Journal of Medicinal Plants Research Vol. 5(21), pp. 5221-5229, 9 October, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Anti-tumor effect of *Archidendron lucidum* (Benth.) against esophageal cancer, colorectal cancer and hepatoma

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Accepted 29 July, 2011

Archidenron lucidum **is an indigenous medicinal plant in Taiwan used for treatment of inflammatory diseases and cancer. This study was aimed to investigate its anti-cancer effect against gastrointestinal and hepatic malignancy. We found that the 50% effective concentrations of its methanol extract (MEAL) against human esophageal cancer CE81T/VGH, hepatoma HA22T/VGH and murine colorectal cancer CT26 cells were less than 5.0 µg/mL *in vitro* whereas those of water extract were greater than 30 µg/mL. Cell cycle arrest at G2/M phase was observed in all three cell lines treated with MEAL. Development of hypodiploidy cells suggests that apoptosis might be one of the cell death pathways of CE81T/VGH cells. Intraperitoneal injection with 1.25 mg/kg MEAL significantly inhibited syngeneic CT26 tumor growth in BALB/c mice without obvious toxicity in terms of changes in body weight, leukocyte count and plasma creatinine and alanine aminotransferase (ALT) levels. Higher dose (2.5 mg/kg) MEAL did not further increase the anti-tumor effect, but resulted in elevation of plasma ALT level. Our results indicate that optimal dose of MEAL might possess the anti-tumor effects against esophageal, hepatocellular and colorectal cancers with a relative safety profile. Accordingly, we are purifying effective and less toxic compounds from MEAL.**

Key words: *Archidendron lucidum* (Benth.), alimentary tract cancer, hepatoma.

INTRODUCTION

Malignant tumor is one of the most common causes of death worldwide and cancer-related mortality is expected to increase considerably. Cancers of gastrointestinal tract and hepatic system are amongst the major causes of morbidity and deaths from cancer. At least 23 to 26% of annual incident cases of cancer patients belong to these categories (Johnson, 2004). Although, the 5 year survival in colorectal cancer has been significantly improved to 62%, esophageal cancer and hepatocellular carcinoma still have dismal prognosis. In United States, the 5 year survival rate is only 8.9 to 12 % after aggressively treatment (Jemal et al., 2004; Hertl and Cosimi, 2005). Clearly, development of new effective therapy in gastrointestinal and hepatic malignancy remains the critically important issue in clinical practice. Natural products have long been recognized as an important source of medicine (Mann, 2002; Neidle and Thurston, 2005). Progress in the medicinal plant research prompts the development of potential anticancer drugs from nature products. During 1983 to 1994, 60 to 80% approved antibacterials and anticancer drugs were derived from natural products. The use of natural

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products has been proved as a major and successful strategy for discovery of new drugs (Cragg et al., 1997; Harvey, 2000; Harvey, 1999). *Archidenron lucidum* is a member of the Leguminosae family, which is indigenous to Taiwan. *Archidenron* genus has about 100 species and most of those species are native in the tropical regions of Asia (Huang and Ohashi, 1993).

People use this herb to control inflammation and various cancers in Taiwan. However, the pharmacological activity of *A. lucidum* has never been studied. Previous studies on various active compounds or constituents of Leguminosae family showed their effect on anti-inflammatory and anti-tumor effect. For example, the crude extract of *Caesalpinia ferrea* had analgesic and anti-inflammatory properties (Nakamura et al., 2002). Ethyl acetate extracts of *Psoralea corylifolia* have shown significant cytotoxic activity against the colorectal cancer HT-29 and breast cancer MCF-7 cells (Mar et al., 2001). Rotenoids isolated from the *Amorpha frutico* have shown potent cytotoxicity against six human cancer cell lines (Li et al., 1993). Triterpenoid saponins from *Acacia victoriae* was shown to inhibit tumor cell proliferation and induce apoptosis (Mujoo et al., 2001). In this study, we examined the effect of water and methanolic extract of *A. lucidium* on growth and cell cycle distribution of three gastrointestinal and hepatic cancer cell lines *in vitro*. The anti-tumor effect and the safety profile *in vivo* were evaluated by using a syngeneic CT26 implantation model.

MATERIALS AND METHODS

Plant material

A. lucidum (Benth.) was collected in Taipei, Taiwan. It was identified and certified by a chemist Prof. Muh-Tsuen Kao (National Taiwan University, Taipei, Taiwan).

Preparation of extract

Ten kilogram of whole plant was air-dried. The crushed plant was then grounded to powder. The water extract of *Archidenron lucidium* (WEAL) was prepared by boiling to 100°C in 10-fold weight of water for 1 h. Then the solution was passed through a 0.45 mm filter paper and dried by freezes dryer. The MEAL was prepared by using 10-fold weight of methanol emersion for 24 h. After filtration, the extract was concentrated with rotary evaporators and freezes dryer. These extracts were then subjected to test the anti-tumor activity *in vitro* and *in vivo*.

Chromatographic analysis

The High-performance liquid chromatography (HPLC) system used was a Shimadzu HPLC (Kyoto, Japan), consisting of LC-20AD binary pumps, a SIL-20AC auto-injector, and a SPD-M20A VP spectrophotometric diode-array detector. The analytical column used was a reversed-phase Inertsil ODS-2 column (250 ×4.6 mm i.d, 5 µg particle sizes, GL Science, Japan). The mobile phase consisted of (A) 10 mM sodium dihydrogen phosphate containing 10% acetonitrile (v/v) and (B) 100% acetonitrile, filtered and degassed prior to use. Separation was carried out gradiently by increasing (B) from 0 to 80% within 60 min at 25 ± 1°C, a flow rate of 1.0 mL/min. The ultraviolet (UV) detection was at 254 nm. Twenty microliter of *A. lucidum* extract prepared in methanol and dilute to 1 mg/ml was injected into the HPLC system.

Cell culture

Three cancer cell lines were used in this study. The murine colorectal adenocarcinoma cell line CT 26 was obtained from the American type culture collection (ATCC). The human hepatocellular carcinoma HA22T/VGH was kindly provided by Prof. Chung-Ming Chang (Institute of microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan). Human esophageal squamous cell carcinoma CE81T/VGH cell line was purchased from Biosources collection and research center (Food Industry Research and Development Institute, Hsinchu, Taiwan). CT26 was cultured in RPMI1640 medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT). CE81T/VGH and HA22T/VGH were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. The cultures were grown in a humidified 5% CO_2 incubator at 37°C.

Trypan blue exclusion test for estimation of cell growth

Initially, 5 x 10^4 cancer cells were seeded in 6 well plates. After overnight incubation, cells were cultured in media containing various concentrations of WEAL or MEAL for 3 days. The attached cells were then trypsinized and counted by the method of trypan blue exclusion test under a light microscope (Olympus). All experiments for measurement were triplicated. The EC_{50} values were calculated by GraphPad Prism 4 software (San Diego, CA).

Flow cytometry

Cells were incubated in medium containing various concentrations (0, 5, 10 µg/mL) of MEAL for 24 h. The harvested cells (10^6) were fixed with 70% ethanol at 4°C for 1 h. Cell were wash with phosphate buffer saline (PBS) and then resuspended in a solution containing 3.4 mM sodium citrate, 20 µg/mL propidium iodide and RNase A at room temperature in dark for another 1 h Cell cycle analysis by deoxyribonucleic acid (DNA) content was performed using a FACS Calibur flow cytometer (Becton Dickinson, San Diego, CA). The data were analyzed using ModFit software (Becton Dickinson, San Diego, CA).

Quantitation of apoptosis

To analysis the percentage of apoptotic CE81T/VGH cells after MEAL treatment, Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) co-staining method was used (R and D Systems, Minneapolis, MN). Analysis was carried out as described previously (Chen et al., 2008). Viable cells take up neither dye (FITC-/PI-). Both early ($FITC^+/PI^-$) and late ($FITC^+/PI^+$) apoptotic cells were counted after 0 and 5 µg /mL MEAL treatment for 24 h.

Micrographs and morphology of cell death

Cell morphology of CT26 and CE81T/VGH with 0 and 10 µg/mL MEAL for 3 days were observed after Liu's staining. The micrographs were taken by 400 x light microscope (Olympus).

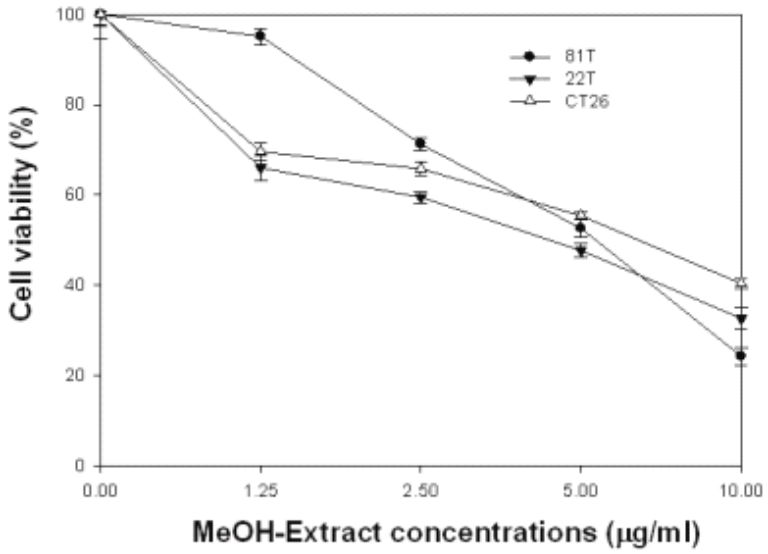

Figure 1. Liquid chromatogram of methanolic extraction of *A. lucidum*.

Animal model

All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of Mackay Memorial Hospital. All animal care and husbandry were conducted in accordance with the Guide for the Care and Use of Laboratory Animal. Following arrival, the animals were kept in our animal facilities for acclimatization for about 7 days, during which they had free access to food and water until 18 h prior to being used in animal study. Male BALB/c mice with age between 6 to 8 weeks were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and housed in a rodent facility at 22 ± 1°C with a 12 h light-dark cycle. 10^6 CT26 cancer cells in 0.1 mL PBS were subcutaneously implanted in the right gluteal region. After 12 days, the tumors approximately grew up to 0.5 cm in diameter and were subjected to further experiment.

In vivo therapeutic studies

Animals were grouped with 5 to 7 mice in each. MEAL at doses of 1.25 and 2.5 mg/kg was intraperitoneally (IP) injected everyday for 50 days. Mice treated with equal amounts of vehicle were used as control. On the 50^{th} day, the mice were all sacrificed under adequate anesthetization with phenobartibal (50 mg/kg IP). The size of implanted tumor was measured by the same observer. Calipers were used to measure the largest (a) and smallest (b) diameters, and the tumor volumes were estimated according to the formula $0.5ab^2$. Change of tumor size was calculated as measured size minus the original size in the same mice in 12^{th} day after tumor implantation.

Safety profile

Three parameters (body weight, leukocyte count, plasma levels of creatinine and ALT were used to monitor the safety profile. The total body weight of each mouse was determined every other day by a single observer. The leukocyte count was estimated by retro-orbital blood sampling every other day during the whole study period. The plasma levels of creatinine and ALT were measured by a SYNCHRON LX20 spectrophotometer (Beckman Coulter, San Diego, CA) by heart blood sampling after sacrificed.

Statistical analysis

We used Sigma Stat software (Version 2.03, SPSS Inc., Chicago, IL) to perform the statistics. Data were expressed as mean ± standard error of mean (SEM) or percentage. Analysis of variance (one way ANOVA) was used to compare tumor size, body weight, serum ALT, creatinine and leukocyte count among the groups and controls. Student t-test was used to compare percentage of apoptosis. The *p* values of 0.05 or less were considered as statistically significant.

RESULTS

Chromatography analysis

A gradient liquid chromatography coupled to photo-diode array detection method was used to analyze the MEAL (Figure 1). According to HPLC profile, most ingredients in the MEAL possess high to moderate hydrophilicity, because these peaks all appear within 30 min before the mobile phase reaches 50 % acetonitrile.

Inhibition of tumor cell growth by extracts of *A. lucidium*

Both MEAL and WEAL inhibited the growth of esophageal cancer CE81T/VGH, hepatoma HA22T/VGH and colorectal cancer CT26 cells in a concentration-dependent manner (Figure 2). The estimated 50% effective concentration (EC_{50}) of MEAL for CE81T/VGH, HA22T/VGH and CT26 cells were 5.4, 3.8 and 5.0 μg/mL,

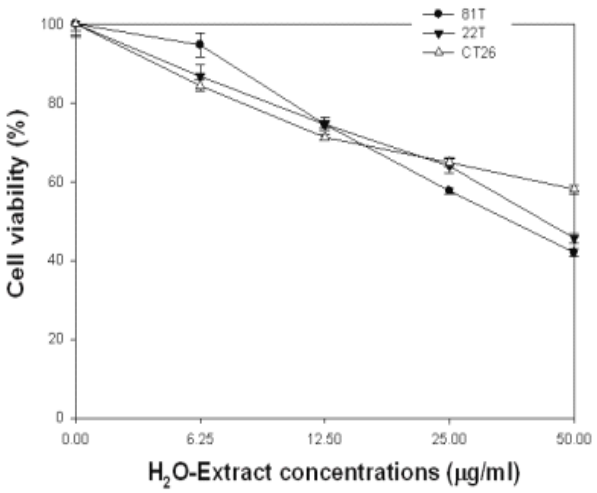

Figure 2. Effect of methanolic extraction of *A. lucidum* on the viability of tested cancer cell lines. These data are expressed as mean ± SEM from 5 separate experiments.

respectively. The potency of WEAL was less than that of MEAL with EC_{50} 32.5, 41.7 and greater than 50 µg/mL for these three cell lines, respectively.

Cell cycle analysis

Arrest of cell cycle at G2/M phase was observed in all three cell lines treated with MEAL, which was most obvious in esophageal cancer CE81T/VGH cells (Figure 3). The percentages of CE81T/VGH cells at G2/M phase are 14.8 ± 2.1, 22.3 ± 3.6 and 46.7 ± 4.6% for group of control, 1.25 and 2.5 µg/mL, respectively. Moreover, the percentage of sub-G1 population of CE81T/VGH cells was 0.3 ± 0.1, 4.4 ± 0.2 and 10.1% ± 0.7 for group of controls, 5 and 10 µg/mL (Figure 3). Thus, apoptosis might be one of the cell death pathways of CE81T/VGH cells because the moderate increase in the amount of hypodiploidy cells.

Apoptotic analysis

After 0 and 5 µg/ml MEAL treatment for 24 h, the percentage of apoptotic CE81T/VGH cells increased over 4 times and had statistics significance (*p= 0.004*). According to Annexin V-FITC and PI co-staining analysis, the data of combine early and late apoptosis were 1.9 ± 1.4 and 9.1 ± 1.1% separately (Figure 4).

Cell morphology change

After treatment with MEAL (10 µg/ml for 3 days), the morphology of CE81T/VGH (Figure 5) and CT26 cancer cells were observed by using Liu's stain and light microscope. The ratio of nucleus to cytoplasm decreased, especially in CT26 cells. Nuclear condensensation and fragmentation with a few apoptotic bodies were more obviously in CE81T/VGH cells. Cell membrane blebbing and smaller contour were evident after treatment. No obvious multinucleated cells, nor cytoplasmic vacuoles, could be observed. Base on the morphological changes, it is possible that apoptosis is the major cell death pathway in CE81T/VGH cells treated with MEAL.

In vivo anti-tumor effect of MEAL

By using syngeneic tumor implantation model, we found that IP injection of animal with 1.25 and 2.5 mg/kg MEAL significantly inhibited CT26 tumor growth in BALB/c mice (Figure 6). The tumor volume at day 50 was 675.6 ± 135.5 mm^3 in control group, 222.5 ± 80.5 mm^3 in lower dose group (1.25 mg/kg) and 291.4 ± 21.4 mm^3 in higher dose group (2.5 mg/kg).

Safety profile after MEAL of *A. lucidium* IP injection

Mean body weight at the end of therapy was 24.0 ± 0.7 g in control group, 27.7 ± 0.6 g in lower dose group (1.25 mg/kg) and 21.3 ± 0.8 g in higher dose group (2.5 mg/kg) without significant difference between groups. The nadirs of leukocyte counts in these three groups were 12.7±2.0, 10.6±1.3 and 10.3±1.8 (x 10^3 cell mm^{-3}) in day 38, 41 and 32. There is no significant leucopenia both compare with control groups and the data in the beginning of each group. No obvious decline in nadir of leukocytes of MEAL-treated groups was noted with comparison to the controls.

At the day 50, all mice were sacrificed for checking liver and renal function. Plasma level of creatinine among control, 1.25 and 2.5 mg/kg groups were 0.2 ± 0.1, 0.3±0.1 and 0.2±0.1 mg/dL. There were no significant changes in plasma levels of creatinine among all the groups. For liver function, there was no significant difference between the plasma levels of ALT in control and lower dose group (1.25 mg/kg MEAL; 38.5 ± 5.9 IU/L). However, the serum level of ALT elevated from 67.0 ± 13.9 IU/l in control group to 434.3 ± 79.6 IU/L at the higher dose group (2.5 mg/kg MEAL). It follows that liver toxicity could be observed in mice received higher dose of MEAL.

DISCUSSION

Our results suggest that the methanolic extract of *Archidenron lucidium* (MEAL) possesses the growth inhibitory activity against human esophageal cancer CE81T/VGH, human hepatoma HA22T/VGH and murine

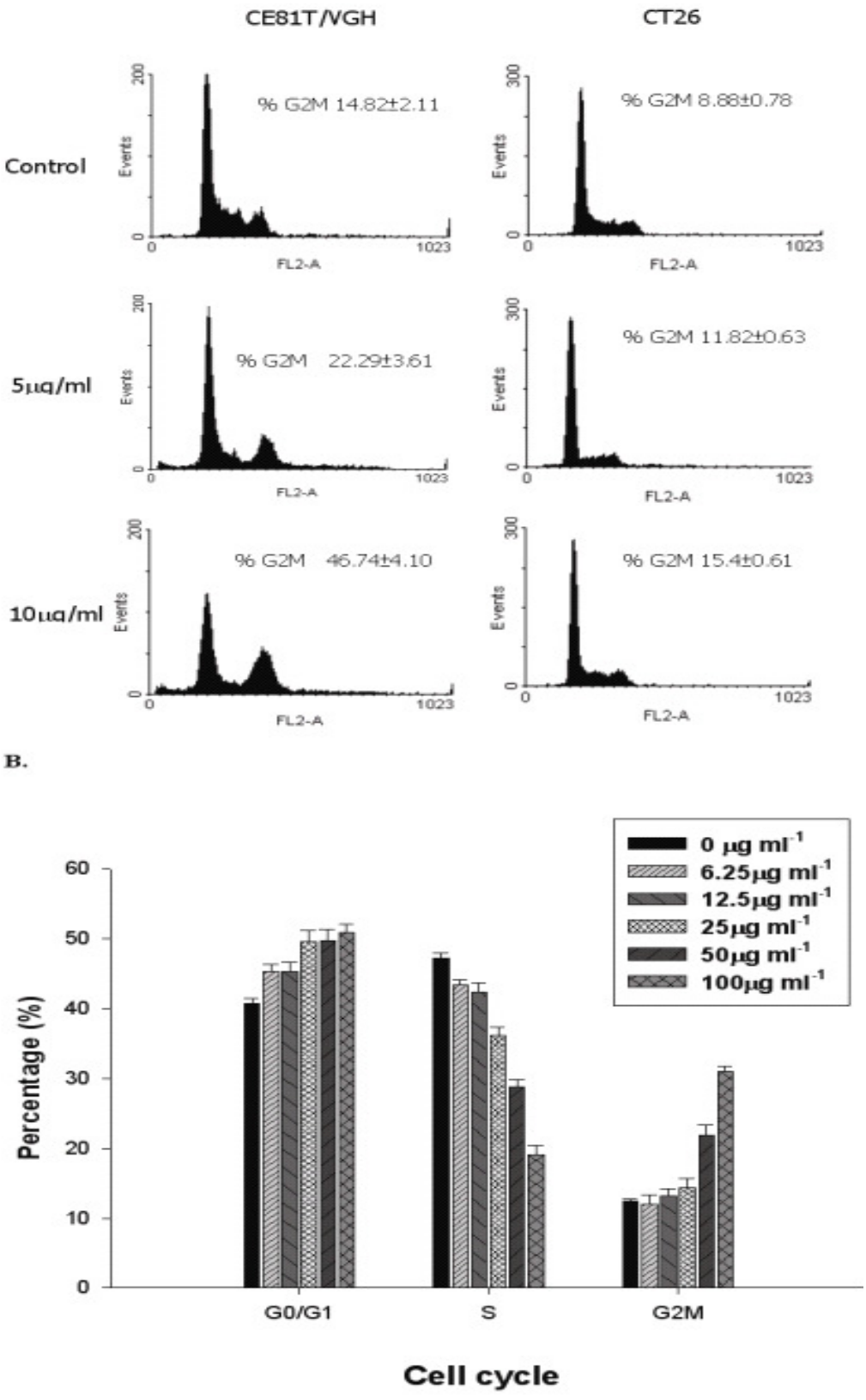

Figure 3. Cell cycle analysis of after treatment with methanolic extraction of *A. lucidum* (MEAL). DNA histograms for esophageal cancer CE81T/VGH and colorectal cancer CT26 cells after 0, 5 and 10 μg/mL MEAL treatment for 24 h were demonstrated. These data are expressed as mean ± SEM from 4 separate experiments.

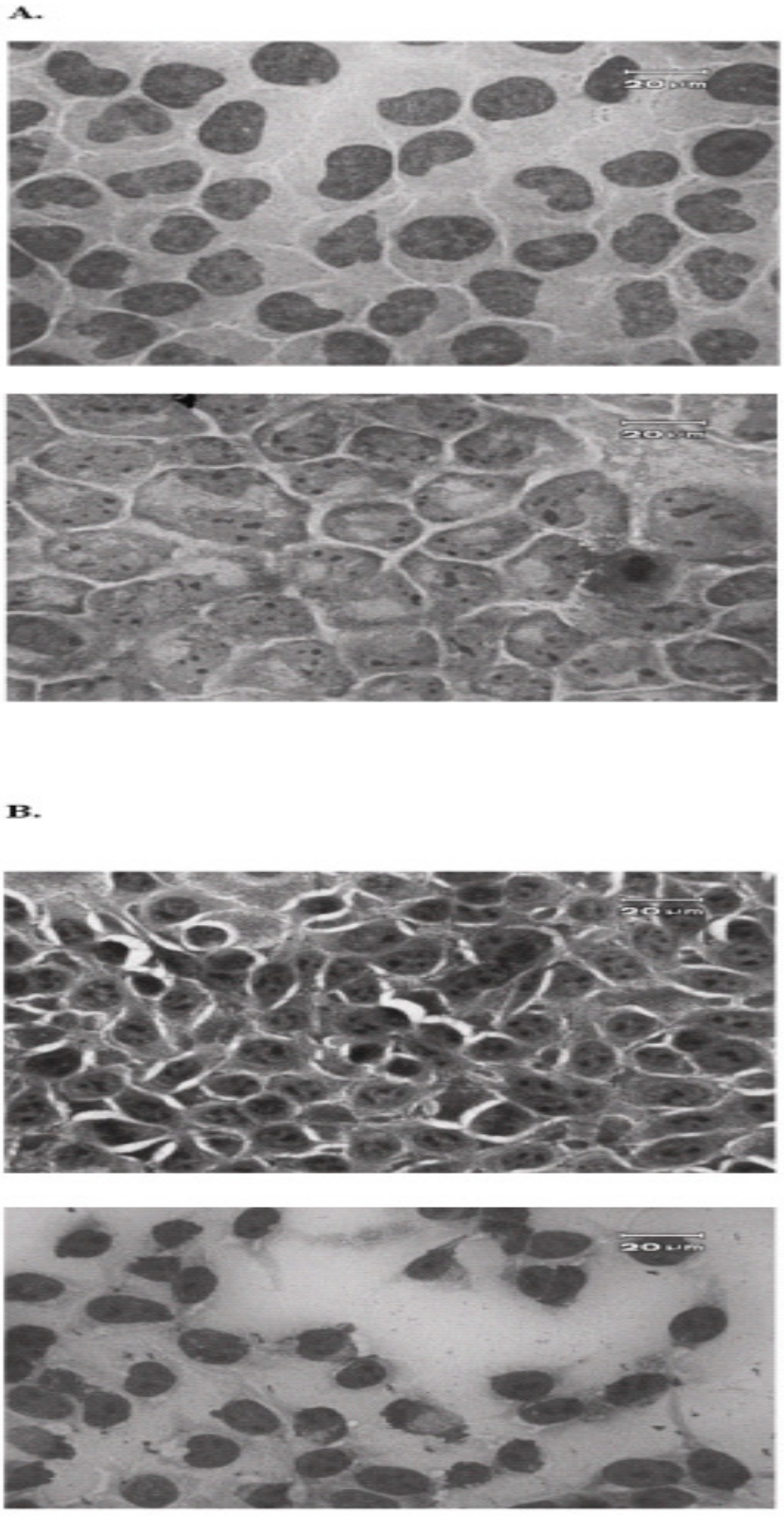

Figure 4. Apoptotic analysis of after treatment with methanolic extraction of *A. lucidum* (MEAL). Annexin V-fluorescein isothiocyanate and propidium iodide staining demonstrate the percentage of apoptotic cells after 0 and 5 µg/mL MEAL treatments for 24 h. These data are expressed as mean ± SEM from 3 separate experiments.

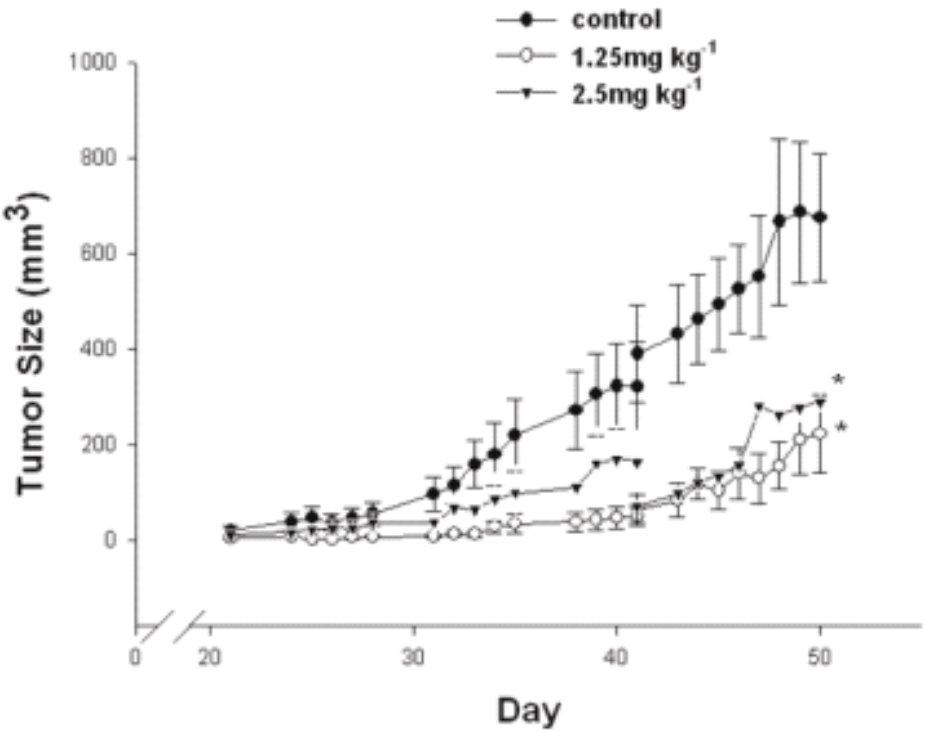

Figure 5. Morphology of esophageal cancer CE81T/VGH cells with (A) and without (B) treatment with 10 μg/mL MEAL for 3 days. Cells were stained with Liu's stain and observed under a light microscope (400 x). (Arrow head, chromatin condensation; scale bar: 20 m)

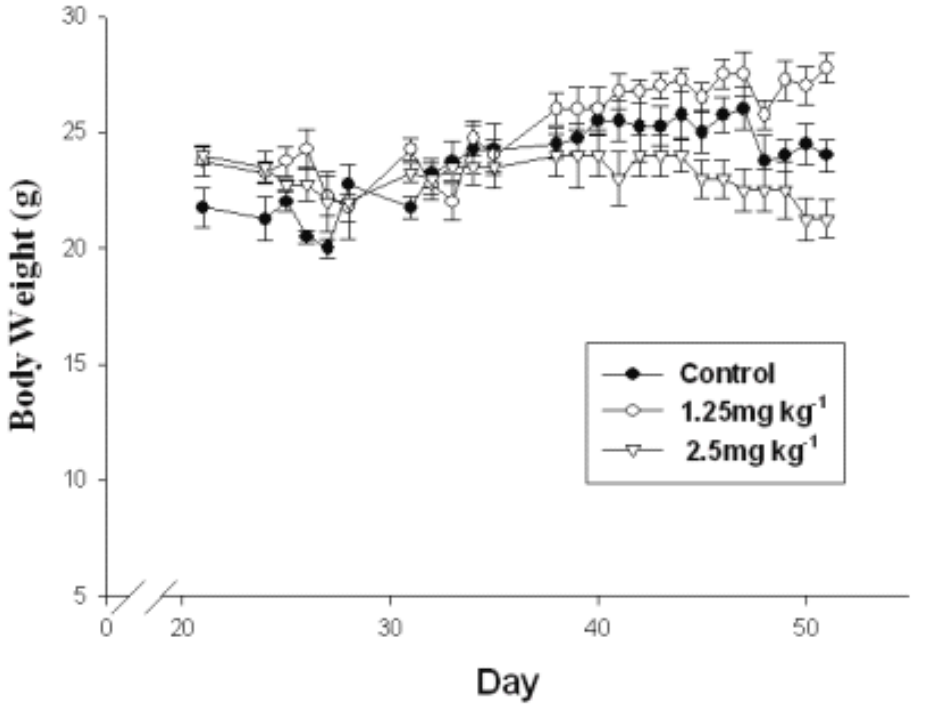

Figure 6. Effect of methanolic extraction of *A. lucidum* on tumor growth *in vivo*. There are three groups, with 5 to 7 mice in each, in these experiments. These data are expressed as mean ± SEM. * $p < 0.05$ when compared with control group.

colorectal cancer CT26 cells *in vitro* and has an effect on arresting cell cycling at G2/M phase. In syngeneic tumor implantation model, optimal dose of MEAL suppressed the growth of CT26 colorectal tumor without significant toxicity. Besides, apoptosis might be the major pattern of cell death in CE81T/VGH cells based on the morphological changes, accumulation of hypodiploid cells and Annexin V-FITC staining. Safety profile is an important issue while evaluating novel agents as candidates for new drug development. In our experiment, tumor size could be effectively controlled by lower dose (1.25 mg/kg) MEAL. Higher dose (2.5 mg/kg MEAL) did not further increase the anti-tumor effect. It seems a therapeutic plateau exists in these dose settings. No significant changes were observed between all three groups in terms of body weight, leukocyte counts and renal function. It means no obvious cachexia, bone marrow suppression and nephrotoxicity were found within these dosage (1.25 and 2.5 mg/kg). However, administration of higher dose of MEAL (2.5 mg/kg) significantly resulted in elevation of serum ALT level. The hepatotoxicity had not been reported before and should be closely monitored in the further study while purifying active compounds. Most patients with hepatocellular carcinoma had poor liver reserve at diagnosis (Farazi and DePinho, 2006).

It implicates that the application of MEAL in treatment of hepatocellular carcinoma may be limited due to a relative narrow therapeutic window for hepatotoxicity. Assessment for the other kinds of toxicity remains necessary in the future. Some compounds purified from natural products are effective but most of the clinical application is limited due to toxicity. Camptothecin has been shown to display anti-tumor activities and tested clinically in 1970s. It was discontinued because of severe side effects (Wall and Wani, 1995). Afterward, Topotecan and irinotecan, water-soluble derivatives of camptothecin, were successfully developed and proven active with less toxicity in clinical trials (Pommier, 2006). It implicates that once the inhibitory effect of MEAL on growth of hepatoma cells is considered promising, the further isolation of active compounds and possible chemical modification may be needed. Concurrent chemoradiation therapy played very important role in treatment of esophageal and colorectal cancer (Blanke et al., 1999; Liao et al., 2004; Layke and Lopez, 2006). Pure compounds derived from nature products (ex. Paclitaxel) with G2/M arresting effect have been investigated as radiosensitizers in clinical practice (Mote et al., 1996). For example, paclitaxel-based chemoradiation therapy has been shown highly active in advanced esophageal cancer (Blanke et al., 1999). The concurrent chemoradiation has been recommendded as standard treatment for unresectable esophageal cancer. It suggests MEAL may have a potential to be developed as radiosensitizer for concurrent chemoradiation. Apoptosis, necrosis, and the other patterns of cell death have been characterized in various types of cancer cells responded to chemical compounds. In our study, apoptotic pathway may be one of the major pathways of cell death induced by MEAL, especially in the case of esophageal cancer CE81T/VGH cells.

Conclusions

The MEAL possesses the growth inhibitory activity against human esophageal cancer CE81T/VGH, human hepatoma HA22T/VGH and murine colorectal cancer CT26 cells *in vitro*. It is also able to arrest cell cycling at G2/M phase and induces apoptosis in CE81T/VGH cells.

In syngeneic tumor implantation model, optimal dose of MEAL suppressed the growth of CT26 colorectal tumor without significant toxicity.

ACKNOWLEDGEMENTS

The authors are grateful to Professor Muh-Tsuen Kao (National Taiwan University, Taipei, Taiwan, Republic of China), for botanical classification and identification of *A. lucidum* (Benth.). This work was supported in part by MMH grant No. 9579 of Mackay Memorial Hospital.

Abbreviations

MEAL, Methanolic extract of *Archidenron lucidium*; **ALT,** alanine aminotransferase; **WEAL,** water extract of *Archidenron lucidium*; **UV,** ultraviolet; **HPLC,** high performance liquid chromatography; **ATCC,** american type culture collection; **DMEM,** dulbecco's modified eagle's medium; **PBS,** phosphate buffer saline; **DNA,** deoxyribonucleic acid; **PI,** propidium iodide; **FITC,** fluorescein isothiocyanate; **IP,** intraperitoneally; **SEM,** standard error of mean.

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