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Solanum nigrum Linn. Water Extract Inhibits Metastasis in Mouse Melanoma Cells in Vitro and in Vivo

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Metastatic melanoma is an aggressive skin cancer notoriously resistant to current cancer therapies. Thus, new treatment strategies are urgently needed. *Solanum nigrum* Linn., commonly used in Oriental medicine, has showed antineoplastic activity in human cancer cell lines. The aim of this study was to evaluate the inhibitive effect of *S. nigrum* Linn. water extract (SNWE) on melanoma metastasis and dissect the underlying mechanisms of SNWE actions. B16-F1 cells were analyzed for migrating and invasive abilities with SNWE treatment, and several putative targets involved in metastatic melanoma were examined. In parallel, primary mouse xenograft and lung metastasis of melanoma models were established to examine the therapeutic potential of SNWE. The results indicated SNWE significantly inhibited B16-F1 cell migration and invasion. Meanwhile, decreased Akt activity and PKCα, Ras, and NF-κB protein expressions were detected in dose-dependent manners. In line with this notion, >50% reduced tumor weight and lung metastatic nodules were observed in 1% SNWE fed mice. This was associated with reduced serum MMP-9 as well as Akt activity and PKCα, Ras, and NF-κB protein expressions. Thus, this work indicates SNWE has potential application for treating metastatic melanoma.

KEYWORDS: Invasion; migration; melanoma; metastasis; *Solanum nigrum* Linn. water extract

INTRODUCTION

Melanoma is the most dangerous form of skin cancer. It is the third most common human malignancy, accounting for most skin cancer deaths. Ultraviolet (UV) radiation exposure is one of the major etiologic factors contributing to the increasing incidence of melanoma over recent decades. If metastatic melanoma develops, it is relatively resistant to current therapeutic regimens, carrying a very poor prognosis, with a 5 year survival rate of <5%. Therefore, new treatment strategies are urgently needed (*1*−*3*).

Metastasis is a complex process comprising a series of events involving multiple tumor−host interactions (*1*, *2*). Cell migration is one essential event during cancer progression, making cancer cells metastasize to other areas of the body (*6*). Another important step is tissue invasion, requiring proteolytic degradation of extracellular matrix (ECM) components by matrix metalloproteinases (MMPs), a family of zinc-binding enzymes (*5*, *7*−*9*).

Solanum nigrum Linn., a herbal plant indigenous to Southeast Asia, is commonly used in Oriental medicine. A previous study showed the ripe fruits of *S. nigrum* Linn. induce growth inhibition and apoptosis in breast cancer cells (*3*). In addition, the extract from the whole plant of *S. nigrum* Linn. results in hepatoma cell death through autophagy and apoptosis (*11*, *12*). These pieces of evidence suggest *S. nigrum* Linn. could exert its antineoplastic activity as a cancer chemoprevention agent. However, limited knowledge exists regarding how *S. nigrum* Linn. modulates tumor cell migration and invasion.

It is interesting to speculate if *S. nigrum* Linn. water extract (SNWE) has therapeutic potential in treating metastatic melanoma by targeting tumor cell migration and invasion. Aberrant activations in signaling pathways emerging as important therapeutic targets in melanoma include Ras, Akt, protein kinase Cs (PKCs), and NF-κB signalings (*1*−*3*, *5*, *13*−*16*). Therefore, the purpose of this work was to explore the underlying molecular mechanism(s) of SNWE being an effective chemoprevention agent through regulating melanoma cell migration and invasion in vitro and in vivo. In this study, we provide evidence supporting the contention that SNWE significantly suppresses melanoma metastasis, pointing to its therapeutic potential in treating melanoma.

MATERIALS AND METHODS

Preparation and Composition Analysis of SNWE. The whole plant of *S. nigrum* Linn. was collected from a mountain region in Miaoli country, located in central Taiwan. *S. nigrum* Linn. water extract (SNWE) was prepared as described previously (*4*,*5*). Briefly, the sun-dried *S. nigrum* Linn. was mixed with water for 30 min and subjected to continuous hot extraction at 100 °C for 40 min. The resulting water extract was filtered and subsequently concentrated in a water bath at 90 °C until it became creamy and then dried in an oven at 70 °C. The analysis of SNWE revealed it contains 20.4 ± 0.97% polyphenol with gallic acid and

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quercetin as the standards, 14.9 ± 1.3% polysaccharide, and 4.8 ± 0.4% protein (**Table 1**). To further characterize the polyphenolic composition of SNWE, the polyphenoic extract was prepared from SNWE. One hundred grams of dried SNWE powder was merged in 300 mL of ethanol and heated at 50 °C for 3 h. The extract was filtered and then lyophilized under reduced pressure at room temperature. The powder was resuspended in 500 mL of 50 °C distilled water, followed by extraction with 180 mL of ethyl acetate three times, resolved in 250 mL of distilled water, stored at −70 °C overnight, and then lyophilized. Finally, the resulting powder was resuspended in distilled water to analyze the polyphenolic compositions. The presence and proportion of the main constituents of *S. nigrum* Linn. polyphenolic extract were identified as gallic acid (1.10%), protocatechuic acid (4.56%), gallocatechin (1.38%), caffeic acid (7.18%), gallocatechin gallates (4.74%), rutin (3.00%), quercetin (2.30%), naringenin (4.54%), and unknown components (**Figure 1**). The further identification of the nine components was established from the recorded mass spectra (**Table 2**).

Cell Culture. B16-F1 cells, a mouse melanoma cell line, were cultured in Dulbecco's modified Eagle's medium supplemented with 100 μL/mL bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin sulfate, 0.1 mM MEM nonessential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Table 1. Composition of SNWE[a]

SNWE	%
polyphenol	20.4 ± 0.97
polysaccharide	14.9 ± 1.3
protein	4.8 ± 0.4

[a] SNWE, *Solanum nigrum* Linn. water extract. Gallic acid and quercetin as standards.

Cell Viability Assay. Cells were seeded in 24-well plates at a density of 1×10^5 per well and treated with 0−3 mg/mL SNWE for 24 h. Then, cells were incubated with medium containing 0.5 mg/mL MTT for another 2 h. The viable cell number was directly proportional to the formazan production and measured by spectrophotometry at 563 nm (*12*).

Wound-Healing Assay. B16-F1 cells (8×10^5) were cultured in 6-well plates until they were completely confluent. The cell monolayer was scraped with a sterile yellow micropipet tip, washed with PBS three times, and the culture was incubated in the medium containing 0−0.5 mg/mL SNWE over time to photography (*6*).

Invasion Assay. B16-F1 cells (5×10^5) were pretreated with or without PKCs inhibitors (5 μM Go-6983, 10 μM Ro-320432, and 5 μM Rottlerin) for 24 h. After stimulation with or without SNWE for 24 h, the cells were seeded in the upper well of a Boyden migration chamber, which was separated from the lower well by an 8 μm porous filter coated with matrigel. Ten percent FBS as a chemoattractant was placed in the lower well to facilitate cell migration. After 8 h of incubation, cells migrating to the lower surface of the filter were fixed with methanol, stained with 5% Giemsa solution, and counted (*16*, *17*, *18*).

Western Blot Analysis. Following treatment with 0−0.5 mg/mL SNWE for 24 h, B16-F1 cells were lysed directly in RIPA buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 5 μL/mL Triton X-100, 5 μL/mL Nonidet-P40, 1 μL/mL sodium deoxycholate). Cell lysates were further subjected to Western blot analysis with the indicated antibodies as described previously (*4*, *5*, *7*). Anti-phospho-Akt, anti-Akt, and anti-Ras antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-NF-κ B, anti-PKCα, and anti-PKCδ antibodies were purchased

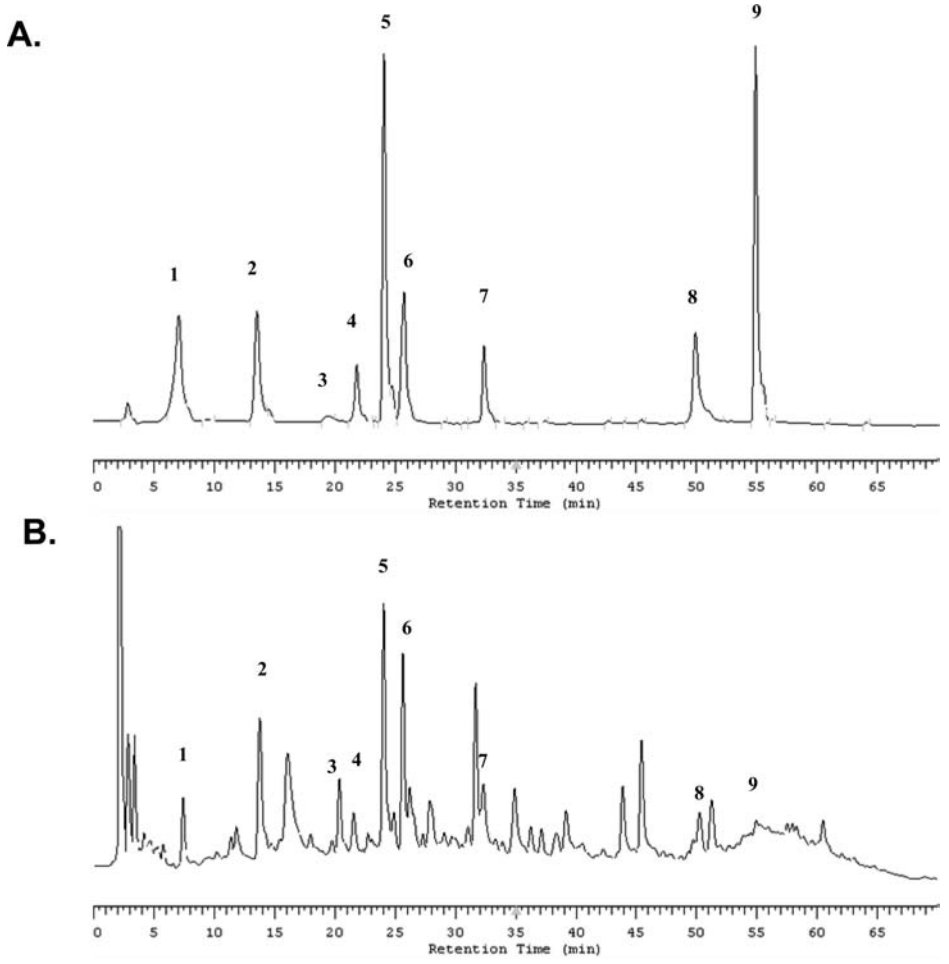

Figure 1. HPLC chromatogram of the polyphenolic components of SNWE. (**A**) HPLC chromatogram of nine kinds of standard polyphenols (1 mg mL^{-1}; 10 μL). Peaks: 1, gallic acid; 2, protocatechuic acid; 3, catechin; 4, gallocatechin; 5, caffeic acid; 6, gallocatechin gallate; 7, rutin; 8, quercetin; 9, naringenin. (**B**) HPLC chromatogram of free polyphenols from SNPE (10 mg mL^{-1}; 10 μL).

Table 2. Characterization of Phenolic Compounds of *Solanum nigrum* Linn.

peak	retention time (min)	assigned identity[a]	recovery (%)	UV λ_{max} (nm)	$[M-H]^-$ *m/z*	LC-ESI-MS^2 [b] *m/z*
1	8.18	GA	1.100 ± 0.32	270, 225	168.9	125.0
2	14.43	PCA	4.555 ± 1.44	222, 259	153.1	108.9
4	22.07	GC	1.378 ± 0.63	272	305.1	216.6
5	24.52	CA	7.179 ± 1.13	256, 354	178.9	135.0
6	26.08	GCG	4.738 ± 0.93	276	457.1	338.3
7	32.64	R	3.001 ± 0.87	256, 354	609.2	301.1, 343.1
8	50.27	Q	2.304 ± 0.48	266, 368	301.3	151.0, 179.0
9	54.87	N	4.539 ± 1.45	231, 288	271.1	150.9, 177.1

[a] GA, gallic acid; PCA, protocatechuic acid; GC, gallocatechin; CA, caffeic acid; GCG, gallocatechin gallate; R, rutin; Q, quercetin; N, naringenin. [b] MS^2 run with 30% collision energy.

from BD Bioscience. Anti-β-actin antibody was purchased from Sigma-Aldrich, Inc.

For in vivo assay, lung tissues were lysed with extraction buffer [20 mM Tris-HCl, pH 8.0, 5 M $CaCl_2$, 1 mmol/L phenylmethanesulfonyl fluoride, 15 mM pepstatin A, and 0.05% (w/v) Brij 35] and homogenized on ice. Following centrifugation at 14000*g* for 40 min (4 °C), the supernatants were harvested for protein quantification using the Coomassie Plus Protein Assay kit (Pierce, Rockford, IL), and then the protein samples were subjected to Western blot analysis.

Xenograft Tumor Assay. Five-week-old BALB/cAnN-Foxn1nu/CrlNarl nude mice were obtained from the National Laboratory Animal Center, Taiwan, housed in cages, and maintained at a temperature of 22 ± 2 °C and a humidity of 65 ± 5% in a controlled animal facility with a 12 h light−dark cycle and allowed ad libitum access to water. First, 1×10^6 B16-F1 cells in 400 μL of matrigel were implanted into the flank of nude mice to result in tumor formation. Then, mice were randomly divided into three groups (5 mice per group) and fed 4 g of daily basal diets containing 0, 0.5, and 1% SNWE for 14 days, respectively. On the last day of the observational period, the mice were sacrificed, and the tumor xenografts were dissected for final wet weight measurement (*8*).

Melanoma Metastasis Assay and Immunohistochemistry Stain. Five-week-old C57BL/6 mice were obtained from the National Laboratory Animal Center, Taiwan. A total of 48 mice were randomly divided into groups A−F (8 mice per group). Groups C−F received injections of 5×10^5 B16-F1 cells into the tail veins; conversely, groups A and B received a saline vehicle alone, and groups A and C were given the control diets. However, groups B, D, E, and F were fed daily experimental diets containing 2, 0.5, 1, and 2% SNWE, respectively. The dietary regimens were continued until the end of the study. The body weights of all mice were recorded every week. All groups were sacrificed 3 weeks after injection of the melanoma cells, and the nodules growing in the livers, lungs, and kidneys were counted and photographed (*7*−*9*). The entire lung was removed and fixed overnight in 10% paraformaldehyde. The fixed segments of lung were further embedded in paraffin and then sectioned and stained with hematoxylin and eosin (HE) for microscopic examination (*7*).

Analysis of MMP Activities. The activities of MMP-2 and MMP-9 were assayed by gelatin zymography as described previously (*7*, *10*). Briefly, sera from tumor-bearing mice were mixed with loading buffer and electrophoresed on 8% SDS−polyacrylamide gel containing 0.1% gelatin at 140 V for 3 h. The gel was then washed twice in Zymography washing buffer (2.5% Triton X-100 in double-distilled H_2O) at room temperature to remove SDS. Following incubation at 37 °C for 12−16 h in Zymography reaction buffer [40 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, and 0.02% NaN_3], the gel was stained with Coomassie blue R-250 (0.125% Coomassie blue R-250, 0.1% amino black, 50% methanol, and 10% acetic acid) for 1 h and destained with methanol/acetic acid/water (20:10:70, v/v/v).

Statistical Analysis. All data were analyzed by Student's *t* test and are presented as the mean ± SD. The difference was considered to be statistically significant at $p < 0.05$.

RESULTS

Cytotoxicity of SNWE on Melanoma Cells. To determine the cytotoxic effect of SNWE on B16-F1 cells, cell proliferation was assessed in the presence of various concentrations of SNWE by MTT assay. The percent of cell survival rate at concentrations of 0.25, 0.5, 1.0, 2.0, and 3.0 mg/mL SNWE was 98.22, 85.33, 37.09, 15.51, and 9.27, respectively. The concentration of SNWE on the inhibition of 50% of B16-F1 cell viability (IC_{50}) was 0.86 mg/mL (**Figure 2**). Because there was no significant decrease in B16-F1 cell numbers below 0.5 mg/mL SNWE treatment (**Figure 2**, $p > 0.05$), 0−0.5 mg/mL concentrations were selected for further evaluation of the antimetastatic effect of SNWE.

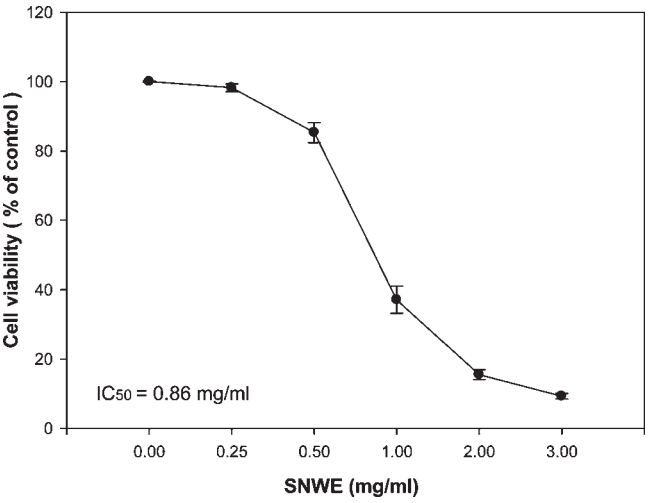

Figure 2. Cytotoxic effect of SNWE in melanoma cells. B16-F1 cells were stimulated with SNWE for 24 h and then subjected to MTT assay. Cell viability is presented as a percentage of control cells. The data are expressed as mean ± SD from three independent experiments. SNWE, *Solanum nigrum* Linn. water extract.

SNWE Attenuates Melanoma Cell Migration. Because cell migration is crucial to the process of tumor metastasis, we first tested if SNWE had an inhibitory effect on B16-F1 cells' migrating ability by wound-healing assay. Compared with the control, SNWE displayed a significant decrease in wound closures by 79.82, 51.89, 48.46, and 43.54% at concentrations of 0.2, 0.3, 0.4, and 0.5 mg/mL, respectively (**Figure 3**, $p < 0.05$). The result indicated SNWE could inhibit mouse melanoma cell migration in a dose-dependent manner.

SNWE Prevents Melanoma Cell Invasion. The notion of SNWE being able to attenuate B16-F1 cell motility raises the possibility of whether SNWE could block the invading ability of melanoma cells. B16-F1 cells stimulated with 0−0.5 mg/mL SNWE for 24 h were seeded in the upper chamber of an 8 μm porous filter coated with matrigel, and the cells migrated to the lower chamber were fixed and counted. Decreases of 73.10 to 29.68% in the invasive ability of B16-F1 cells were observed in response to 0.2−0.5 mg/mL SNWE compared with untreated cells (**Figure 4**, $p < 0.05$), indicating SNWE significantly suppressed melanoma cell invasion.

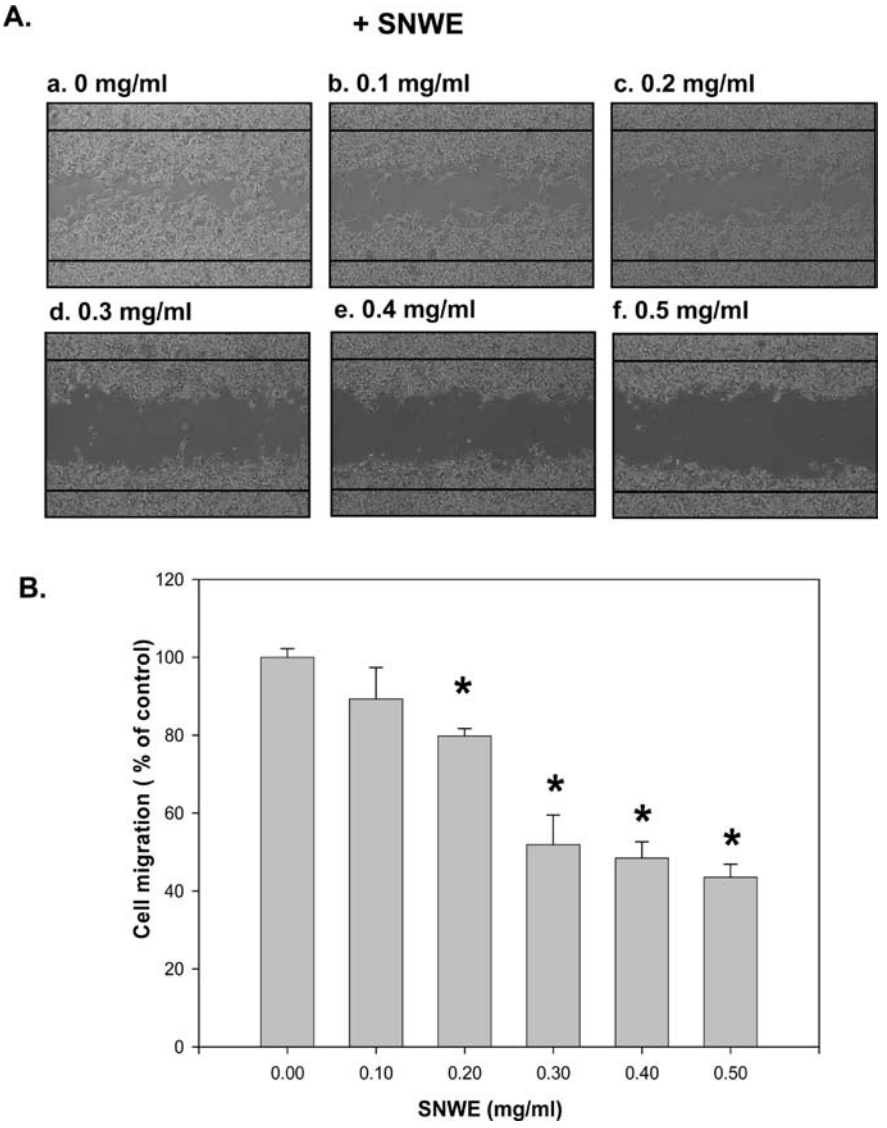

Figure 3. Inhibitory effect of SNWE on melanoma cell migration. B16-F1 cells were grown to confluence and then subjected to injury with a yellow tip. Wound healing was measured in the presence of 0−0.5 mg/mL SNWE for 24 h postinjury. (**A**) The cells migrating into and filling the wounded area were photographed and (**B**) counted to indicate their migratory capability. The data are expressed as mean ± SD from three independent experiments; ∗, $p < 0.05$ compared with the control.

SNWE Suppresses Melanoma Cell Invasion through Regulating PKCα Signaling. PKC is involved in proliferation, survival, angiogenesis, and metastasis as well as tumorigenesis by virtue of its central role in conveying several signaling transduction cascades. To understand the underlying molecule mechanism involved in SNWE-mediated melanoma cell metastasis, we examined if PKC was a putative target of SNWE. B16-F1 cells stimulated separately with SNWE, Go-6980 (general PKC inhibitor), Ro-320432 (PKCα inhibitor), and Ruttlerin (PKCδ inhibitor) were harvested for analyzing cell invasion as described above. Either Go-6983 or Ro-320432 treatment gave rise to a significant decrease in cell number detected in the lower chamber, and this phenomenon was similar to the SNWE action (**Figure 5A**), implying SNWE might suppress the invasive ability of melanoma cells through deactivating PKCα signaling. As expected, decreased PKCα but not PKCδ protein expression was observed in a dose-dependent manner in response to SNWE by Western blotting assay (**Figure 5B**), and the quantified data are further presented in **Figure 5C**,**D**. The evidence suggests PKCα is a putative downstream target of SNWE.

SNWE Decreases Akt Activity and the Protein Levels of Ras and NF-κB in Melanoma Cells. An important signaling pathway involved in cell migration as well as in melanoma tumorigenesis is the PI3K/Akt pathway. Because Akt, an important influence on PI3K, is overexpressed by up to 60% in melanomas, we considered Akt to be a selective target in preventing melanoma tumorgenesis in response to SNWE. SNWE-treated B16-F1 cells were harvested for analyzing AKt activity by Western blot detected with specific phosphorylated Akt antibodies. Significantly decreased Akt activity was found in response to increased concentrations of SNWE (**Figure 6A**,**B**). Meanwhile, the protein levels of Ras, a member of the Rho family of GTPases emerging as key regulators of cell migration and NF-κB, which is overexpressed in melanomas, were reduced in a pattern similar to that described above (**Figure 6A**,**C**,**D**). The result indicates that Akt signaling could be a mechanism basis of SNWE stimulation in melanoma cells.

Effect of SNWE on Melanoma Tumorgenesis. To evaluate the potential preventive effect of SNWE on melanoma development, B16-F1 cells were inoculated subcutaneously in the hind flank of athymic nude mice. All mice inoculated with B16-F1 cells developed primary tumors, with an end-point tumor growth of 14 days and a mean tumor weight of 2.91 g. Interestingly, 0.5 and 1% SNWE diets led to mean tumor weights diminishing to around

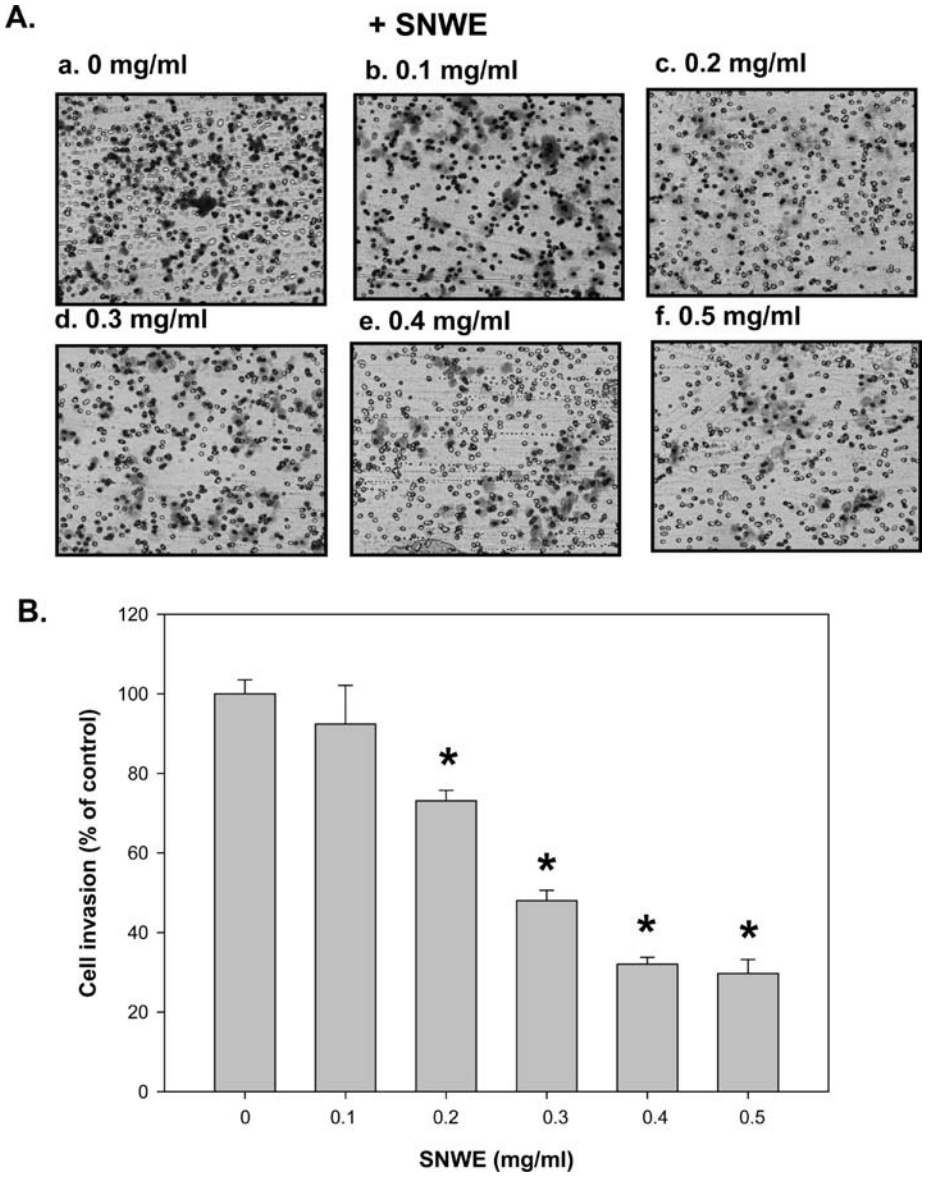

Figure 4. Effect of SNWE on melanoma cell invasion. B16-F1 cells pretreated with indicated concentrations of SNWE for 24 h were seeded on upper chamber with 8 μm porous filter coated with matrigel. The lower chamber contained 10% FBS as a chemoattractant to incubate for 8 h. (**A**) Representative images of B16-F1 melanoma cells adhering to the lower surface of the filters after the invasive process. Cells were stained with Giemsa solution, and images were taken by photography. (**B**) Invading cells per file on the lower surface were counted. The data are expressed as mean ± SD from three independent experiments; *, $p < 0.05$ compared with the control.

1.49 and 1.11 g, respectively (**Figure 7**, $p < 0.05$), indicating SNWE significantly suppresses melanoma tumor growth in vivo.

SNWE Inhibits Melanoma Metastasis to the Lung. The work described above clearly showed SNWE is a potent agent suppressing the migration and invasion of melanoma cells. To further support this hypothesis in vivo, B16-F1 cells were injected into the tail vein of C57BL/6 mice and lung metastasis determined after 21 days as described under Materials and Methods. A 100% incidence of melanoma metastasis was noted in B16-F1 cell-injected mice and melanoma metastasizing to lungs and livers as well as kidney was clearly inhibited, accompanied by increased amounts of SNWE administration (**Table 3**; **Figure 8A**). In this regard, the number of metastatic nodules growing in the lung was counted and showed significant decreases of around 16, 45, and 58% in the presence of 0.5, 1, and 2% SNWE, respectively (**Figure 8B**,**C**). Additionally, the histopathological data of melanoma cells in the lung further supports SNWE being a potent agent to inhibit melanoma metastasis (**Figure 8D**).

Molecular Mechanism(s) Involved in SNWE Actions in Vivo. To further address the molecular mechanism(s) of the blockage of melanoma invasion and metastasis in mice by SNWE, we measured the activity of MMPs, which are recognized as playing a key role in these events. Using Zymography, we found the serum MMP-9 activity of tumor-bearing mice was significantly reduced by 3-fold in the presence of 2% SNWE diet (**Figure 9A**, higher band, lane 8 vs lane 5), whereas serum MMP-2 activity was not obviously decreased (**Figure 9A**, lower band, lanes 6−8 vs lane 5). Moreover, total lysates from homogenized lung tissue were harvested for Western blot, and the result indicated the Akt activity and protein levels of Ras and NF-κB as well as PKC-α were clearly reduced in dose-dependent manners (**Figure 9B**), which were consistent with the above in vitro data (**Figures 5** and **6**). Collectively, our finding demonstrates SNWE significantly inhibits melanoma invasion and metastasis in vitro as well as in vivo, which supports the case for SNWE as a promising agent in treating malignant melanoma.

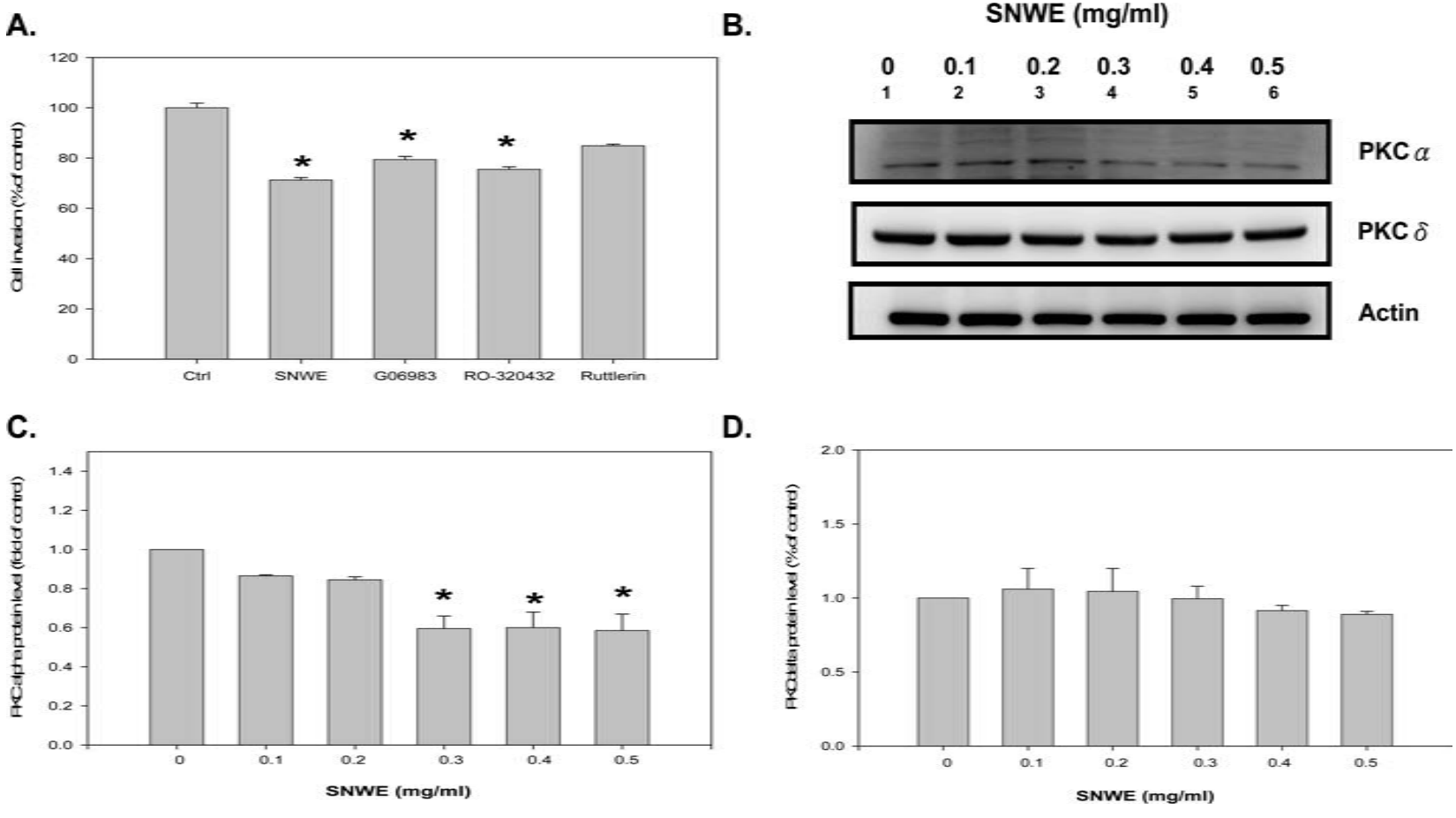

Figure 5. SNWE suppresses melanoma cell invasion through PKCα. (**A**) B16-F1 cells were treated with PKC inhibitors for 24 h and then subjected to invasion assay as described in **Figure 4**. The relative invasive ability is presented as mean ± SD from at least three independent experiments. (**B**) B16-F1 cells were stimulated with 0−0.5 mg/mL SNWE for 24 h. Cells were lysed and subjected to analysis by Western blot with anti-PKCs antibodies as indicated. The quantification of (**B**) PKCα and (**C**) PKCδ expression is presented as mean ± SD from at least three independent experiments; ∗, $p < 0.05$ compared with the control. Expression levels were normalized to β-actin protein level.

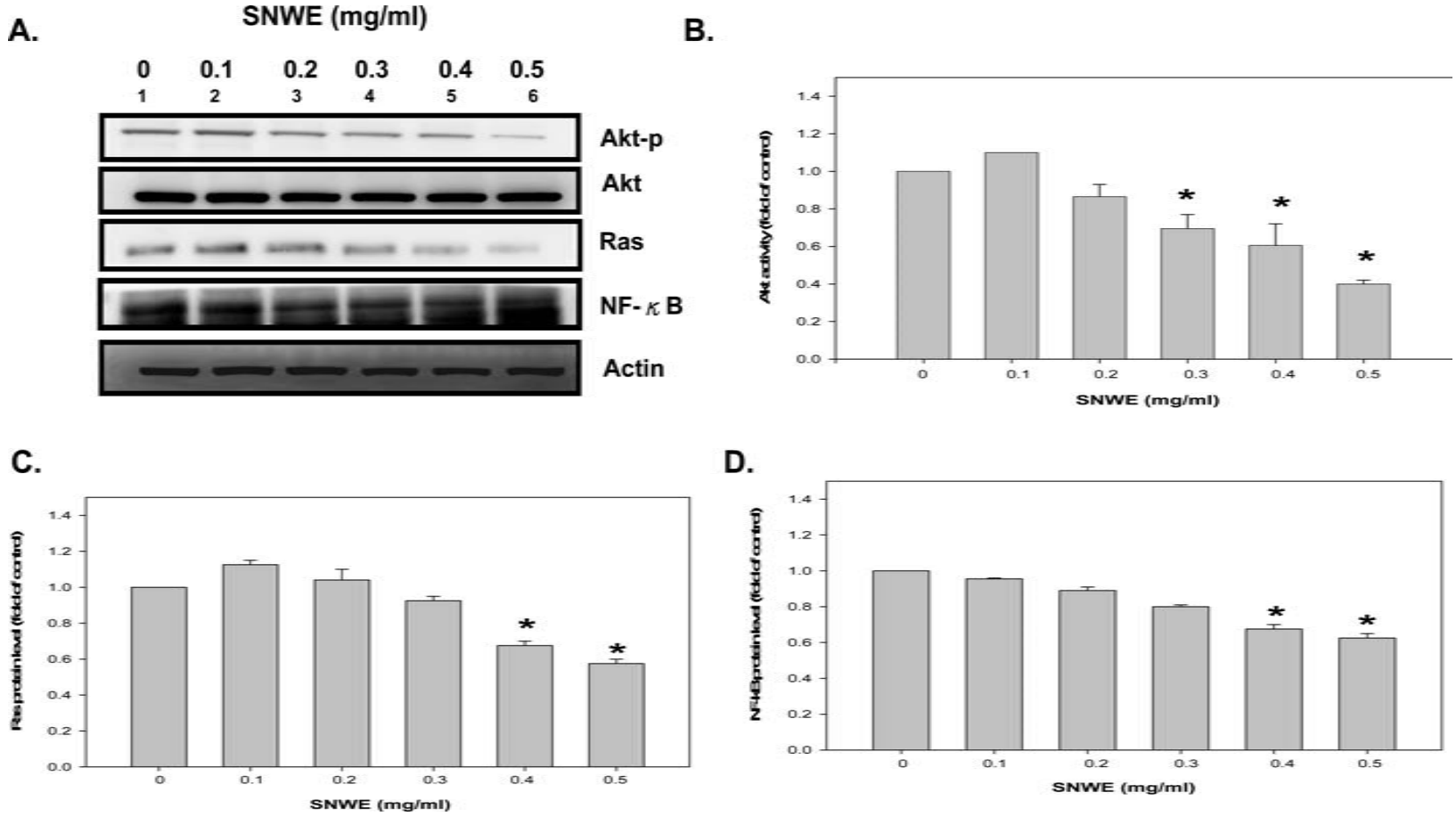

Figure 6. SNWE inactivates Akt and decreases Ras and NF-κB protein levels. B16-F1 cells were stimulated with 0−0.5 mg/mL SNWE for 24 h. Cells were lysed and subjected to analysis by Western blot with antibodies as indicated. The quantification of (**B**) Akt activity, (**C**) Ras, and (**D**) NF-κB expression is presented as mean ± SD from at least three independent experiments; $*$, $p < 0.05$ compared with the control. Expression levels were normalized to the β-actin protein level.

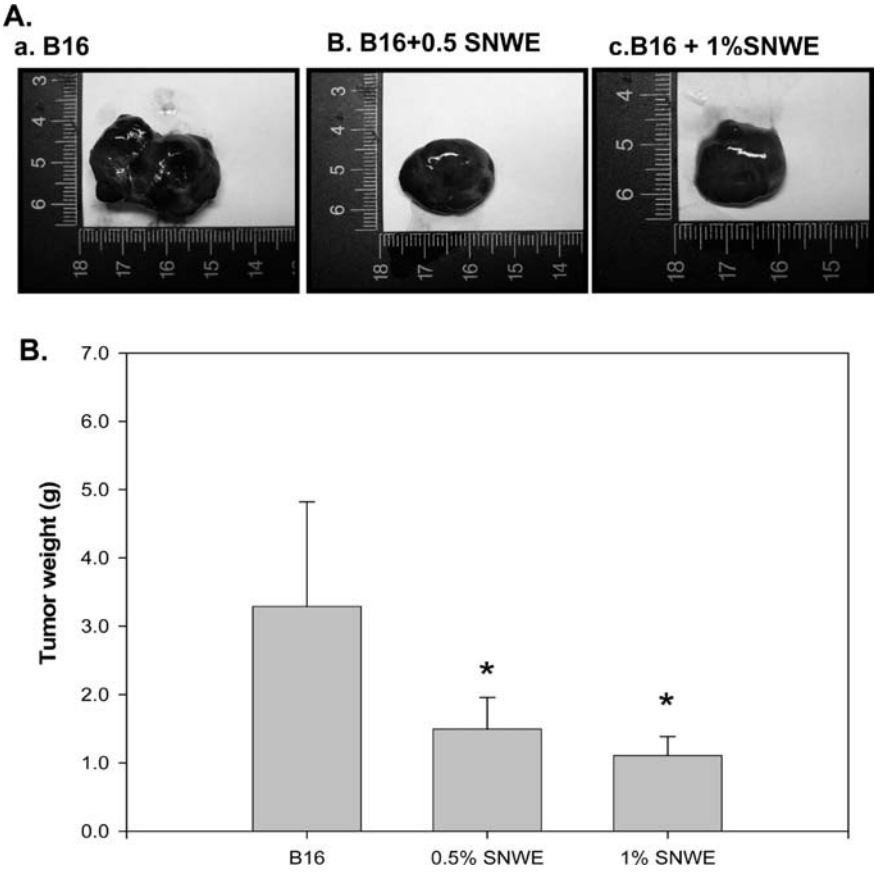

Figure 7. SNWE inhibits melanoma growth in BALB/cAnN-Foxn1nu/CrlNarl nude mice. BALB/cAnN-Foxn1nu/CrlNarl nude mice implanted with B16-F1 xenografts were fed with SNWE as indicated for 14 days. (**A**) Tumor formation was visualized by photography. (**B**) Tumor weight is represented as mean ± SD, $n = 5$; ∗, $p < 0.05$ compared with the control.

Table 3. Incidence of Metastasis in SNWE-Fed Mice[a]

		incidence of metastasis		
	no. of mice	lung	liver	kidney
normal	8	0/8	0/8	0/8
B16	8	8/8	6/8	6/8
B16 + 0.5% SNWE	8	8/8	4/8	4/8
B16 + 1% SNWE	8	8/8	3/8	3/8
B16 + 2% SNWE	8	8/8	1/8	2/8

[a] SNWE, *Solanum nigrum* Linn. water extract.

DISCUSSION

We conducted a series of experiments to examine the potential role of SNWE in treating metastatic melanoma, an aggressive disease that is refractory to conventional chemotherapeutic agents and lacks adequate treatment options. Our finding provides the first demonstration of our knowledge that SNWE could significantly suppress mouse melanoma metastasis both in vitro and in vivo. In the present study, we demonstrated SNWE inhibits cell migration and invasion as well as metastasis in mouse melanoma cells. Suppressing PKCα activity was associated with decreased melanoma cell invasion (**Figure 5A**). Additionally, SNWE administration resulted in reduced PKCα protein expression (**Figure 5B**). This finding suggests SNWE may exert antimetastatic effects by deactivating PKCα. Previous studies reporting PKCα activation have shown increased tumor cell invasion and metastasis, leading to the activation of the MAPKs and PI3K/Akt pathway (*5*). Our results clearly showed SNWE induced decreases in the Akt phosphorylation and Ras protein level (**Figure 6**), implying multiple signaling pathways must be targeted by SNWE to overcome melanoma metastasis (*11*). Whether SNWE suppresses melanoma invasion through PI3/Akt as well as PKCα pathways requires further investigation. NF-κB up-regulation has been proposed as an event that promotes melanoma tumor progression (*1*, *2*). In the present work, the result of SNWE mediating reduced NF-κB expression both in vitro and in vivo offers NF-κB as a potential target for therapeutic intervention.

The absence of cytotoxic effects of SNWE in vitro (**Figure 2**) is consistent with the notion that there was no clear difference in the body weights, food intake, and survival rate between all groups, and no illness was seen in the mice (data not shown). SNWE suppressed tumor growth in a primary mouse xenograft model (**Figure 7**). Meanwhile, using a well-established murine experimental metastasis assay further confirmed SNWE inhibited melanoma cells from metastasizing in the lung (**Figure 8**), revealing the potential of SNWE in treating metastatic melanoma. We found SNWE prevented melanoma cell migration and invasion in vitro at concentrations of 0.2−0.5 mg/mL (**Figures 3** and **4**). In this regard, the concentrations of SNWE suppressing melanoma cell metastasis in vivo were 0.5−2% SNWE, responding to 1−4 mg/kg/day (**Figure 8**), indicating the effect of SNWE could be more effective in vivo than in vitro. Comparably, it was noted that tamoxifen administration reduces the ability of B16BL6 melanoma to metastasize at concentrations of 1−10 mg/kg/day (*2*). Thus, our result pointed out that SNWE is potentially useful as an antimetastatic agent against melanoma. Besides, we observed a significant decrease in the number of lung metastasis nodules in

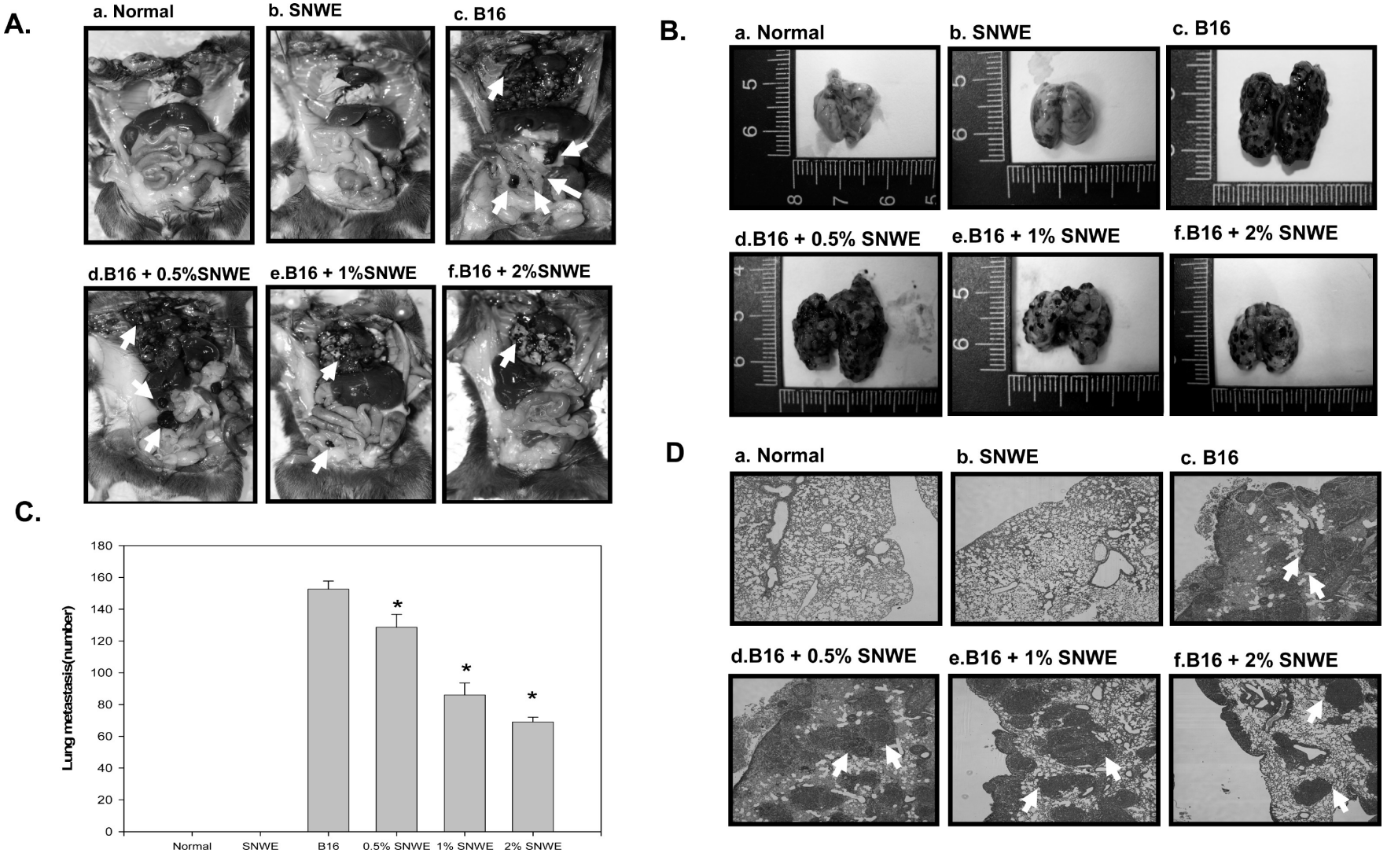

Figure 8. SNWE inhibits melanoma metastasis. B16-F1 cells were injected into the tail veins of C57/BL6 mice fed SNWE as indicated. (**A**) The metastatic nodules (arrow) growing in the lung, liver, kidney, and intestine were photographed at day 21. (**B**) Lungs were removed, photographed, and (**C**) metastatic nodules were counted. The numbers of metastatic nodules per mouse lung represent the mean ± SD from eight mice per group. (**D**) Immunohistochemical staining of lung tissues. Melanoma cell, arrow; original magnification, ×100.

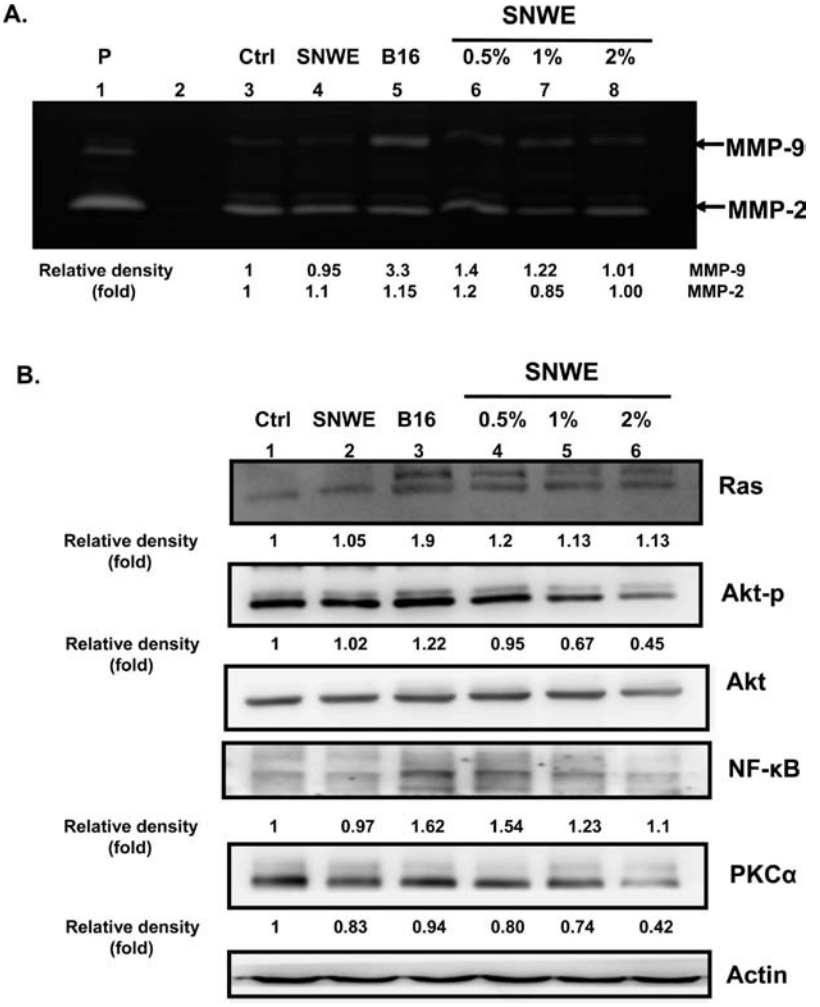

Figure 9. Effects of SNWE on melanoma metastasis and putative targets in vivo. (**A**) Analysis of MMP activities on the sera of B16-F1-bearing mice fed SNWE. B16-F1 cells were injected into the tail veins of C57/BL6 mice fed SNWE as indicated. After scarifice, blood samples were obtained and further loaded onto polyacrylamide gel containing gelatin. Three serum samples randomly chosen from eight mice per group were used for analyzing MMP activities. The representative image of the MMP activities was determined from three independent experiments and quantified by densitometric analysis. (**B**) Lung tissues from tumor-bearing mice were extracted and further subjected to Western blot with the indicated antibodies. Three tissue samples randomly chosen from eight mice per group were used for Western blot, and the three independent experiments results showed a similar tendency. The representative data were quantified by densitometric analysis. Expression levels were normalized to the β-actin protein level.

SNWE-fed mice was associated with decreased Akt activity and expressions of Ras, PKCα, and NF-κB (**Figure 9B**), which further conformed the in vitro result (**Figure 6**). MMPs have been regarded as major molecules that assist tumor cells by cleaving several ECM components during metastasis (*4*, *5*, *7*−*9*, *19*). The data showing SNWE decreased plasma MMP-9 activity in in vivo experiments (**Figure 9A**) agree with the proposed prognostic and diagnostic utility of MMP-9 levels in plasma serum and might be correlated with the significant effect on lung metastasis reduction in tumor-bearing mice (**Figure 8**) (*10*).

A wide range of naturally occurring substances present in our daily diet have cancer-preventing properties, making chemoprevention tend to diet-based intervention (*20*, *21*). In this regard, polyphenolic compounds are plentifully found in fruits, vegetables, and some beverages, which recently have been reported to inhibit cell invasion and metastasis in various cancer cell lines and experimental mice (*22*−*26*) in addition to their antioxidant, anti-inflammation, and antitumor activities (*27*−*29*). *S. nigrum* Linn. contains 20.4 ± 0.97% polyphenol (**Table 1**), and phenolic acid and polyphenol were further identified as two major representative polyphenolic compounds with gallic acid and quercetin as standards in polyphenolic extract (**Table 2**; **Figure 1**). This suggests SNWE significantly suppresses melanoma cell metastasis in vitro and in vivo, and this might due to the actions of polyphenolic components from SNWE.

Taken together, our data demonstrate that SNWE suppresses the formation of lung metastatic nodules in a mouse melanoma model, and this effect seems to be due to the inhibition of tumor cell migration and invasion through Akt and PKCα deactivation. Further investigations are required to analyze the detailed molecular mechanisms by which SNWE exerts it antimetastatic activity for treating melanoma.

ABBREVIATIONS USED

MMPs, matrix metalloproteinases; PKCs, protein kinase Cs; SNWE, *Solanum nigrum* Linn. water extract.

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