$NaNO_2$-treated rats. AAF/$NaNO_2$ treatment also induced fibrosis with fiber extension and collagen accumulation, which further resulted in liver fibrosis and cirrhosis.

These pathological changes (fibrosis and hepatoma) were ameliorated by the SNWE supplement that, especially, showed a dose-dependent effect (**Figure 6C**,**D**). The severity of the liver fibrosis and hepatoma induced by AAF/$NaNO_2$ treatment was scored and is summarized in **Table 1**. As shown, 80% AAF/$NaNO_2$-treated rats presented hepatic carcinogenesis, and the other 20% had liver fibrosis. One percent and 2% SNWE supplement prominently reduced hepatic carcinogenesis to 40 and 20%, respectively, as compared to AAF/$NaNO_2$ treatment. The fibrosis score was also significantly decreased by SNWE supplement in a dose-dependent manner. These results demonstrate that SNWE supplement effectively alleviated the hepatic fibrosis and hepatoma induced by AAF/$NaNO_2$ treatment in rats.

The liver plays pivotal roles in the metabolism of carbohydrates, lipids, and amino acids and detoxification of xenobiotics. Therefore, dysfunction of the liver usually results in accumulation of metabolites and xenobiotics and subsequently leads to hepatic inflammation, fibrosis, cirrhosis, and neoplasia and then may

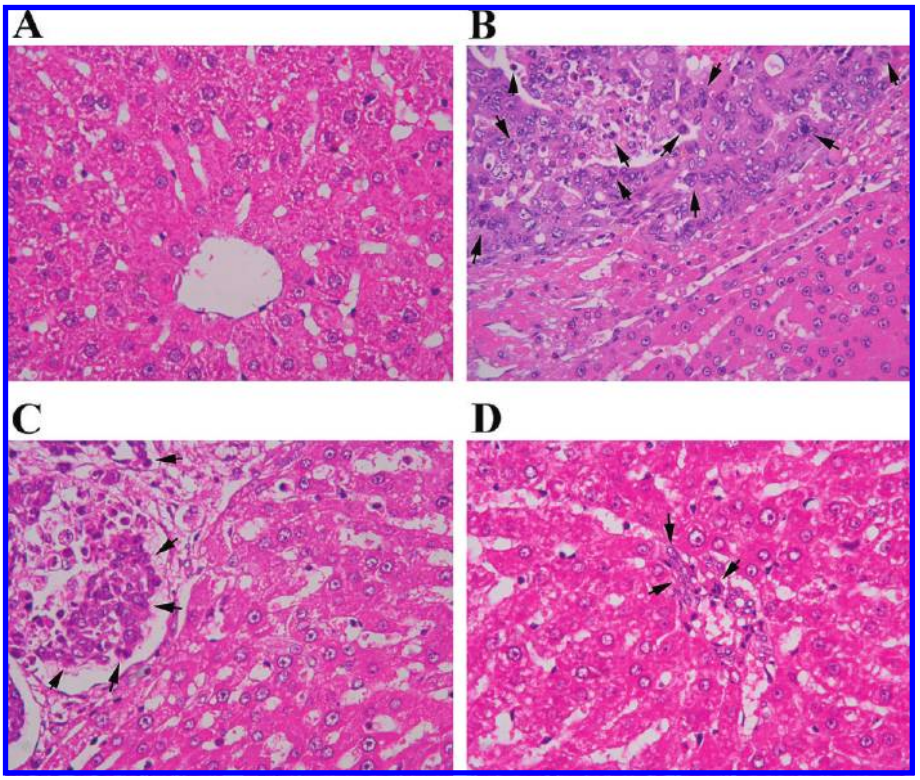

Figure 6. Effects of SNWE supplement on AAF/$NaNO_2$-induced late stage hepatoma. Livers from Wistar rats fed on (**A**) normal diet, (**B**) normal diet containing AAF and $NaNO_2$, (**C**) normal diet containing AAF, $NaNO_2$, and 1% SNWE, and (**D**) normal diet containing AAF, $NaNO_2$, and 2% SNWE were fixed, embedded, and sectioned. The sections were stained with H&E and examined by microscope at 400×. The representative hepatoma cells are indicated by arrows. The scoring for hepatic fibrosis and hepatoma was determined and is summarized in **Table 1**.

Table 1. Scoring System for Hepatic Fibrosis and Hepatoma in Rats Treated with AAF/$NaNO_2$

	fibrosis score[a]					
group	0	+	++	+++	++++	hepatoma[b]
control	100%					0%
AAF		20%				80%
AAF + 1% SNWE			20%	40%		40%
AAF + 2% SNWE		20%	40%	20%		20%

[a] 0, normal histological morphology; +, slightly fibrosis; ++, moderate fibrosis; +++, complete fibrosis; and ++++, serious fibrosis or cirrhosis. [b] Histological features confirmed as hepatoma.

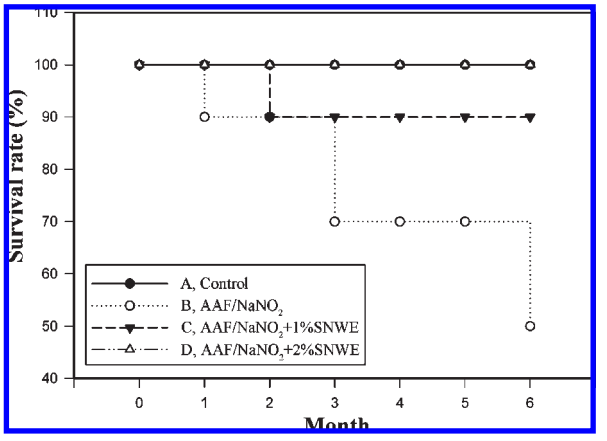

Figure 7. SNWE supplement prolonged the lifespan of Wistar rats fed on AAF/$NaNO_2$. The survival rates (%) for four groups of Wistar rat fed on different diets are indicated. (**A**) Control (normal diet, ●), (**B**) AAF/$NaNO_2$ (normal diet containing AAF/$NaNO_2$, ○), (**C**) AAF/$NaNO_2$ + 1% SNWE (normal diet containing AAF/$NaNO_2$ and 1% SNWE, ▼), and (**D**) AAF/$NaNO_2$ + 2% SNWE (normal diet containing AAF/$NaNO_2$ and 2% SNWE, ▽). Each group contained 10 Wistar rats at the beginning.

further cause the late stage hepatoma (*35*). It is believed that proper treatment in an early stage of hepatic disorder is able to prevent the further progression to irreversible cirrhosis and malignant carcinogenesis. For example, interrupting or reversing hepatic fibrosis could be a plausible approach to avoiding its progression to HCC (*36*). However, the preventive mechanism for liver carcinoma is not yet well-investigated. In this study, we demonstrate that SNWE significantly inhibits the progression of hepatocarcinogenesis, which has been demonstrated by a significant decrease of the late stage hepatoma in the AAF/$NaNO_2$-treated rats.

SNWE Supplement Ameliorated the Survival Inhibited by AAF/$NaNO_2$ Treatment. Because AAF/$NaNO_2$ treatment effectively induced lethal hepatoma, the mortality of rats treated with AAF/$NaNO_2$ continuously increased during the 6 month experiment. As shown in **Figure 7**, AAF/$NaNO_2$ treatment decreased the survival rate of rat up to 50% at the end of experiment. Notably, 1% and 2% SNWE supplement significantly increased the survival rate to 90 and 100%, respectively, at the end of experiment. The findings indicate that SNWE supplement prominently ameliorated the survival rate, suggesting that SNWE supplement may elongate the lifespan of AAF/$NaNO_2$-treated rat through the effective alleviation of hepatic injury and the inhibition of further progression into lethal hepatoma.

In conclusion, this study demonstrates that SNWE is able to decrease the AAF-induced liver damage and to increase the activity of GST and the expression levels of Nrf2 and the downstream antioxidant proteins in the liver. Moreover, SNWE also

suppresses the AAF/$NaNO_2$-induced hepatoma and the resulting mortality. These findings strongly indicate that SNWE supplement may provide potent protection against carcinogen-induced hepatic injury, early stage hepatocarcinogenesis, and even late stage hepatoma.

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Received May 28, 2009. Revised manuscript received August 3, 2009. Accepted August 10, 2009. This study was supported by a grant (NSC96-2321-B040-001) from the National Science Council, Taiwan.